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# The Effect of Toxic Cyanobacteria (Blue-Green Algae) on Water Plants and Animal Cells

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## Abstract

The eutrophication of the Sulejow reservoir dam in Poland is connected with the problem of toxicity of cyanobacterial blooming (blue-green algal blooming). The main species responsible for hepatotoxic "algal bloom" formation is *Microcystis aeruginosa.* The aim of this study is to evaluate the influence of the toxic cyanobacterial extract containing microcystins on the growth and morphology of a water plant *(Spirodela oligorrhiza)* and animal cells (rat hepatocytes). A higher concentration of cyanobacterial extract (MC-LR = 343 μg/dm$^3$) reduced the number of fronds by about 50% in comparison with the control. The extract affected the reduction mass of fronds and the concentration of chlorophyll. The activity of the constitutive acid phosphatase decreased. The first morphological changes in rat hepatocytes typical of apoptosis were observed after 30 minutes of incubation with the cyanobacterial extract. The hepatocytes underwent cell membrane blebbing (MC-LR = 100 μg/dm$^3$). The next 30 minutes of incubation caused an increase in the percentage of deforming cells of more than 50% (MC-LR = 100 μg/dm$^3$). High chromatin condensation and apoptotic bodes were observed in 90% of cells after 120 minutes (MC-LR > 500 μg/dm$^3$). The results of studies confirm the high toxic and cytotoxic effect of blue-green algal blooming from Sulejow reservoir on both plants and animals.

**Keywords:** *Cyanobacteria* (blue-green algae), *Microcystis aeruginosa, Aphanizomenon flos-aquae* microcystin-LR (MC-LR), *Spirodela oligorrhiza,* rat hepatocytes, morphological changes.

## Introduction

As in other countries, the problem of toxicity of cyanobacterial bloom (blue-green algal bloom) is of considerable importance in Poland. The main reason of toxic cyanobacterial bloom formation is intensive antropogenic eutrophication of water bodies, observed especially in lowland reservoirs. A consequence and characteristic of the eutrophication of inland waters is the massive growth of *Cyanobacteria* (blue-green algae), which can be called a "cyanobacterial bloom" [23]. Microcystins are dangerous hepatotoxins, which can be produced by some strains of *Cyanobacteria* generally those such as *Microcystis, Anabaena, Oscillatoria {e.g.* microcystin-LR) [1, 6,11,12, 29]. These substances are natural endotoxins, and their high concentration in water can result from cell lysis. Their mortal influence via liver cell damage was observed mainly in animals [4, 28]. The signs of hepatotoxicosis in

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animals after exposure on cyanobacterial bloom containing microcystins include weakness, reluctance to move about, anorexia, pallor of the extremities and mucous membranes [1, 8, 24]. Humans who have swallowed contaminated water may experience headaches, fever, diarrhoea, abdominal pain, nausea and vomiting. After swimming in contaminated water, humans may get itchy and irritated eyes and skin, as well as other hayfever-like allergic reactions. Moreover, the death of haemodialysis patients in Brazil from the presence of microcystin-LR in dialysis water was documented by Jochimsen in 1998 [14].

The presence of toxins in the blue-green algal bloom in the Sulejow reservoir (located in central Poland) were analysed in phytoplankton samples. Two species of *Cyanobacteria* were detected as bloom creators, *Microcystis aeruginosa* and *Aphanizomenon flos-aque.* In each sample microcystin-LR was detected, and in some of them up to 3 different kinds of microcystins (microcystin-YR, microcystin-RR, microcystin-WR) were also present. In the Sulejow reservoir, a high concentration of MC-LR and other microcystins (more than 100 ig/g dry weigh of phytoplankton) was detected in May, August and September 1994. The highest concentration of MC-LR (191 µg/g d.w. of phytoplankton) and the other microcystins (438 µg/g d.w. of phytoplankton) was found on 23 September 1994.

