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Determination of microcystins and nodularin (cyanobacterial toxins) in water by LC–MS/MS. Monitoring of Lake Marathonas, a water reservoir of Athens, Greece

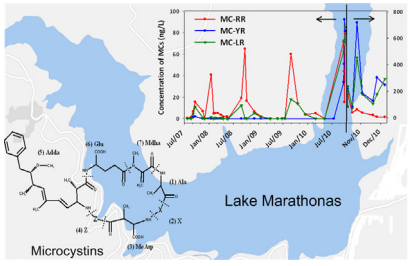
Triantafyllos Kaloudis [a,*], Sevasti-Kiriaki Zervou [b], Katerina Tsimeli [b], Theodoros M. Triantis [b], Theodora Fotiou [b], Anastasia Hiskia [b,*]

[a] Quality Control Department, Athens Water Supply and Sewerage Company (EYDAP SA), Oropou 156, 11146 Galatsi, Athens, Greece
[b] Laboratory of Catalytic – Photocatalytic Processes (Solar Energy – Environment), Institute of Advanced Materials, Physicochemical Processes, Nanotechnology & Microsystems, National Center for Scientific Research "Demokritos", Patriarchou Grigoriou & Neapoleos, 15310 Agia Paraskevi, Athens, Greece

HIGHLIGHTS

- Method for the determination of MCs and NOD in water by SPE and LC–ESI-MS/MS.
- New patterns of MC-LA fragmentation have been observed.
- Method validation by assessment of specificity, linearity, accuracy and precision.
- MCs monitoring in Lake Marathonas (July 07–December 10).
- MC-LR, MC-RR and MC-YR were detected.

GRAPHICAL ABSTRACT



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ABSTRACT

A method for the determination of the hepatotoxic cyanotoxins microcystins (MCs, i.e. MC-LR, MC-RR, MC-YR, MC-LA) and nodularin (NOD) in water was developed using liquid chromatography with electrospray ionization triple quadrupole mass spectrometry (LC–ESI-MS/MS) after solid phase extraction (SPE). New patterns of fragmentation of MC-LA were observed under the experimental conditions used. The method was fully validated to meet accreditation criteria. Mean recoveries at three concentration levels (0.006, 0.1 and 1 μg L^{-1}) ranged between 70 and 114% with %RSD values generally below 20%. Detection limits were 2 ng L^{-1} for all hepatotoxins. The method was applied to study the occurrence of MCs and NOD in Lake Marathonas, a water reservoir of Athens, over a period from July 2007 to December 2010. The protein phosphatase inhibition assay (PPIA) was additionally used for fast screening of samples. MC-YR, MC-LR and MC-RR were detected and found to vary seasonally with consistent peaks during early autumn, having maximum concentrations of 717, 451 and 174 ng L^{-1}, respectively. The results of this study constitute the first report on the presence, concentration levels and seasonal variations of MCs in Lake Marathonas. None of the target cyanotoxins were detected in treated drinking water samples during the period of the study.

1. Introduction

Cyanobacteria are a group of prokaryotic organisms that occur worldwide in fresh, brackish and saline waters. Under favorable conditions, cyanobacteria can multiply rapidly to form blooms in water [1]. A lot of cyanobacteria species and strains are toxigenic,

* Corresponding authors. Tel.: +30 2106503643.
E-mail address: hiskia@chem.demokritos.gr (A. Hiskia).

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i.e. able to produce a diverse range of potent cyanotoxins [2,3]. Toxic cyanobacterial blooms can be especially detrimental to human and animal health, aquatic habitats, aquaculture enterprises and tourism economy [4–6].

Microcystins (MCs) comprise a large group of cyanotoxins with cyclic heptapeptide structure and hepatotoxic activity that are produced by cyanobacteria belonging to the genera *Microcystis*, *Anabaena*, *Plankothrix*, *Nostoc* and *Snowella* [7,8]. MCs seem to be the most widespread cyanotoxins that are present in fresh and brackish waters [9]. To date there are over 89 structurally characterized MC variants [10]. The chemical structure of MCs is shown in Fig. 1a. MC variants are named as MC-XZ where X and Z are two variable L-amino acids; X is commonly leucine (L), arginine (R), or tyrosine (Y) and Z arginine (R) or alanine (A). Nodularins (Fig. 1b) are similar to MCs hepatotoxic cyclic pentapeptides that are considered to be produced solely by cyanobacterial strains of the brackish/marine genus *Nodularia* [11,12]. However, recently nodularin (NOD) has been detected in freshwater organisms where it is accumulated from freshwater benthic cyanobacterial mass [13]. NOD is the main variant of nodularins, having arginine as the Z variable amino acid.

In recent years there is a growing concern regarding the health effects of MCs and NOD, because they are hepatotoxic and act as tumor promoters, through the inhibition of protein phosphatases 1 and 2A, which play a key role in cell regulation [14,15]. The unique Adda amino acid in their structure (Fig. 1a and b) is largely responsible for their toxicity [16,17].

