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RESEARCH PAPER

Quantitative determination by screening ELISA and HPLC-MS/MS of microcystins LR, LY, LA, YR, RR, LF, LW, and nodularin in the water of Occhito lake and crops

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Abstract The occurrence of harmful cyanobacterial blooms in surface waters is often accompanied by the production of a variety of cyanotoxins, and these toxins are designed to target in humans specific organs on which they act. When introduced into the soil ecosystem by spray irrigation of crops, they may affect the same molecular pathways in plants having identical or similar target organs, tissues, cells, or biomolecules. There are also several indications that terrestrial plants, including crops, can bioaccumulate cyanotoxins and present, therefore, potential health hazards for humans. During this project, for monitoring purposes, water samples were collected from lake Occhito, in which there was an algal bloom (*Planktothrix rubescens*) in 2009, and from three tanks which acted as hydraulic junctions. In addition, crop samples irrigated with water from the three tanks mentioned above were also picked. Finally, the characterization of principal cyanobacteria was performed, to determine the presence of cyanotoxins such as microcystins and validate a method of screening ELISA for the determination of microcystins in vegetable samples and a confirmatory method by HPLC-ESI-MS/MS.

Keywords *Planktothrix rubescens* · Microcystins · Crops · Lake Occhito · ELISA · HPLC-ESI-MS/MS

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Introduction

The contamination of organic and inorganic pollutants in water bodies with low hydrodynamic causes eutrophication, whose biological response is to increase the algal biomass. In this case, the development of toxic species of cyanobacteria in a lacustrine ecosystem is the inevitable point of arrival of the contribution of excessive nutrients and the cause of contamination and accumulation of algal toxins as a result of algal bloom biomass [1, 2]. In general, contamination by cyano bacteria is an example of how an environmental problem will also become a problem of food safety, both for the presence of cyanotoxins in the waters, and by the fact that some crops could be irrigated with potentially contaminated water from the same biotoxins. The toxins with most frequent detection are the microcistyns (MCs), a family of more than 90 variants of toxins (MC-LR, MC-RR, MC-YR, etc.), known hepatotoxic, tumor promoters, and probable human carcinogens. MCs have a common structure (Fig. 1) containing three D-amino acids (alanine, β-linked *erythro*-β-methylaspartic acid and α-linked glutamic acid), two variable L-amino acids, R1 and R2, and two unusual amino acids, *N*-methyl dehydroalanine (Mdha) and 3-amino-9-methoxy-10-phenyl-2,6,8-trime thyldeca-4,6-dienoic acid (Adda) [3]. Humans can also be exposed to MCs by consumption of vegetables irrigated with water containing toxic cyanobacteria [4]. Indeed, it has been reported that MC-LR could be absorbed by roots and be translocated from roots to shoots in seedlings of agricultural plants [5]. A second study, that used different species too, revealed a high level of MCs accumulation in lettuce [6]

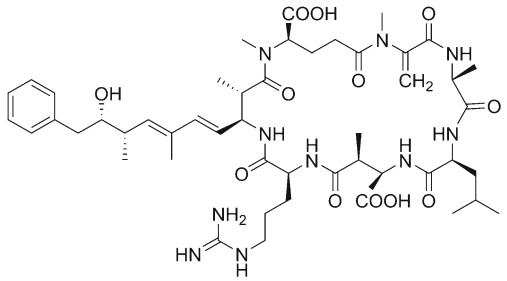

Fig. 1 Chemical structure of MC LR

exceeding the tolerable daily intake of 0.04 μg kg^{-1} of body weight day^{-1} recommended by the World Health Organization [7]. Furthermore, the MCs, by acting as protein phosphatase 1 and 2A inhibitors and inducers of reacting oxygen species (ROS) production, could be involved in several physiological and molecular processes in higher terrestrial plants [8–11]. Therefore, the accumulation of cyanotoxins in the terrestrial food chain is at present remains more worrying, and the proposed quality limits are rare; indeed, many aspects concerning these toxins are particularly scarce, notably those relative to the fate of cyanotoxins toxicity and bioaccumulation on agricultural crops [12–15]. For this reason, the potential risk of these cyanobacteria has led to the development of methods of extraction and analysis of MCs in vegetable samples in order to contemporaneous monitor crops and water lake samples.

Several methods such as bioassay, enzyme-linked immunosorbent assay (ELISA), and liquid chromatography (LC) methods with mass spectrometric or ultra violet (UV) detector have been used for MCs and nodularin detection method for identification and quantification of MC-LR [16–18]. High-performance liquid chromatography (HPLC) with UV detector is an acceptable sensitive method for identification and quantification of MC-LR. Limit of detection (LOD) by UV detector in determination of MCs is reported to be below 1 μg/L, which is suitable for detecting trace amounts of MCs in water samples. Achieving good separation and sensitivity of an HPLC method is related to several parameters such as mobile phase components, HPLC condition, including temperature, flow rate, and column features, e.g., length, silanol activity, and materiel of stationary phase [19].

In the present study, a method of screening test ELISA and a confirmatory method by HPLC-MS/MS were developed and validated to quantify microcystins in water and crop samples.

The main aim of this project was to determine microcystins and nodularin content in water samples from lake Occhito and from three tanks (Finocchito, Pozzilli, and Tavoliere) which act as hydraulic junction, situated at the boundaries between the two Italian regions Molise and Apulia. After the emergence of the March–April 2009, when following heavy rainfall, there were incidents of flooding of the river Fortore, and effluent into the lake, and was made necessary the opening of the Occhito dam bulkheads with spill in sea water containing the algae of *Planktothrix rubescens* species. In addition, crop samples irrigated with water from the three tanks mentioned above were also picked for the quantification of microcystins and nodularin by ELISA and HPLC-MS/MS methods.