The cyanobacterial toxin group of microcystins are known to affect a number of processes in plant tissues, and their presence in water used for irrigation may have considerable impact on the growth and development of crop plants. Microcystins have also been detected in the tissues of exposed plants, suggesting that the uptake of these toxins by edible plants may have significant implications for human health. We have ever more information about the effect of microcystins on terrestrial and aquatic animals, but knowledge about the effects of microcystins on higher aquatic plants is still scarce [7, 22].

Microcystins are primarily hepatotoxic because they use a carrier such as the bile-acid carrier of liver cells to pass through the cell membrane [3, 4, 6, 9, 24]. In hepatocytes, the microcystins inhibit the action of protein phosphatase 1 and 2A (PP1 and PP2A) and activate phospholipase $A_2$ and cyclooxygenase [7, 10, 13, 15, 36]. The inhibition of phosphatases causes hyperphosphorylation of cytoskeletal proteins, the consequence of which is the breakdown of intermediate filaments of the cell cytoskeleton [8, 30, 33]. This process causes deformation of the hepatocytes and the development of blebs; these changes are characteristic of the early apoptotic phase.

The objective of this study was

(1) to demonstrate the potential influence of the cy anobacterial extract containing microcystins from Sulejow reservoir on the growth and acid constitution and cellular phosphatase activity in *Spirodela* and chloro phyll (a+b) content, and

(2) to investigate the morphological changes in rat hepatocytes as an apoptotic effect of the cyanobacterial hepatotoxic extract containing microcystin-LR and 3 other variants of microcystins (microcystin-YR, micro cystin-WR and microcystin-RR) from the Sulejow reser voir.

## Experimental Procedures

### Chemicals

All chemicals were produced by Sigma (St. Louis, Mo., USA).

### Growth of *Spirodela oligorrhiza*

An axenic culture of *Spirodela oligorrhiza (Lemnaceae)* was grown in liquid medium [2], under constant illumination (6.8 Wm-2) and at a temperature of 24°C. The growth of the cultures was measured every 24 hours over 4 days by screening the number of fronds, and measuring the weight and chlorophyll concentration after the end of the bioassay.

A water extract of freeze-dried material obtained from natural cyanobacterial bloom and dominated by *Microcystis aeruginosa* was homogenised and sterilised using a membrane filter. HPLC analyses revealed the presence of MC-LR in the bloom sample. The concentration of MC-LR was 169 µg/g of d.w. plankton. A dilution series was made from this extract, which gave equivalents of 20, 40 and 80 mg d.w. of bloom material, and doses of 86 ug/$dm^3$ 172 µg/$dm^3$ and 343 µg/$dm^3$ MC-LR in $dm^3$ of liquid medium. The bioassay was with 3 replications.

### Extraction of Cyanobacterial Bloom Samples for Hepatocytes Assay

Methanol (400 µl) was added to 10 mg d.w. of freeze-dried material obtained from natural cyanobacterial bloom and dominated by *Microcystis aeruginosa* and homogenised for 20 seconds, three times on ice. The sample was then left for 1.5 hours at 4°C, after which it was centrifuged at 5000 rpm for 5 minutes. The supernatant was transferred to a new tube and dried with SpeedVac for 2 hours. The cyanobacterial extract was dissolved in pure DMSO and then diluted in a hepatocyte incubation buffer (concentration of DMSO was maximally 1% v/v).

HPLC analyses revealed the presence of MC-LR in the bloom sample.

### Isolation and Culture of Rat Hepatocytes

The hepatocytes were isolated from male Wistar rats (120-200 g) by *in vitro* collagenase perfusion [21]. Cell number and viability were measured by trepan blue staining.

Viability was about 90%-95%.

### Analysis of Hepatocytes

Hepatocytes were incubated for 30, 60 or 120 minutes with cyanobacterial extract containing MC-LR at 100, 250, 500, 1000, and 2000 µg/$dm^3$. The control cells were incubated without cyanobacterial extract in the same conditions. The cell samples (10 µl) were mixed with 9 volumes of 2% glutaraldehyde buffered with

0.1 M Na-cacodylate and containing 1 µg/ml of Hoechst 33342 DNA-specific dye. Cells were analysed using a Zeiss Axiophot microscope with fluorescent light and interference contrast. The changes in cell morphology were calculated for each concentration of MC-LR per 100 hepatocytes. Data analysis was carried out using the Sigma Plot computer packages.

