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Cyanobacteria and cyanotoxins in fishponds and their effects on fish tissue

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ABSTRACT

Cyanobacteria can produce toxic metabolites known as cyanotoxins. Common and frequently investigated cyanotoxins include microcystins (MCs), nodularin (NOD) and saxitoxins (STXs). During the summer of 2011 extensive cyanobacterial growth was found in several fishponds in Serbia. Sampling of the water and fish (common carp, *Cyprinus carpio*) was performed. Water samples from 13 fishponds were found to contain saxitoxin, microcystin, and/or nodularin. LC–MS/MS showed that MC-RR was present in samples of fish muscle tissue. Histopathological analyses of fish grown in fishponds with cyanotoxin production showed histopathological damage to liver, kidney, gills, intestines and muscle tissues. This study is among the first so far to report severe hyperplasia of intestinal epithelium and severe degeneration of muscle tissue of fish after cyanobacterial exposure. These findings emphasize the importance of cyanobacterial and cyanotoxin monitoring in fishponds in order to recognize cyanotoxins and their potential effects on fish used for human consumption and, further, on human health.

1. Introduction

Fish have an important role in maintaining the stability of water ecosystems due to the position which they occupy in food chains. In addition they serve as a human food source. On the other hand, cyanobacteria are an important component of the diet for many fish (Zurawell et al., 2005). Cyanobacteria are photosynthetic microorganisms that subsist in diverse terrestrial and aquatic environments, including fishponds, throughout the world. In many fishponds cyanobacteria constitute the major part of the phytoplankton biomass during the summer and autumn in temperate latitudes, frequently forming mass occurrences known as water blooms. They can easily adapt to certain conditions usually encountered in fishponds such as reduced light conditions, high temperatures, nitrogen depletion in the upper layer, high degree of eutrophication and decrease in the numbers of large phytoplanktivorous filter-feeders (*e.g. Daphnia*) subject to intensive predation by fish (Sevrin-Reyssac and Pletikosic, 1990). These organisms can produce a number of metabolites through their secondary metabolism, some of which can be extremely harmful. Cyanobacterial toxins (cyanotoxins) can be both retained in cyanobacteria and released into the water during their senescence and lysis. Many aquatic organisms, especially fish, are then directly exposed to dissolved cyanotoxins.

Living in the aquatic environment, fish can in a variety of ways come into contact with cyanobacteria and their toxins, which may affect their growth, development, histology, reproduction and survival (Li et al., 2004; Palikova et al., 2004, 2007; Deng et al., 2010; Svirčev et al., 2015). Exposure of fish to cyanotoxins can occur in two ways: the first way is active introduction by the oral route through drinking and the consumption of cyanobacterial cells and other organisms that have accumulated cyanotoxins. Another potential way is passive, *via* direct contact of the gill epithelium with the surrounding water containing toxins. Both types of exposure can occur in natural conditions (Malbrouck and Kestemont, 2006).

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After ingestion, MCs require active transport into cells *via* organic anion transporting polypeptides (OATPs) (Boaru et al., 2006). The involvement of OATPs in the uptake of MCs has been demonstrated in humans, mice, rats and fish (Fischer et al., 2005, 2010; Meier-Abt et al., 2007; Hagenbuch and Gui, 2008; Lu et al., 2008; Feurstein et al., 2009; Steiner et al., 2014). OATPs are detected in various tissues of zebrafish (Popovic et al., 2010). Therefore, MCs may be distributed and accumulated in various fish organs (Cazenave et al., 2005; Xie et al., 2005; Chen et al., 2006; Romo et al., 2012). They are known to accumulate in tissues of other aquatic organisms including zooplankton (Karjalainen et al., 2008), mussels (Mazur-Marzec et al., 2007), molluscs (Shumway, 1995), *etc.* Consumption of aquatic organisms which have accumulated cyanotoxins may pose a potential risk to human health (Xie et al., 2005; Peng et al., 2010; Papadimitriou et al., 2010; Berry et al., 2012). Therefore, it is necessary to control cyanotoxin concentrations in fishponds where fish are grown for human consumption.

The aim of this paper was to assess the presence of frequently found cyanotoxins MCs and/or NOD, STX and other PSP toxins in water samples from blooming fishponds in Serbia, and to assess the accumulation of MCs in fish muscle tissue. A further goal was to determine the histopathological effects of cyanobacteria and their toxic metabolites on various organs of fish grown in ponds where cyanobacterial blooms and cyanotoxin production were recorded. Studies of this kind have not been performed in Serbia where an epidemiological connection between exposure to cyanotoxins in drinking water and the prevalence of several forms of cancers has been proposed (Svirčev et al., 2013, 2014a). Since a major part of the domestic fish consumed in Serbia is farmed and cyanobacterial mass occurrences are common in fishponds, the current study provides novel data about exposure to cyanotoxins in the local conditions.

2. Material and methods

2.1. Qualitative and quantitative determination of cyanobacterial communities and chlorophyll a

Mass occurrences of cyanobacteria in the investigated fishponds were observed from May to September of 2011. Water samples were collected from 13 ponds (P1–P13) during the summer of 2011. Samples for the examination of cyanobacteria in the plankton community were collected from the middle of the ponds with a Ruttner's bottle of 1 l volume. Afterwards the samples were stored in bottles of 100 ml and fixed with 4% v/v formaldehyde. Identification and counting of cyanobacterial phytoplankton were done with a Leica inverted microscope using the Utermöhl method (Utermöhl, 1958).