Eutrophic water conditions combined with surface water temperatures of 15–30 °C, that are frequently encountered in Mediterranean waterbodies, favor bloom formations [18]. Cyanobacterial water blooms have been observed in Greek surface waters [1,19–24] and cyanobacterial genera (*Microcystis*, *Anabaena*, *Anabaenopsis*, *Aphanizomenon*, *Cylindrospermopsis*, *Planktothrix*, *Limnothrix*) with known toxin producing taxa have been identified [20,21,24–26]. MC-LR and MC-RR were the most abundant variants detected in biomass samples during these blooms, while MC-LA, MC-YR and demethylated derivatives of microcystins-LR and -RR have also been detected [22,25,26]. However, limited quantitative data have been reported, using analytical techniques with low confirmatory potential.

In a preliminary study (May 2007–December 2008) in Lake Marathonas, a drinking water reservoir in the area of Athens, microcystins-RR, -LR and -YR were detected at concentrations up to 0.060, 0.029 and 0.009 $\mu g\,L^{-1}$, respectively [27]. The diversity of cyanobacterial phylotypes in Lake Marathonas was also investigated in October 2007 and September 2008, to reveal phylotypes of potentially toxin producing strain of *Microcystis aeruginosa* [28]. Although no cyanotoxins were detected in the treated drinking water of Athens [29] the occurrence of potentially MC producing cyanobacteria has triggered the initiation of a monitoring program for MCs in the lake. The World Health Organization (WHO) has proposed a provisional limit of 1 $\mu g\,L^{-1}$ in drinking water for the most common MC variant, MC-LR [30]. Following this guideline, many countries have established maximum acceptable concentrations (MAC) for MCs in drinking water [31]. Documentation of conformance to these limits necessitates the development and thorough validation of sensitive and accurate analytical methods. These methods should be able to separate, identify, and quantify individual MCs since MC variants differ in toxicity. In the past, methods based on reversed-phase high-performance liquid chromatography with diode array or electrochemical detection have been proposed [32–34]. Moreover, bioassays, enzyme linked immunosorbent assays (ELISA) and protein phosphatase inhibition assays (PPIA) have been applied for the detection of MCs [35,36]. Especially, PPIA that is now available as a kit can be used as a fast screening tool, although data regarding the applicability of this kit for screening have not yet been published in the literature. PPIA provides important toxicological information regarding the bioactivity of MCs and NOD, since detection is based on functional activity rather than on recognition of chemical structure. However, methods created on the basis of the detection cited above do not offer unequivocal and definitive analytes identification, which is a necessary feature in confirmatory assays.

The advent of the electrospray ionization (ESI) source coupled to LC has provided a facile method of ionizing non-volatile substances, such as cyanotoxins, for mass spectrometry detection (LC–ESI-MS) [37,38]. Although, the successful characterization of several cyclic peptides has been achieved by this approach, the methodology needed to be improved for the confirmation of MCs in complex matrices. During the last years, LC–ESI-MS/MS methods are used for achieving greater sensitivity and specificity [39–42]. Tandem mass spectrometry allows the signal-to-noise ratio to be substantially improved in the detection of cyclic peptide cyanotoxins or selected MC variants and it is also possible to combine the improved selectivity of LC–MS/MS with increased sensitivity by using multiple-reaction monitoring (MRM) in which selective transitions are monitored. However, the reported validation data are still limited and there is not yet a standardized method for this analysis using LC–MS/MS.

The present study had two main objectives. The first one was the development and validation of a method suitable for detection, identification and quantitative determination of the most common cyanobacterial hepatotoxins in water at low $ng\,L^{-1}$ level. The scope of method validation covers the requirements of ISO 17025 accreditation and the criteria of European guidelines [43,44]. As the presented method is already included in the scope of accreditation of the NCSR "Demokritos" laboratory by the Hellenic Accreditation System (ESYD) that has international recognition, it could serve as a rough guide for other laboratories wishing to be accredited for the analysis of MCs in water. Furthermore, the validation data provided could support the development of European or international standards for the analysis of MCs in water based on LC–MS/MS that is currently the most reliable method for sensitive quantitative analysis of these compounds. The second objective was to study the occurrence of MCs in Lake Marathonas, a drinking water reservoir of the area of Athens that seasonally suffers from cyanobacterial blooms, while no data regarding the presence of MCs have been reported in the literature. Moreover, results using a new commercially available PPIA kit in real samples are also presented and discussed in terms of its applicability for screening of the target hepatotoxins. This study constitutes the first report on the presence, concentration levels and seasonal variations of MCs in Lake Marathonas.

2. Experimental

2.1. Study area description and sample collection

Lake Marathonas is located north-east of Athens and has a surface area of 2.4 square kilometers, a watershed of 132 square kilometers, a maximum capacity of 41 million m^3 of water and an operational volume of 34 million m^3. The reservoir operates as a backup source for the water supply system of the greater Attica region and as a primary regulating reservoir.

Samples of water from Lake Marathonas were collected from July 2007 to December 2010. Sampling sites are described in Fig. 2.

2.2. Chemicals and apparatus

Certified standard solutions (~10 $mg\,L^{-1}$ in methanol) of MC-LR, MC-RR, MC-YR, MC-LA and NOD were supplied by DHI Water & Environment (Copenhagen, Denmark) and were stored at −20 °C.