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Short Communication

Monitoring and Evaluation of Contamination by microcystins in Lake Occhito

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Abstract

The occurrence of harmful cyanobacterial blooms in surface waters is often associated with the production of a variety of cyanotoxins, which are able to target specific human organs where they badly act. During this project, as a monitoring purpose, water samples were collected from both Lake Occhito, where an algal bloom (*Planktothrix rubescens*) was observed in 2009, and three tanks, acting as hydraulic junction (Finocchito, Pozzilli and Tavoliere). Next, the characterization of the main cyanobacteria was performed, in order to search cyanotoxins such as microcystins, followed by the quantitative determination by ELISA and HPLC-MS/MS technique. As a result, we observed that *Planktothrix rubescens*, currently known as a producer of microcystins, presented a flowering of 1.3 x 10^6 cells l^{-1} in March 2016. Moreover, the screening ELISA detected concentrations of microcystin LR over the limit of detection in 41samples, with a concentration range between 0.1 to 2.1 ng mL^{-1}. Subsequently, concentrations above the limit of quantification for the only microcystin LR were confirmed by HPLC-MS/MS technique in 37 water samples, with concentrations range between 0.007 to 0.18 ng mL^{-1}. However, many environmental and epidemiological studies are needed to define the risk assessment related to toxic eutrophication events in Italy.

INTRODUCTION

The contamination of water bodies with low hydrodynamics due to organic and inorganic pollutants induces a biological response known as eutrophication, which leads to an increase in the algal biomass [1-4]. In fact, the inevitable consequence of excessive nutrients in a lacustrine ecosystem is the growth of toxic species of cyanobacteria, which may cause contamination and accumulation of algal toxins [5-8]. In general, contamination by the toxin producing cyanobacteria is an example of how an environmental issue might also become a problem for human safety. The most frequently detected toxins are the microcystins (MCs), a family of more than 90 toxic variants (MC-LR, MC-RR, MC-YR etc.), known as being hepatotoxic, tumor promoters and probable human carcinogens. MCs have a common structure (Figure 1), containing three D-amino acids (alanine, β-linked *erythroid*-β-methyl aspartic acid, α-linked glutamic acid), two variable L-amino acids, R1 and R2, and two unusual amino acids, *N*-methyldehydroalanine (Mdha) and 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid (Adda) [9-11]. The main aim of this project was to determine the content of MCs in water samples from Lake Occhito and from the three tanks (Finocchito, Pozzilli and Tavoliere) which act as hydraulic junction and situated between two Italian regions (Molise and Puglia). In March 2009, because of intense rainfalls, the flooding of the river for to reoccurred and the opening of Lake Occhito bulk heads was necessary, with the inevitable flowing into the sea of water containing the algae of *Planktothrix rubescens* species. Considering the potential risk due to the diffusion of *Planktothrix rubescens* and the sea contamination, we decided to carry out the characterization of cyanobacteria such as MCs type and consequently the quantification by ELISA and HPLC-MS/MS.

MATERIALS AND METHODS

Chemical and reagents

Abraxis (Warminster, PA, USA) supplied a mixed solution of a single congener of microcystins (MC-LR, MC-LY, MC-LA, MC-YR, MC-RR, MC-LF, MC-LW) at the concentration of 10 μg mL^{-1} and

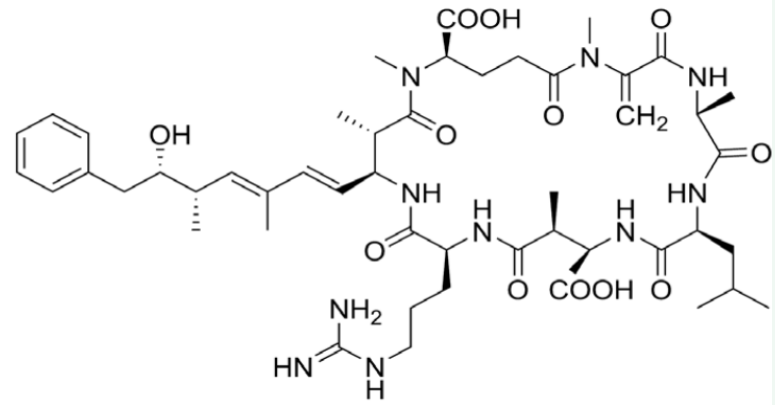

Figure 1 Chemical structure of MC LR.

