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# Cytotoxic effects and changes in cytokine gene expression induced by microcystin-containing extract in fish immune cells – An *in vitro* and *in vivo* study

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ABSTRACT

Blooms of cyanobacteria producing very toxic secondary metabolites (especially microcystins) are potent environmental stressors, hazardous not only to aquatic animals but also to public health. The purpose of this study was to investigate the effects of an extract containing microcystins on immune cells isolated from the common carp (*Cyprinus carpio* L.). In the present study it has been found that the extract induced apoptosis and inhibited *in vitro* lymphocyte proliferation. In addition, the results indicated the possible role of oxidative stress in this cytotoxicity and apoptosis. The *in vivo* investigations showed that the extract containing microcystins had greater suppressive effects on the essential functions of immune cells (intracellular reactive oxygen species production and lymphocyte proliferation) than the pure toxin alone. Moreover, immersion of fish in the toxic extract caused changes in the mRNA levels of various pro- and anti-inflammatory cytokines in carp leukocytes, while after exposure to the pure toxin, only IL1-β expression was markedly up-regulated. The observed modulatory effects on immune cells could have important implications for the health of planktivorous fish, which feed more frequently on toxic cyanobacteria.



## 1. Introduction

The increased reproduction of cyanobacteria is a consequence of anthropogenic pollution and the eutrophication of water bodies, and may also be connected to climate changes. Global warming and the heating of surface waters change their characteristics and ecosystem processes. Increased temperature, enhanced surface stratification and stimulation of photosynthesis by elevated $CO_2$ levels provide more optimal conditions for the growth of harmful cyanobacteria, promoting their growth over other phytoplankton groups (e.g., diatoms and green algae) [1,2]. Not all species of cyanobacteria produce toxins. It is known that some of them can be either toxic or non-toxic, while some can produce several different toxins at the same time [3,4]. The toxins produced by cyanobacteria, cyanotoxins, cause significant problems in aquatic biota, and pose serious hazards to both wild and domestic terrestrial animals. Water and food chain contamination with these toxins may also cause health problems for humans [5,6]. Several species of cyanobacteria produce different kinds of toxins, which are classified based on the mode of their toxicity: hepatotoxins, neurotoxins, cytotoxins, dermatoxins and irritant toxins [7]. Moreover, depending on their chemical structure, they can also be divided into three classes: cyclic peptides, alkaloids and lipopolysaccharides [8].

The main hepatotoxins produced by cyanobacteria are microcystins (MCs) - cyclic heptapeptides containing both D- and L amino acids, N-methyldehydroalanine (Mdha) and a β- amino acid side-group, 3-amino-9-methoxy-2-6,8-trymethyl,10-phenyldeca-4,6-dienoic acid (Adda), which is involved in their toxic properties [9]. MCs comprise a group of approximately 80 structural variants produced by freshwater cyanobacteria genera, such as *Microcystis, Anabaena, Plankothrix, Anabaenopsis, Hapalosiphon* and *Nostoc* [5,7].

In many eutrophic surface waters worldwide, concentrations of MCs are higher than their safe level, which is 1 μg $L^{-1}$ for the most toxic MC variant, microcystin-LR (MC-LR) [10,5]. The total (intracellular and dissolved) cyanotoxin levels have been particularly high recently in surface waters of Japan, China and Portugal (concentrations above 10 μg $L^{-1}$). MC concentrations as high as 25 000 μg $L^{-1}$ have been reported in samples of cyanobacterial scums in Germany [7].

MCs are potent and specific inhibitors of serine/threonine-specific protein phosphatases 1 and 2A (PP1 and PP2A) [11]. Protein phosphatases are responsible for phosphoprotein dephosphorylation and act as modulators of protein kinases. The balance

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between kinase and phosphatase activity constitutes a crucial regulatory mechanism in various types of eukaryotic cells, including immune cells [12,13]. MCs exert their toxicity trough general over-phosphorylation of cellular proteins that can affect a variety of basic cellular processes ranging from cell growth, differentiation, cytoskeletal dynamics and cell adhesion, cell-to-cell communications to the overall regulation of gene expression [14].

Phosphorylation and dephosphorylation of regulatory proteins is a universal regulatory mechanism that controls all major metabolic and signalling processes, some of which are also associated with apoptosis [15]. Apoptosis is classified as organised programmed cell death. The execution of apoptosis is mediated by caspases, which are cysteine proteases that are activated through either the receptor-mediated extrinsic or the mitochondrial-mediated intrinsic apoptotic pathways. The extrinsic pathway is initiated by the interaction between death ligands such as FasL and death receptors such as Fas at the cell surface, leading to a downstream signalling cascade that results in the activation of caspase-8. The intrinsic pathway is triggered by signals that cause the release of cytochrome *c* from the mitochondria into the cytosol. In the cytosol, a complex known as the apoptosome (formed from apoptotic protease activating factor-1 (Apaf-1), procaspase-9 and cytochrome *c*) activates the executioner caspases 3, 6 and 7. Both these pathways lead to the activation of a sequence of cellular, morphological and biochemical changes such as: rounding and retraction of cells, compression of cytoplasmic organelles, membrane blebbing, chromatin condensation, cytoskeleton disruption, DNA fragmentation and apoptotic body formation [16].

