Microchemical Journal 130 (2017) 329–335

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

First evidence of MC-HtyR associated to a *Plankthothrix rubescens* blooming in an Italian lake based on a LC-MS method for routinely analysis of twelve microcystins in freshwaters

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ARTICLE INFO

Article history:
Received 1 August 2016
Received in revised form 11 October 2016
Accepted 11 October 2016
Available online 13 October 2016

Keywords:
Cyanobacteria
Planktothrix rubescens
Cyanotoxins
LC-MS
Microcystin-HtyR
Freshwaters

ABSTRACT

A recent Italian regulatory level for microcystins (MCs) in drinking water was set at 1.0 μg/L, intended as the sum of all variants that can be determined using commercially available standards. A selective multi-residue method for analyzing twelve variants of MCs (MC-RR, MC-YR, MC-LR, MC-LA, MC-LW, MC-LF, MC-LY, [D-Asp^3]-MC-RR, [D-Asp^3]-MC-LR, MC-WR, MC-HilR, MC-HtyR) in surface and drinking waters was optimized and validated in accordance with the Italian implementation of the Drinking Water Directive 98/83/EC. The proposed method was robust as proved by using nodularin as quality control, with inter-matrices reproducibility better than 17% and matrix effects not significantly dependent among different water samples. LODs were in the range of 0.003–0.030 μg/L for all the analytes, allowing the quantitative analysis of selected MCs at levels lower than 1/10 of the proposed new Italian parametric values. The method was tailored for the routine analysis of MCs in the frame of risk management related to MCs production during toxic algal blooms.

The optimized analytical protocol was then applied to the analysis of water samples collected in Occhito Lake (Apulia, Italy), used as a source of drinking water, after an extraordinary bloom of *Planktothrix rubescens*. In October 2010, the presence of MC-HtyR was detected here with maximum concentration level of 0.025 μg/L. To our knowledge, this is the first report on MC-HtyR presence, obtained with a confirmatory method, associated to a *P. rubescens* bloom in surface waters.

1. Introduction

Cyanobacteria (blue-green algae) are microscopic photosynthetic organisms that form a common and naturally occurring component of most aquatic ecosystems. These photosynthetic prokaryotic organisms can cause adverse effects when present in water systems. Of the approximately 150 known genera of cyanobacteria, only about 40 include species involved in production of cyanotoxins, so they are divided into producers and non-producers [1]. These toxins have caused mortality in animals and illness in humans and have a deleterious effect upon drinking water quality [1]. The most common cyanobacterial genera, particularly in freshwater, include *Microcystis*, *Cylindrospermopsis*, *Planktothrix*, *Anabaena* and *Anabaenopsis*, while microcystins (MCs) and cylindrospermopsin are the most widespread algal toxins in Europe.

MCs are a group of hepatotoxic cyclic heptapeptides produced by a number of cyanobacterial genera. Structurally, all MCs consist of the generalized structure cyclo(-D-Ala^1-X^2-D-MAsp^3-Y^4-Adda^5-D-Glu^6-Mdha^7-), where X and Y are variable L-amino acids, D-MeAsp is D-erythro-methylaspartic acid and Mdha is *N*-methyldehydroalanine. Adda, i.e. (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, is the peculiar amino acid charged for the toxicity of this class of algal toxins. Substitutions of the variable L-amino acids at positions 2 and 4 give rise to at least 21 known primary MC analogues and alterations in the other constituent amino acids result in >100 MCs reported to date.

MC-HtyR is a variant containing a homotyrosine (Hty) in position 2 and arginine (R) in position 4 (Fig. 1). While demethylated variants of MC-HtyR have been reported to be produced by *Planktothrix agardhii* [2,3] or *rubescens* [4,5], *Anabaena* spp. [6,7] and *Oscillatoria agardhii* [8], the presence of the methylated congener, presumably associated to *Anabaena* strains, has rarely been reported [6,9]. Toxicological information, similarly to all variants other than MC-LR, is quite scarce and inconsistent. One paper reports that the inhibition ability regarding PP2A of the [Asp^3]-[ADMAdda^{5}Dhb^7]-MC-HtyR was comparable with one related to MC-LR, although without covalent bond [10]. Another [11] describes the PP2A inhibition of the [D-Asp^3]-MC-HtyR, [D-Asp^3, (*Z*)-Dhb^7]-MC-HtyR and [D-Asp^3, (E)-Dhb^7]-MC-HtyR variants, with an estimated equivalent activity of about one third with respect to that of the main congener MC-LR. The same demethylated variants of MC-

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http://dx.doi.org/10.1016/j.microc.2016.10.012
0026-265X/

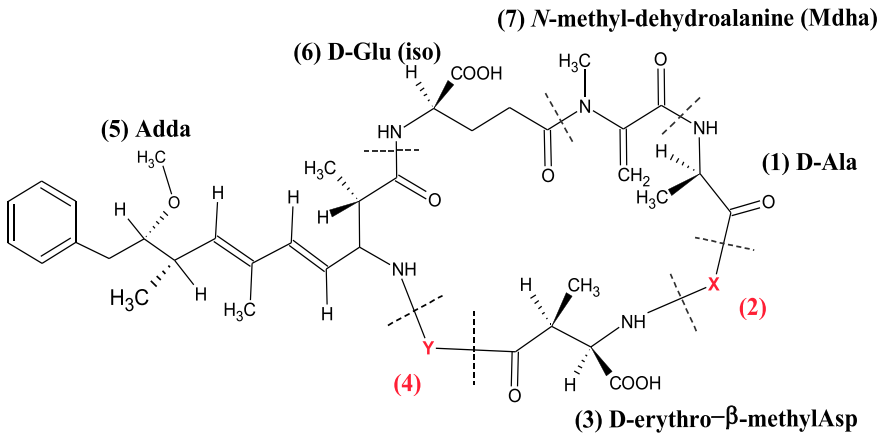

Fig. 1. General structure of microcystins, with X and Y variable amino acids. For MC-HtyR, X = homotyrosine (Hty) and Y = arginine (R).