nodularins (NOD) at 10 µg mL^{-1}. HPLC grade methanol (MeOH) and the analytical standard of leucine enkephalin (ENK) were purchased from Sigma-Aldrich (Milan, Italy). HPLC grade water was produced using a MilliQ system. Waters (Milford, MA, USA) provided HLB SPE Waters OASIS cartridges. All the reference materials were of analytical grade purity. ELISA analyses were performed using the Microcystins Plate EnviroGard Kit (Strategic Diagnostics Inc., Newark, DE, USA). Finally, for the characterization of cyanobacteria, an inverted microscope (LeitzLabovert FS; Zeiss Axiovert 100) was used.

Sampling

Lake Occhito, created for drinking purposes, is the largest artificial reservoir in Italy, gathering the water coming from the River Fortore. Its mean depth is 90 m, its surface area is 13km^2 and its long axis is 12km. In our research, a total of 111 samples were collected, using a 2.5 l Ruttner bottle, from June 2015 to May 2016. The samples were withdrawn from three different points of the lake (lake junction, lake center, lake tributary), and for each point samples were taken from both surface water and deep water (at a depth of 10-15 meters). Furthermore, samples from the surface water of the three tanks (Tavoliere, Finocchito, Vasca D) were collected. The map of the sampling points of both Lake Occhito and the three tanks is shown in Figure (2).

Characterization, ELISA and HPLC- MS/MS methods

Characterization: For the characterization of cyanobacteria, within 24 hours of collection, lake samples were put in the settling chambers, where they lied for at least 48 hours [12,13]. Subsequently, for the identification of cyanobacteria, a microscopic examination was carried out using the inverted microscope LeitzLabovert.

ELISA method: Lake samples were frozen at -20°C and thawed just before the analysis by ELISA test for MCs detection. This procedure favored cell lysis and consequently the possible release of MCs by the cyanobacteria, if present. The analytical method to determine microcystins in lake water samples was previously validated according to the decision 2002/657/CEE [14].

HPLC-MS/MS method: Lake samples, previously analyzed by ELISA test, were also analyzed using HPLC-ESI-MS/MS instrumental method [15]. The pre-analytical procedure for lake water samples is quite similar than the protocol for a neurotoxin domoic acid in water samples [16]. An aliquot of lake water was acidified with 2% of formic acid and was then spiked with 50 mg of the ENK internal standard in a 500 mL volumetric flask; the sample was then brought to volume with sample. Cyanotoxins were then extracted by solid phase extraction (SPE) using Oasis HLB cartridge (6 cc, 200 mg). The SPE column was conditioned with 5mL of MeOH followed by 5 mL of acidified ultra pure water. The water sample, previously prepared as above, was passed through the SPE column at 40/50 mL min^{-1} using a vacuum manifold, followed by 5mL of ultrapure water. Cyanotoxins, adsorbed on the cartridge, were eluted drop wise with 5 mL of MeOH into a glass vial. Then, the sample was diluted 1:5 with ultrapure water in an amber vial by auto sampler and analyzed using HPLC-MS/MS with electro spray ionization interface (ESI) and triple quadrupole.

RESULTS AND DISCUSSION

The presence of some species of diatoms, Coniugatoficee, Cloroficee, dinoflagellates and cyanobacteria, was detected. The latter, accounting for 44.3% of the total, were made up of *Oscillatoria brevis, Oscillatoria splendens, Oscillatoria putrid, Pseudolyngbya limnetic Limnothrixredeckei, Planktothrix rubescens, Aphanizomenonflos-aquae, Chrysosporum ovalisporum, Dactylococcopsis fascicularis.* The water lake temperature was between 20°C and 25°C in the warmer seasons, while during the cold season it was between 10°C and 15°C. *Planktothrix rubescens,* the only species currently known as a producer of microcystins, presented a flowering of 1.3 x 10^6 cells l^{-1} in March 2016. Therefore, it was deducted that *Planktothrix rubescens* formation is favored during cold seasons. For the analysis of the results, it is important to consider that the upper limit of the concentrations of MCs in drinking water must be equal to 1 µg L^{-1}, as dictated by the WHO (World Health Organization) [17]. In addition, the guidelines of the EPA (Environmental Protection Agency USA) [18] have established for MCs a TDI limit (tolerable daily intake) for humans, pointing respectively 0.006 mg kg-1 bw day-1 for acute injury and 0.003 mg kg-1 bw day-1 for chronic damage. The results of the ELISA method,