Analysis of dysfunction in the piscine immune system as a testing strategy for environmental risk assessment has been performed very rarely. It is particularly important to determine the consequences of chronic exposure to low concentrations of cyanotoxins, and the interactions among different classes of the toxins. There have been several reports on the influence of MCs on mammalian lymphocyte and phagocyte function (summarized by Rymuszka and Sierosławska [17]) but only a few studies on the toxins' interaction with fish immune cells, and impairment of immune homeostasis by cyanotoxins [18–25]. Moreover, studies on zebrafish larvae have revealed that MC-LR up-regulated the transcription of several genes essential for early lymphoid development and production of heat shock proteins, potentially causing changes in the functioning of the immune system [26].

The first aim of this study was to examine the *in vitro* effects of crude cyanobacterial extracts containing MCs on fish immune cells. This involved exploring whether toxic changes in the cells induced by the extract were apoptotic and/or necrotic. The second aim was to determine the *in vivo* effects of pure MC-LR or crude cyanobacterial extracts containing MCs on the esssential functions of fish immune cells, such as lymphocyte proliferation and the production of reactive oxygen species (ROS) by phagocytes. To better understand the immunotoxic mechanisms of cyanotoxins, the mRNA expression levels of pro- and anti-inflammatory cytokines, including IL-1β, TNF-α, IL-10 and TGF-β, were analysed in carp leukocytes.

## 2. Material and methods

### 2.1. Toxin

The MC-LR standard (purity>96%) was from Alexis Biochemicals (San Diego, USA). A stock solution containing the toxin at 1 mg $mL^{-1}$ was prepared in dimethylsulphoxide (DMSO, POCh, Poland). The final DMSO concentration in exposed and control groups was <0.01%.

### 2.2. Preparation and analysis of extract from cyanobacterial scum

The cyanobacterial scum used for the experiments was collected from a highly eutrophic dam reservoir near Kraśnik located 45 km away from Lublin (SE Poland). The study was conducted over the Summer (from July to October) in 2008 when the algae were in the intensive phase of growth. The bloom samples were harvested using a nylon net (200 mm mesh size). Phytoplankton were identified and counted at 400× magnification using an inverted microscope. During the study period, *Microcystis* spp., *Aphanizomenon* sp., and *Planktothrix* sp. were dominant in the reservoir. The collected samples were freeze-dried and stored at −20 °C until MCs quantification. Prior to use, samples were divided into several portions (10 mL). The cyanobacterial cells were ultrasonicated on ice (twice for 5 min, 70 W, Sonoplus Bandelin) in 75% methanol acidified with 2 M HCl, and centrifuged at 10 000× *g* for 5 min at 10 °C. Supernatants were collected and the pellets were resuspended and dissolved with methanol. Debris was removed by centrifugation at 10 000× *g* for 5 min at 10 °C. Prepared extracts were evaporated to dryness and kept in the freezer until used for toxin analysis and cytotoxicity assays.

MCs in samples were determined using a commercially available enzyme-linked immunosorbent assay (ELISA, EnviroGard, USA) according to the manufacturer's protocol. The assay did not differentiate between microcystin-LR and other MC variants. The assay uses antibodies against MC-LR and is able to detect this structural variant of MC, but it is also known that these polyclonal antibodies show similar cross-reactivities with other MC variants (eg. MC-RR, MC-YR). In the study, the results were given as total MCs levels. Analysis of the crude extract revealed the presence of MCs at a concentration of 1.4 mg $L^{-1}$.

### 2.3. Animals

All procedures carried out on animals were performed in accordance with the Local Committee of Ethics on animal experimentation (approval number 9/2009). Common carp (*Cyprinus carpio* L.), weighing 250–300 g, were purchased from a commercial farm. The fish were acclimatized for a week in a recirculation system with filtered water tanks (100 L) at 20 °C and fed with commercial diets once a day prior to use in the study. The water quality parameters - pH (6.8–7.2), hardness (67 mg $L^{-1}$ $CaCO_3$), and dissolved oxygen (6.2–7.1 mg $L^{-1}$) content - were checked regularly. During the study, no mortality was observed, or changes in fish behaviour. Before obtaining materials for cell isolation, animals were anaesthetised with 0.2% Propiscin (Żabieniec, Poland) diluted in water.

### 2.4. Separation of immune cells from blood and head kidney

Carp blood was collected by puncturing the caudal vein, and diluted 1:1 in phosphate buffered saline without $Ca^{2+}$ and $Mg^{2+}$ (PBS, Biomed, Poland). Lymphocytes were isolated on a Gradisol L gradient, and phagocytes, predominantly (>80%) granulocytes and monocytes, on a Gradisol G gradient (Aqua-Medica, Poland) by centrifugation at 450× *g* for 30 min at 4 °C. After centrifugation, the leukocyte layer at the interface was collected and washed twice with RPMI-1640 (Lonza, Belgium; the RPMI was made up to the correct osmolarity for carp (270 mOsmol $kg^{-1}$) by adding water).

The head kidney was removed and pushed through a nylon mesh (100 μm), and the cells were suspended in RPMI-1640. The cell suspensions were layered on a discontinuous gradient of Percoll (1.02, 1.06, 1.07 and 1.08 g $mL^{-1}$; Sigma, Aldrich) to obtain fractions enriched for different leukocyte subsets and centrifuged at 400× g for 30 min at 4 °C. Leukocytes in the density range from