## Results

### The Effect of Cyanobacterial Extract and on Water Plants

After only 24 or 48 hours, the morphology of *Spirodela oligorrhiza* started to alter in the experiment with cyanobacterial extract. A higher concentration of extract promotes a reduction in the number of fronds (Fig. 1). In every dilution, the extract affected the reduction mass of fronds (from 1.0 mg in control to 0.35 mg FW in culture with extract), and the concentration of chlorophyll in 100 mg FW (fresh weigh) of fronds in comparison with the control plants. The contents of all chlorophyll (a+b) are significantly reduced by about 40% for the 80 mg concentration of MC-LR, and by about 47% for 40 mg and 30% for 20 mg in comparison with the control (Fig. 2). Some of the fronds showed chlorosis. The activity of cellular phosphatases was decreased at the concentrations from 86 to 343 µg/ $dm^3$ of MC-LR (Fig. 3).

### The Effect of Cyanobacterial Extract on Rat Hepatocytes

The morphological changes in hepatocytes were analysed after 30 minutes. As in previous studies [20] the blebbing of hepatocytes were seen; MC-LR > 100 µg/ $dm^3$ ( Fig. 5A). The next 30 minutes saw an increase in the percentage of deforming cells over 90%, MC-LR > 500 µg/ $dm^3$ (Figs. 4B and 5B). Highly condensed chromatin and apoptotic bodies were observed in 90% of hepatocytes after 120 minutes, MC-LR > 500 µg/ $dm^3$ (Figs. 4C and 5C).

## Discussion of Results

In the present work, the potential toxic and cytotoxic effects of cyanobacterial bloom (blue-green algal bloom) on water plants and animal cells (rat hepatocytes) were documented.

The extract of the Sulejow reservoir cyanobacterial blooms inhibited *Spirodela* growth; this process is connected with the decrease in phosphatase activity and reduction of chlorophyll (a+b) concentration (Figs. 2 and 3). Moreover the extract of "algal bloom" mainly caused root growth inhibition together with a reduction in the number of roots and fronds (Fig. 1). Probably the microcystins produced by the blue-green algae do not change a plant's primary DNA structure, or the putative changes occur only in a very small part of the genomic

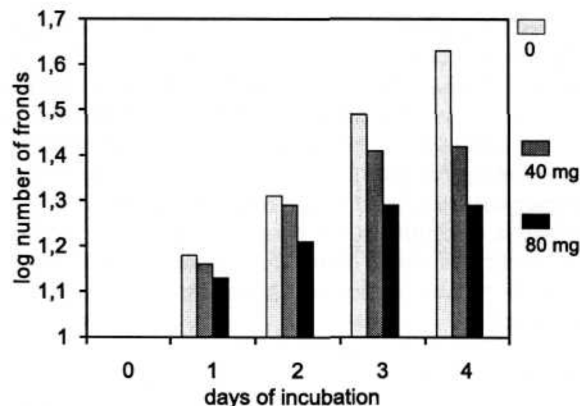


Fig. 1. Growth of *Spirodela oligorrhiza* in 4N medium with different concentration of cyanobacterial extract.

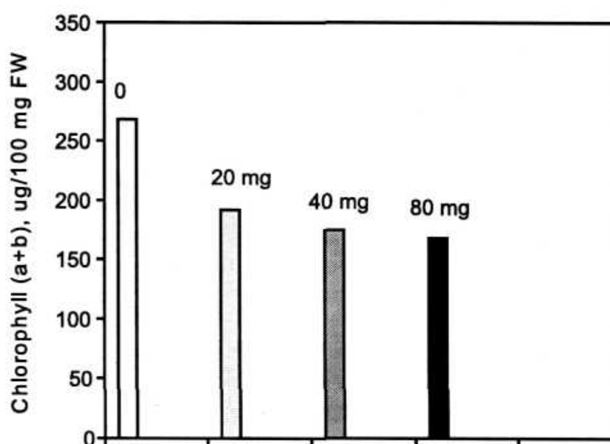


Fig. 2. Concentration of chlorophyll (a+b) in *Spirodela oligorrhiza* after 4 days of incubation in medium with different concentration of cyanobacterial extract (0-control, 1-20 mg/35 ml, 2-40 mg/35ml, 3-80 mg/35ml).