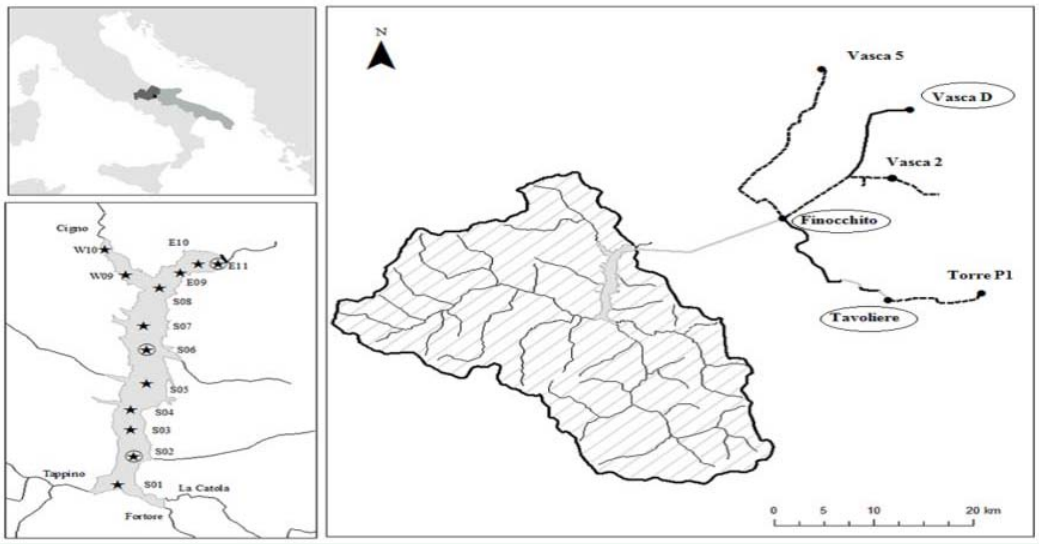

Figure 2 Map of the sampling points of the Occhito basin and collection tanks.