Water samples for chlorophyll *a* (Chl *a*) determination were concentrated by filtering 0.5 l of water through a 0.45 μm membrane filter. Filters were folded into centrifuge tubes and Chl *a* was extracted overnight with 90% acetone at 4 °C and in darkness. Extracts were centrifuged and measured spectrophotometrically (APHA, 1995). Measurements were done in duplicate and the results were expressed as mean values. The trophic state determination was done according to Felfoldy (1980).

2.2. Quantitative determination of cyanotoxins in ponds water and fish meat

For cyanotoxin detection in water samples enzyme-linked immunosorbent (ELISA) assays were used. In order to ensure release of cyanobacterial intracellular contents including cyanotoxins if present, water samples were freeze-thawed and sonicated, and then centrifuged at 5000 rpm for 15 min. Resulting supernatants were used for two ELISA assays: microcystins-ADDA and saxitoxins (STXs). The Abraxis Microcystins-ADDA ELISA (Microcystin/Nodularins ADDA ELISA, Abraxis LLC, Pennsylvania, USA) is an immunoassay for the quantitative and sensitive congener-independent detection of MCs and NOD. The STXs ELISA (Saxitoxin (PSP) ELISA, Abraxis LLC, Pennsylvania, USA) is an immunoassay for the quantitative and sensitive detection of STXs. According to the ELISA KIT manufacturers, the quantitation range for the microcystins-ADDA ELISA assay is 0.10–5 ppb (μg l^{-1}), and for the STXs ELISA 0.015–0.4 ppb (μg l^{-1}). The ELISA plates were read using a microplate reader (Asys Expert Plus UV, Biochrom, UK).

Fish *Cyprinus carpio* grown in 10 constantly blooming ponds throughout the summer were sampled and analyzed for the presence and effects of cyanotoxins. For cyanotoxin detection in fish muscle samples LC–MS/MS was performed in order to investigate possible human exposure through consumption of fish meat. Furthermore, histological analyses (five individuals on average) of liver, kidney, gills, intestines and muscles were done. Individual ponds from which the fish were sampled are not indicated because of practices in fish farming which involve relocation of the fish between ponds. Sampled individuals had an average weight of 354 g (55–758 g), an average total length of 270 mm (155–360 mm) and standard length of 217 mm (125–285 mm).

In order to detect potential accumulation of cyanotoxins, 400 mg of freeze-dried muscle tissue was taken. Pure methanol (3 ml) was added to the samples, which were sonicated and then centrifuged at 3300 × *g* for 10 min and the supernatants retained. In order to remove interfering lipids hexane (6 ml) was added to the supernatants and then discarded after phase separation. Samples were evaporated, 10% methanol (5 ml) was added, followed by sonication and passage of the material through reversed-phase cartridges (OASIS HLB Cartridge 200 mg, Waters) to retain the cyanotoxins. Cartridges were then eluted with 100% methanol (3 ml), followed by evaporation and dissolving the residues in 75% aqueous methanol (200 μl). After vortexing, the samples were filtered (0.2 μm GHP ACRODISC 13, PALL), centrifuged (at 10,000 × *g* for 10 min), and the supernatants were then diluted ten-fold with 75% methanol to ensure good dissolution of the analytes and to avoid problems with ion suppression in the MS instrument. The samples were then analyzed by LC–MS/MS using an Agilent 1290 Infinity Binary LC system (Agilent Technologies) coupled to an Agilent 6460 triple-quadrupole mass spectrometer. The toxins were separated on a Supelco Ascentis C18 HPLC column (Bellefonte, PA, USA); 50 mm × 3 mm I.D., 3 μm particle diameter, protected by a 4 × 2 mm C8 guard column. The mobile phase consisted of solvents A: 99% water; 1% acetonitrile; 0.1% formic acid and B: acetonitrile; 0.1% formic acid.

Reference samples for the identification of selected MCs were an extract of *Microcystis wesenbergii* NIES-107 (National Institute of Environmental Studies, Tsukuba, Japan), which produces mainly dm-MC-RR, MC-RR, dm-MC-YR, MC-YR, dm-MC-LR, MC-LR (Meriluoto et al., 2004); and an extract of *Microcystis aeruginosa* PCC 7820 (Institute Pasteur, Paris, France) which produces MC-LR, MC-LY, MC-LW and MC-LF (Lawton et al., 1994). Cyanobacterial extracts were analyzed in four different concentration levels and detection ranges are presented in Table 1. The accuracy of the instrument calibration was assured by comparing the MC-LR and MC-RR concentrations in the in-house reference materials to those of certified reference materials. Certified Reference Materials CRM-MCLR and CRM-MCRR were obtained from the Biotoxin CRM Program of National Research Council Canada (Halifax, Canada). The rest of the MCs (for which certified reference materials were unavailable) were quantified as MC-LR equivalents on a HPLC-coupled photodiode array detector set at 238 nm (SOP_TOX-IC_AAU_06F; Meriluoto and Codd, 2005) and then used in LC–MS work.