Dissolved and intracellular microcystins in lake water during *Planktothrix rubescens* algal bloom: HPLC quantification and crustacean acute toxicity test

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INTRODUCTION

The algal bloom due to cyanobacteria, such as *Microcystis*, *Anabaena*, and *Planktothrix*, may cause serious environmental problems. Cyanobacteria can produce a large spectra of toxins that can have effects on human health and/or on aquatic living organisms. Lately, *Planktothrix rubescens is* the dominant species in a large number of subalpine European lakes (Briand et al, 2005) but it also proliferates in Southern Italy (Mattei and Stefanelli, 2008). *P. rubescens* can produces mainly microcystin, an highly hepatotoxic cyclic heptapeptides, of which about 80 different structures are known. Microcystins differentiate each other on the basis of two L-amino acid (X and Z in figure 1) and of little changing on amino acid structures, such as removal of one or more methyl group. Their name reflects these structural differences, i.e. MC-LR has leucine (L) in position X and arginine (R) in position Z. The toxicity of *P. rubescens* is usually determined by the production of some different microcystins, (Fastner et al., 1999) typically demethylated microcystins, like [D-Asp^3]MC-RR, a microcystin without methyl group in the D-asparagine amino acid. Microcystin toxicity is mainly due to the "Adda" amino acid, tipically produced by cyanobacteria and by few other organisms. The Adda is the functional group that causes toxic effects, because it allows the binding of the toxin to the protein phosphatases causing enzymes inhibition and liver diseases.

In 2009, a massive seasonal bloom of *P. rubescens* was observed in Lake Occhito, a main reservoir used for crop irrigation located in Southern Italy (Fig. 2). The algal bloom was monitored for quantifying algal toxin content in the lake water to verify the possible health risk. Microcystins dissolved into the water were separated from intracellular ones by filtering lake samples. Toxin content was detected and quantified, using high performance liquid chromatography (HPLC-DAD). Acute toxicity tests (24 h exposure) with *Thamnocephalus platyurus* were performed using endocellular and extracellular water extracts.

Fig. 2 – Lake Occhito in Southern Italy

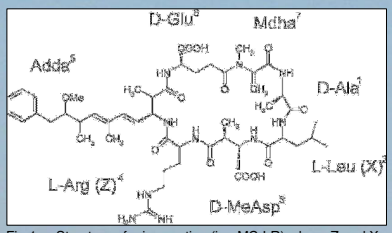

Fig.1 – Structure of microcystins (i.e. MC-LR) where Z and X indicate the position of variable aminoacids

STUDY AREA AND METHODS

Lake Occhito is an artificial lake with a dam built on the Fortore River between Puglia and Molise regions. The reservoir has a volume of 3.33*10^8 m^3, a surface area of 13 km^2 and a maximum depth of 40 m. Its waters are mainly used for irrigation of about 1100 km^2 of agricultural soils, but also as drinking water production for the Foggia Province. Sampling campaigns were undertaken between April 2009 and April 2010, collecting water from lake and from the main tanks of the irrigation networks. Microcystins dissolved into the water were separated from algal cells by filtering (1,2 µm; Whatman GF/C) lake water samples (1 L). After frozen-defrozen treatment over night, intracellular toxins were extracted twice (10 mL, 15 min, room temperature) by sonication of filters into methanol/water (9:1 v/v) and water, respectively. Extracts were concentrated, jointed, and filtered (0,45 µm; Spartan 30/B) to a final volume of 10 mL. Dissolved microcystins were extracted and concentrated using a C_{18} (500 mg/3 mL; Bakerbond SPE) column. Water sample (1 L) was passed through cartridge at a flow rate of 5-10 mL/min. After a washing step (MilliQ/methanol, 80:20), cartridges were eluted with methanol + TFA (trifluoroacetic acid, 0.1 %) and methanol/MilliQ (75:25 v/v) + TFA (0.1 %). Extracts were concentrated under N_2 flux (Turbovap II, FKV) to a final volume of 1 mL. Extracts were analysed with a Diode-Array 1050 HPLC (Hewlett Packard, USA) equipped with an automatic sampler and a column Luna (5 µm, C18(2), 100 Å, 250x3.0 mm; Phenomenex, USA). For quantitative analysis, external standard method was used at a wavelength of 238 nm. Calibration curve was obtained using a linear regression analysis in a range of concentration from 0.2 to 1.6 µg/mL. LOQ was 0.1 and 0.3 µg/L for dissolved and endocellular microcystins, respectively (Guzzella et al, 2010). Toxicity test were carried out with the Thamnotoxkit FTM (MicroBioTests Inc, Belgium). Lethal effects of microcystins after acute exposure (24 h) were evaluated with the anostracan crustacean *Thamnocephalus platyurus*. Dilution series of samples were tested starting from concentrated extracts. A dose-response relationship was used to calculate the 24hEC$_{50}$: the microcystin concentration able to inhibit 50 % of *T. platyurus* nauplii after 24 h of exposure.

RESULTS AND DISCUSSION

[D-Asp^3] microcystin-RR was the only microcystin detected in the lake samples. The microcystin was identified by comparison of retention time and UV adsorbance spectrum with a certified standard (Fig. 3). Microcystin extracellular concentration in the lake was never above the WHO limits for drinking waters (1 µg/L). Maximum level of dissolved microcystin was 0.7 µg/L on April 2009 sample at S06 sampling station (Fig. 4). In the same sample the highest endocellular concentration (30.8 µg/L) of [D-Asp^3]MC-RR was measured. As predictable, endocellular toxin was 92-97% of the total microcystin content. Results showed that toxin was mainly present into the cells; after algal cell dying, microcystin was released into the medium where dilution in water and biodegradation reduce its concentration. So that the extracellular content of microcystin depends on the physiological state of the alga; an aged population of *P. rubescens* is expected to show greater levels of dissolved toxins.