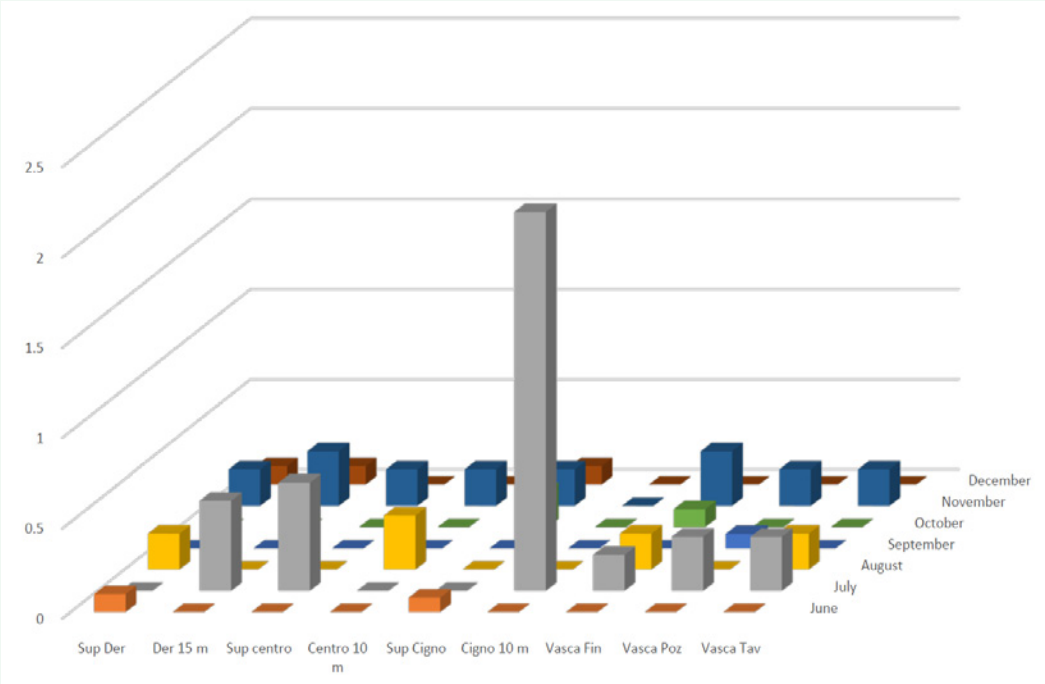

Figure 3 MCs concentrations (ng ml^{-1}) in water samples carried out in 2015, after ELISA test. The samples were collected at the lake junction, lake center, lake tributary and the three tanks.

referred to the samples taken during a timeframe of 15 months, between March 2015 and May 2016, have shown that concentrations of MCs were over the limit of detection in 41 samples, with a concentration range between 0.1 to 2.1 ng mL^{-1}. MCs concentrations were related with the month in which the water lake samples were collected (Figure 3,4). Concentrations above the limit of quantification, for the only MC-LR, were confirmed by HPLC-MS/MS technique for 37 water samples, with concentrations range between 0.007 to 0.18 ng mL^{-1}.Chromatograms related to the internal standard, MC-LR and MC-RR, obtained from a lake sample taken in November, are shown in Figure (5).

CONCLUSION

Considering the MCs extracellular contents, the toxin detected in waters in most samples did not correlate with the cyanobacterial cell density [19]. This lack of correlation could be expected considering the variety of water bodies, but it could also depend on the phase of the bloomorit was the result of both adsorption and biodegradation. Therefore, biodegradation would appear to be the main fate for most cyanotoxins in aquatic systems and the

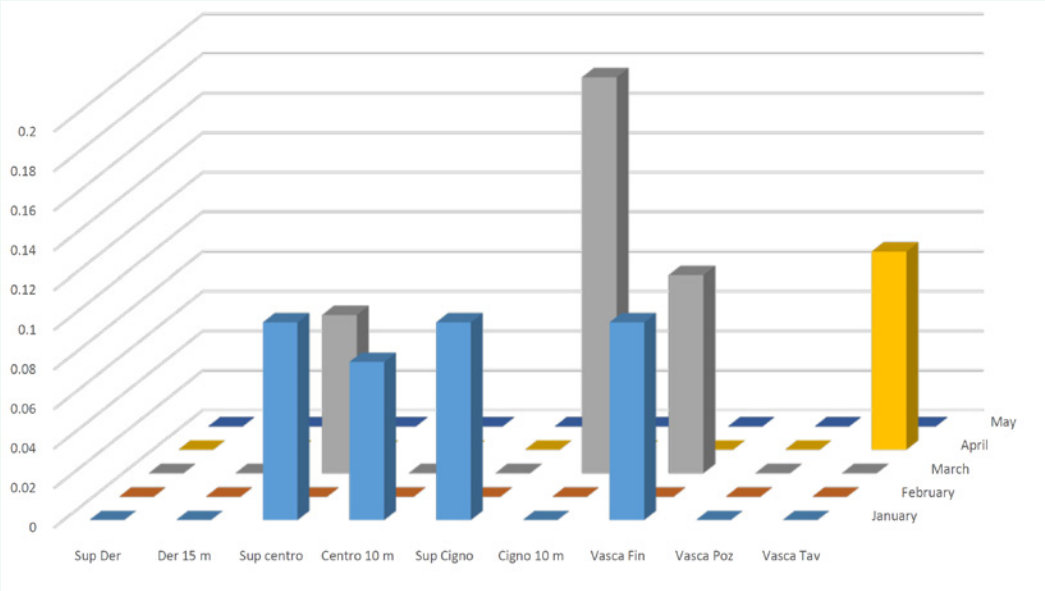

Figure 4 MCs concentrations (ng ml^{-1}) in water samples carried out in 2016, after ELISA test. The samples were collected at the lake junction, lake center, lake tributary and the three tanks.