HtyR were found to be more toxic than MC-LR on cultured rat hepatocytes [12].

In order to protect consumers from the adverse effects of cyanotoxins, the World Health Organization (WHO) has recommended a provisional guideline value of 1 μg/L for MC-LR equivalents in drinking water [13], while no definitive values have been proposed by WHO for other specific MC variants.

After an extraordinary bloom of *Planktothrix rubescens* observed in early 2009 in Occhito Lake, used as a source of drinking water in Southern Italy, an intensive monitoring programme for determining cyanotoxins was conducted over two years [14]. This case study of the Occhito Lake supported the decision to adopt a regulatory parameter for MCs in Italy. Indeed, Italy has recently proposed [15] a regulatory value of 1.0 μg/L for total content (intracellular + extracellular) of MCs in drinking water, intended as the sum of all congeners that can be quantified, and "at least the congeneric elements for which there are commercially available analytical standards must be detected". This new parameter for water quality requires official confirmatory methods that can determine as many variants as possible. Although several analytical methods based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for analysis of MCs in freshwaters are reported in the literature [16–22], to our knowledge no simultaneous confirmatory detection of all commercially available variants (nowadays twelve certified standards) has been described, nor fully validated in surface and drinking waters.

2. Materials and methods

2.1. Chemicals and reagents

The analytical standards MC-RR, MC-YR, MC-LR, MC-LA, MC-LW, MC-LF, MC-LY, [D-Asp^3]-MC-RR, [D-Asp^3]-MC-LR, MC-WR, MC-HilR, MC-HtyR and nodularin, used as surrogate internal standard (IS) for all cyanotoxins, were purchased from Enzo Life Sciences, Inc. (Farmingdale, New York, USA). Microcystins may be toxic if inhaled, absorbed through skin, or swallowed. They may cause irritation to the respiratory tract, eye, skin and liver damage. Dilute solutions should be handled with laboratory gloves, eye protection, and protective clothing. If solids are weighed, this should be done with suitable ventilation and respiratory equipment.

HPLC grade acetonitrile, methanol and dichloromethane were obtained from Carlo Erba, (Milan, Italy). Ultra-pure water, purified to 18.2 MΩ cm, was obtained with a Milli-Q plus PF system from Millipore (Molsheim, France). Trifluoroacetic acid (TFA) was from Sigma-Aldrich (St. Louis, MO, USA). All other solvents and chemicals were of analytical grade (Carlo Erba, Milan, Italy), and were used as supplied.

Extraction cartridges filled with 0.5 g of Carbograph 4 were supplied by LARA (Rome, Italy). A 125 mm diameter Black Ribbon 589 paper filter was purchased from Schleicher & Schuell (Legnano, Italy).

2.2. Standard solutions

The individual stock standard solutions were prepared by dissolving each compound with at least 2 mL of methanol. After preparation, all stock solutions were stored at −18 °C in the dark to minimize analyte degradation. Working standard solutions of analytes and IS were obtained by suitable diluting stock solutions with mobile phases, to a final concentration of 1 μg/mL and 5 μg/mL respectively (Table S1). When unused, all working standard solutions were stored at 4 °C in the dark, and renewed after two working weeks.

2.3. Site description

The Occhito Lake (41°34′48″N 14°56′42″E) is an artificial reservoir in the province of Foggia (Southern Italy, Apulia Region) and is fed by a river and two streams: the Tappino, Cigno and La Catola. The lake area is about 13 km^2 and its depth reaches 70 m. It has a storage capacity of over 330 million m^3 of water, 250 million m^3 of which are used for human consumption [14,23]. About 60% of this water is used for the irrigation of about 1100 km^2 of farmland, while about 20% is destined for the supply of drinking water (about 800,000 inhabitants served) to surrounding municipalities of Foggia Province.

2.4. Water samples collection

During fourteen months of monitoring (January 2010–May 2011), water samples were taken monthly from the Occhito Lake at the following point-stations:

1) intake of the WTP - sampling depth 1 m (raw water, −1 m)
2) close to the dam - sampling depth 34 m (dam, −34 m)
3) close to the dam - sampling depth 16 m (dam, −16 m)
4) close to the dam - sampling depth 1 m (dam, −1 m)
5) central lake - sampling depth 24 m (centre, −24 m)
6) central lake - sampling depth 12 m (centre, −12 m)
7) central lake - sampling depth 1 m (centre, −1 m)

All water samples were transported in ice chests to the laboratories, where they were stored at −18 °C in order to determine the total cyanotoxins content after one cycle of freezing-thawing.