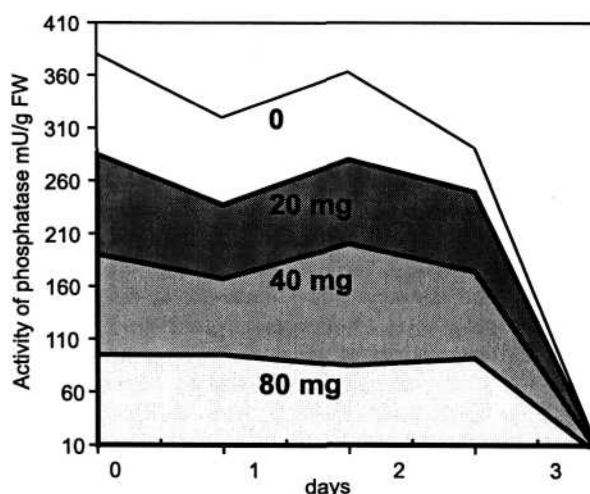


Fig. 3. Activity of phosphatase in *Spirodela oligorrhiza* cultivated at the different concentrations of cyanobacterial extract. Cone: 0 -control, 20 mg, 40 mg, 80 mg.

sequences [25]. However, it seems probable that DNA modifications occur in higher-level structures, which significantly influence the level of gene-expression. In future experiments, more specific genome DNA fragments should be used in Southern hybridisation, as should techniques allowing for the observation of changes occurring at a higher structure level [25]. The experiment presented above was a preliminary for a more precise analysis on the possibility of using *Spirodela oligorrhiza* as a quick bioassay for the evaluation of *Cyanobacteria* (blue-green algae) toxicity.

The higher plants, especially from the *Lemnaceae* family, are widespread and fast-growing (doubling time 1-4 days) due to its high sensitivity to a number of chemical factors, including heavy metals, detergents, growth regulators and herbicides [15, 18]. For this reasons, *Spirodela* is a good object to study for aquatic toxicity tests, and can be a useful object for toxin detection [5, 19, 32]. Weif et al. in 2000 [34] showed the influence of microcystin-RR on *Lemna minor* L.; the plants were exposed to several concentrations (0.1 - 5 mg per litre). A significant reduction of plant growth and formation of fronds were noticed at concentrations of 3 and 5 mg MC-RR per litre during cultivation for 6 days. Our report indicates that the cyanobacterial toxin MC-LR can be taken up from the surrounding medium by the aquatic macrophyte *Spirodela,* and raises the possibility that the microcystin may contribute to the reduction in macrophyte populations in eutrophic water bodies which are dominated by cyanobacteria. These results have been correlated with studies by other authors, who demonstrated that microcystins inhibits the growth of plants [16, 17, 34] and decreased activity of enzymes as a protein phosphatase. The production of *Cyanobacteria* could affect aquatic plant establishment and growth by changing the physical environment of germinating and established plants and the production of toxin, which could affect plant metabolic processes [5].

The morphological changes in animal cells, rat hepatocytes after cyanobacterial extract exposure, indicated an apoptotic process. This mode of cell death is very important because it regulates the balance between cell death and cell proliferation, and it assures proper tissue homeostasis [27, 35, 31]. Hepatocytes are the main target for toxins of blue-green algae; therefore, apoptotic changes can be observed there earlier (30 min) than in other cells (Fig. 5). A selective transport system characteristic of hepatocytes enables the preferential uptake of cyanotoxins by these cells [11, 26]. In this work, as in previous publications [20], the morphological hallmarks characteristic for apoptosis such as blebbing of the cell (early phase), condensation and marginalisation of chromatin and the creation of apoptotic bodies (late phase) after exposure to cyanobacterial extract containing MC-LR were observed (Fig. 4).