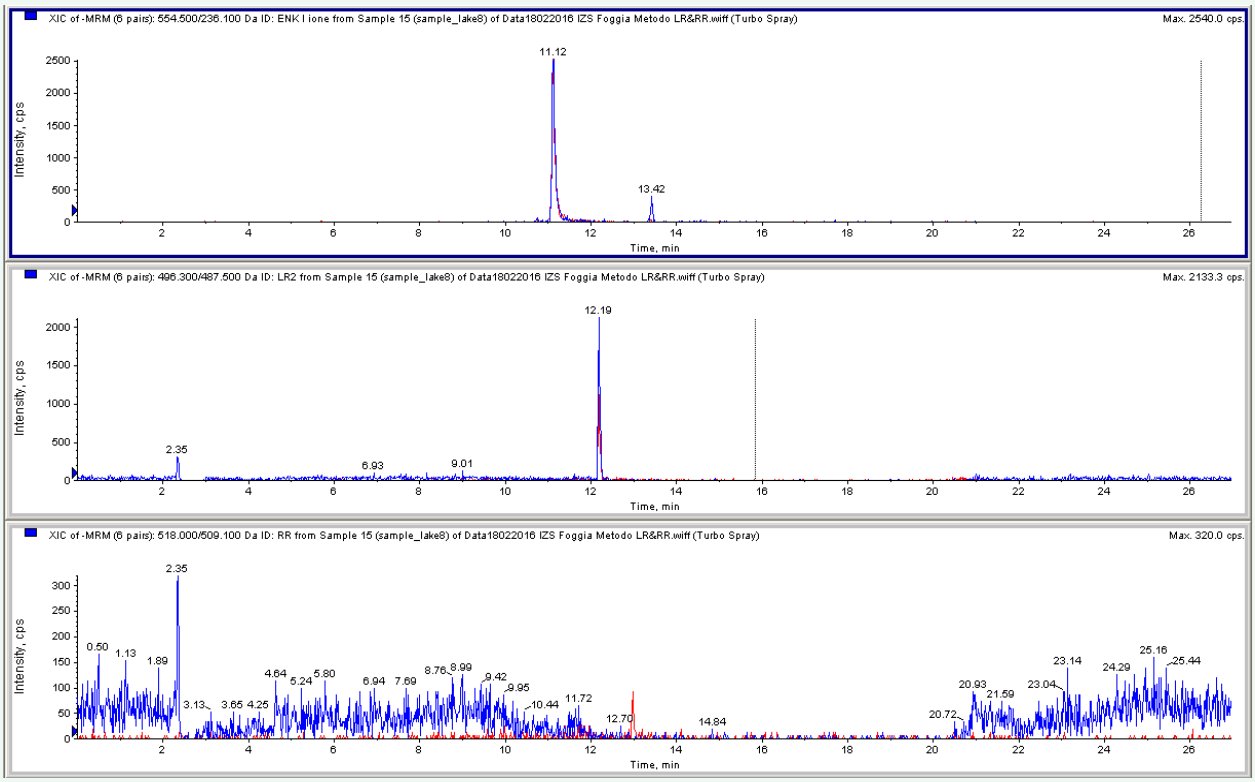

Figure 5 Chromatograms relative to the internal standard transactions (first chromatogram), MC LR (second chromatogram) and MC RR (third chromatogram), obtained by a lake sample taken on November.

relative performance of this process would be very site specific and dependent upon local sediment characteristics and microbial activity [20]. In fact, sampling carried out in the period of decline, with the lowering of cell numbers and the corresponding release of toxins in waters during cell lysis, could account for the extracellular MCs levels [21]. In regards to the results of the monitoring, we found that some values of MCs concentrations in Lake Occhito, after ELISA test, were over the TDI values suggested from EPA. By contrast, HPLC-MS/MS method did not confirm values of MCs concentration that resulted over TDI

guidelines after ELISA test. It can be deduced that with the ELISA technique we detected higher concentrations as the sum of MCs. The chromatography coupled with mass spectrometry, however, quantifies separately the various isomers of MCs present in the samples. Therefore, very low values detected by ELISA technique in most cases were not confirmed by HPLC-MS/MS technique. In conclusion, more extended environmental and epidemiological studies are needed to define the risk assessment related to toxic eutrophication events in Italy, but some possible major routes for human exposure may be considered.

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