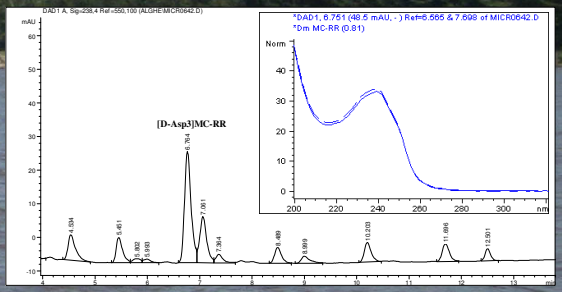

Fig.3 – Chromatogram of endocellular extract and UV absorbance of the peak at 6.75 min compared with [D-Asp^3]MC-RR standard

During 2009, toxin level decreased from June to October and finally concentrations were always near or below the LOQ. The lake water is transported into a 16 km long gallery to the Finocchito tank where it is transferred to the distribution net. At Finocchito, toxin concentrations were always lower than that detected in the lake; during 2009, microcystins were about 50 % of those measured at E11 sampling site, near to the dam where is located the water plug. The difference between lake water results and those of the Finocchito tanks depends also on the fact that the water for the irrigation distribution is taken at 10-25 m of depth, while the algal density usually decreases below the 10 m of depth. Accordingly to the life cycle of *P. rubescens*, concentrations of the endocellular microcystin at Finocchito on January 2010 increased again reaching the maximum value of 1.80 µg/L , while dissolved concentrations were always lower the LOQ.

The lake sample collected on April 2010 showed low levels of toxins in endocellular and extracellular extracts. On the basis of detected concentrations, an alert threshold of total microcystin concentrations > 10 µg/L was proposed and only samples that overcame this value were tested for their toxicological effects. *T. platyurus* is an anostracan crostacean, very sensitive to algal toxins. Only the April 2009 sample collected in the lake and concentrated 10 x was positive to the toxicity test showing a 12.5 % of inhibition at 24 h exposition. This showed that acute toxic effects on *T. platyurus* become measurable when the [D-Asp^3]MC-RR concentration overcome a concentration of 300 µg/L. The results are in agreement with the dose-response curve obtained starting from a standard solutions of [D-Asp^3]MC-RR (Fig. 5); in this case the 24hEC$_{50}$ was calculated in 845 µg/L. [D-Asp^3]MC-RR has a toxicity between the toxicity of MC-RR (2130 µg/L) and that of MC-LR (100 µg/L) (Toronke et al. 2000). None samples collected on June 2009 showed acute toxic effects with the Thamnotoxkit.

CONCLUSION

P. rubescens bloom on April 2009 into Lake Occhito confirms diffusion of this cyanobacterium in Southern Italy. Microcystins production can be effectively monitored using HPLC method that allows to detect WHO limits within the 24 h since sampling. *T. platyurus* test is useful to evaluate the potential toxicity of [D-Asp^3]MC-RR.

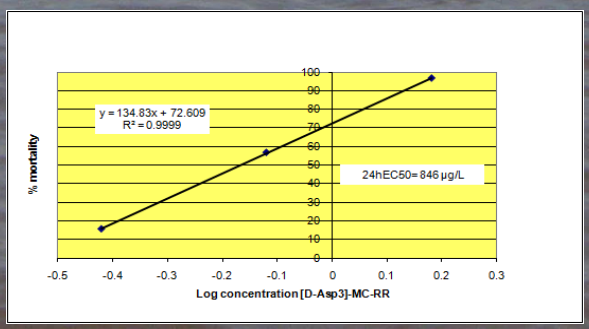

Fig.5 – Dose-response curve of *T. platyurus* to [D-Asp^3]MC-RR

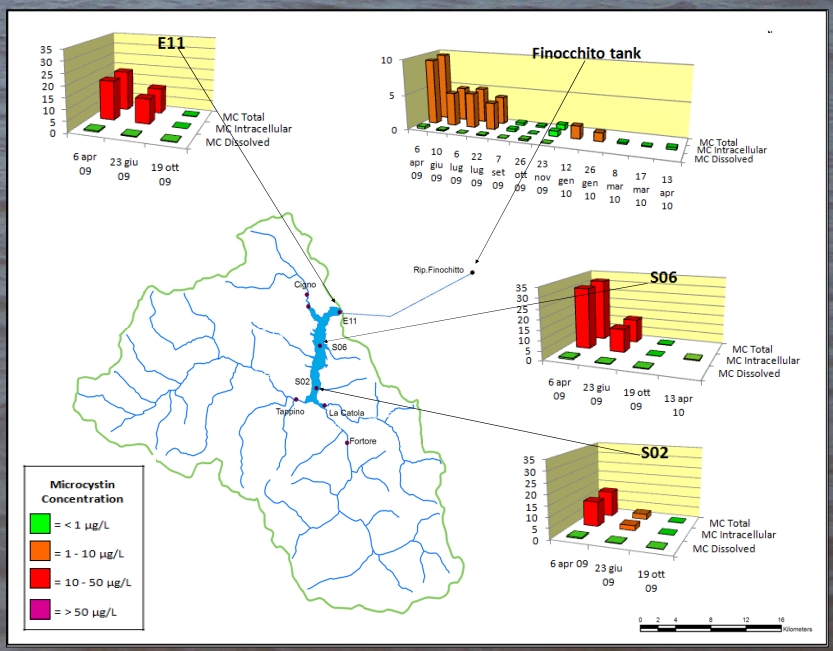

Fig.4 – Drainage basin of Lake Occhito and microcystin concentration (µg/L) in the Lake and in Finocchito tank

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