In summary, the results obtained in our studies showed the high toxic and cytotoxic influence of cyanobacterial extract containing microcystins on water plants and animal cells. The undesirable effect on the natural ecosystem is connected with the possibility of microcystins accumulating in phytoplankton, zooplankton, freshwater mussels, freshwater clams and freshwater fish [8]. Further transfer of the blue-green algae

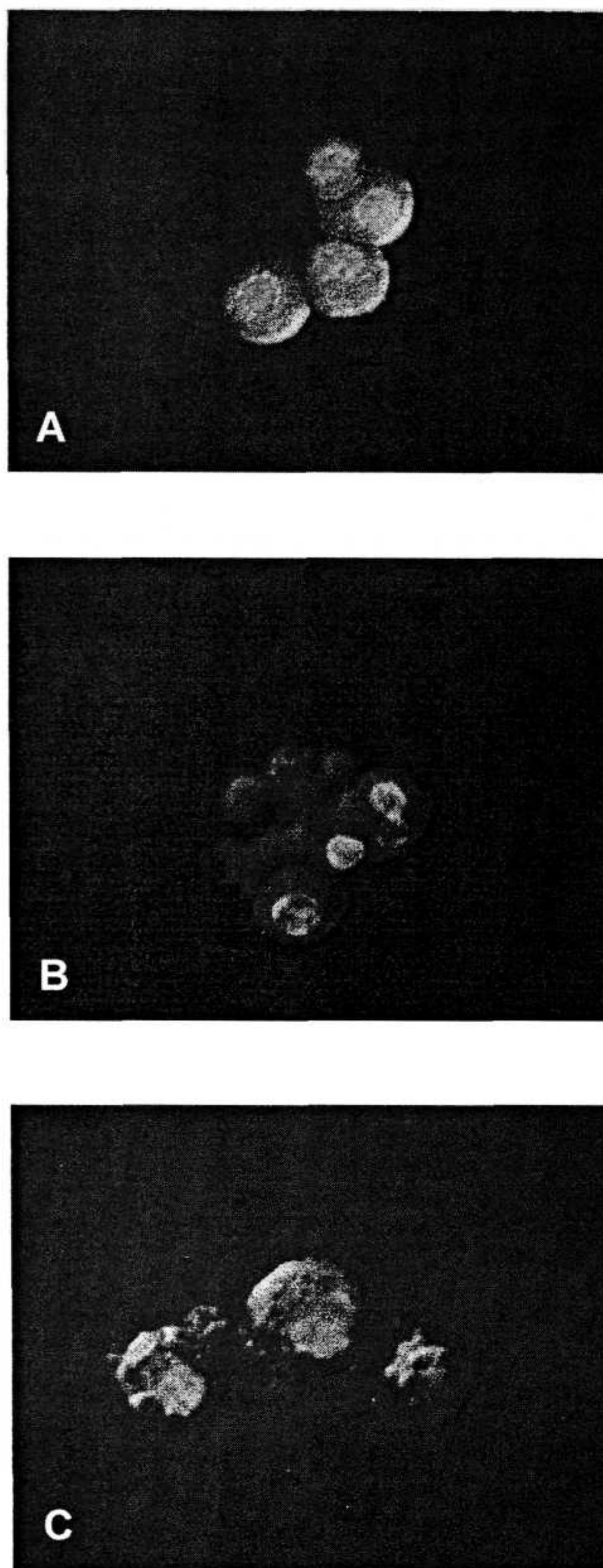


Fig. 4. (A, B, C). Microscopic analysis. Rat hepatocytes incubated with cyanobacterial extract (MC-LR = 1000 μg/dm$^3$) A - control cells without cyanobacterial extract; B - cell with cyanobacterial extract (60 minutes), blebbing of cells is visible; C - cells with cyanobacterial extract (120 minutes), shrinkage of cells, condensation and margination of chromatin and apoptotic bodies are observed; magnification 1000x. Cells were stained using Hoechst 33342 dye.

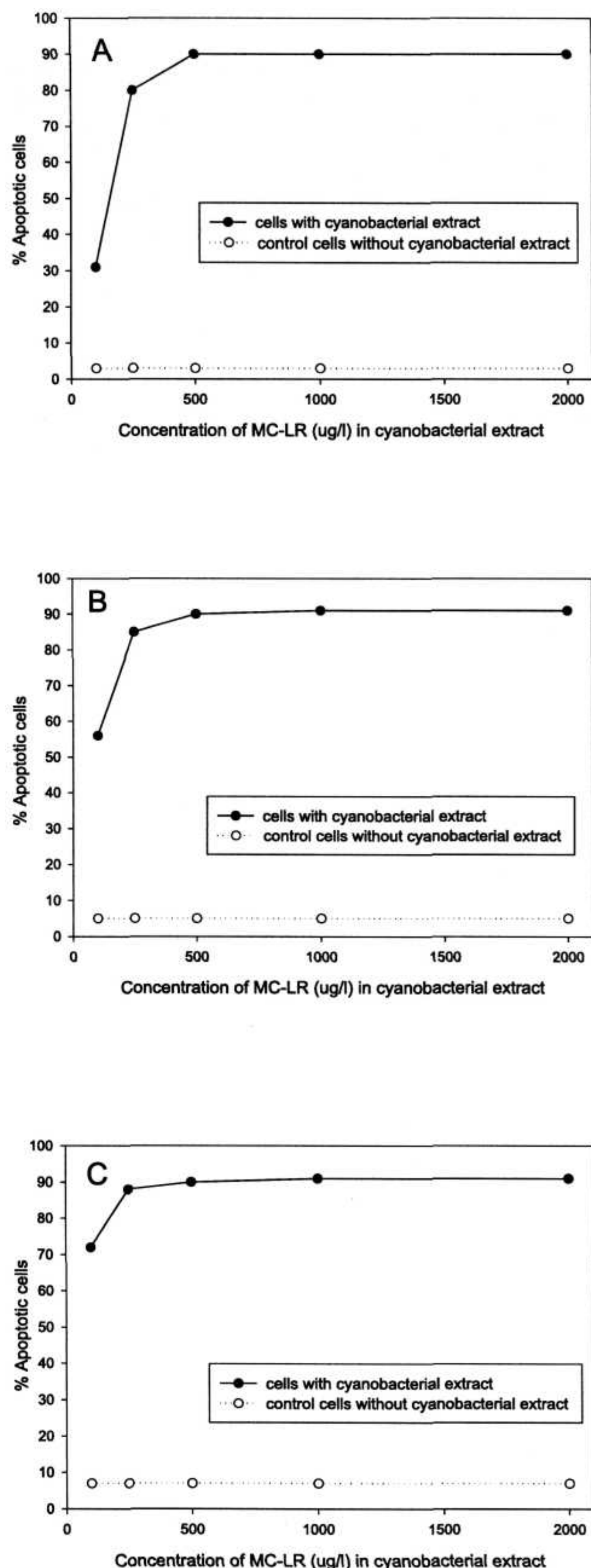


Fig. 5. Apoptotic effect of cyanobacterial extract containing microcystin-LR on rat hepatocytes after incubation with hepatotoxin: A - for 30 minutes, B - 60 minutes, C - 120 minutes. The cells with apoptotic changes were determined by fluorescence and differential interference contrast light microscopy.

toxins through the food chain is possible. Additionally, humans may swallow contaminated water during swimming or drinking. Therefore a bioassay conducted with water plants - *Spirodela* together with other methods - as an *in vitro* hepatocyte assay could be useful for estimating the influence of the toxic "algal blooms" on the natural ecosystem as a potential source of food and drinking water.

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