Materials and method

Chemical and reagents

A mix solution of 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 nodularins (NOD) at 10 μg mL^{-1} as concentration were supplied by Abraxis (Warminster, PA, USA). All the reference materials were of analytical grade purity. 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 Milli-Q system. HLB SPE Waters OASIS cartridges were purchased by Waters (Milford, MA, USA).

Sampling

Lake Occhito, created for drinking purposes, is the largest artificial reservoir in Italy by damming the River Fortore; its mean depth is 90 m, its surface area is 13 km^2, and its long axis is 12 km. Therefore, a total of 184 samples were collected for the study. Then, 111 water samples from 3 stations in the lake and from the 3 tanks (Tavoliere, Finocchito, Vasca D) were withdrawn monthly, using a 2.5 l Ruttner bottle, from June 2015 to May 2016. Then, 73 crop samples were collected from countryside between San Severo, Lesina, and Lucera. In these countries, agricultural crops were irrigated with water coming from the three tanks mentioned above. In Fig. 2, it shows the map of the sampling points of the Occhito basin and collection tanks. Both water and crop samples were collected in a refrigerator and in the absence of light to prevent microcystins and nodularin degradation [20, 21].

ELISA method

Crop samples analysis An aliquot of vegetable sample (5 g of wet weight) was homogenized and extracted with 10 mL MeOH using vortex system for 10 min. Then, the sample was centrifuged for 5 min at 4000 rpm and the supernatant decanted and filtered on a paper filter. The extraction was repeated on the residue, the sample was centrifuged, and the supernatant filtered on the same filter previously used. The filter were washed three times with 5 mL of MeOH; the obtained solution was gathered, then reduced to a small volume (1–2 mL) by rotary evaporator (Büchi, Switzerland) at 35 °C,

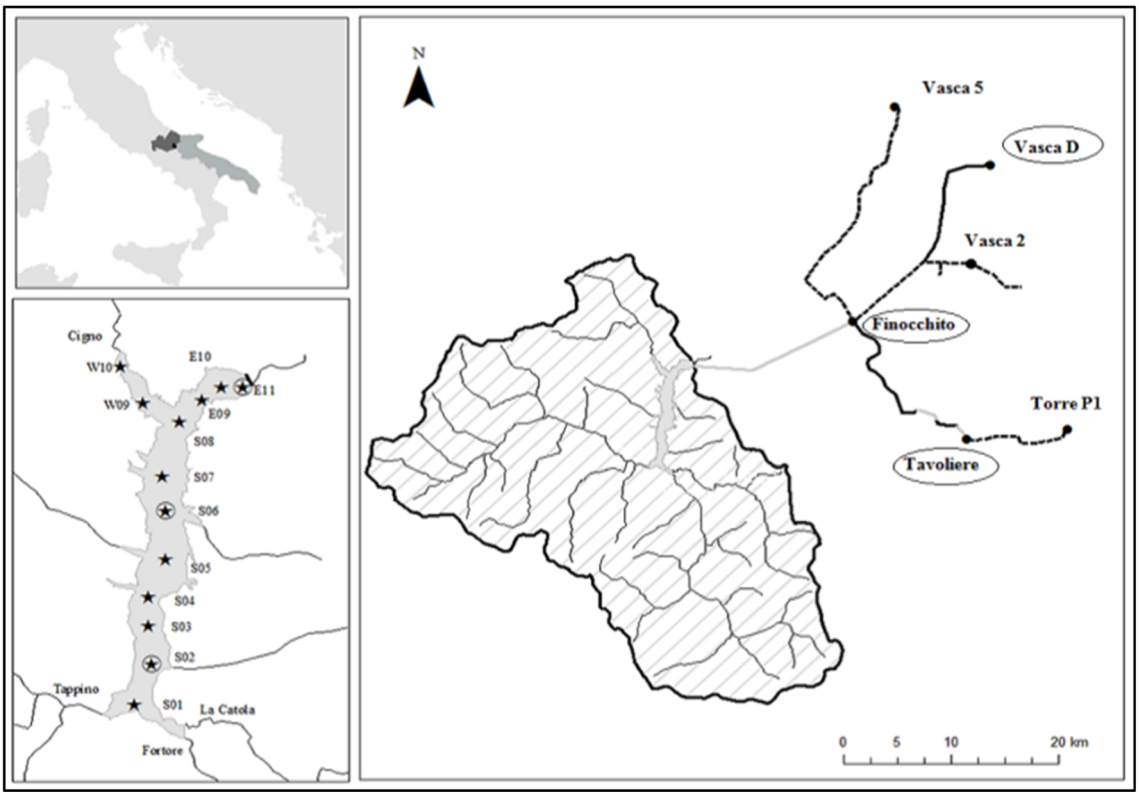

Fig. 2 Map of the sampling points of the Occhito basin and collection tanks

and diluted to 5 mL with MeOH. Furthermore, 1 mL of the extract (corresponding to 1 g) were then added with 1 mL of distilled water and loaded onto a HLB SPE Waters OASIS cartridge, preconditioned with 1 mL MeOH followed by 1 mL of distilled water. The column was washed with 1 mL of 5 % MeOH in distilled water. MCs were eluted by 1 mL of MeOH. The MeOH eluate diluted in 1 mL distilled water was stored at −20 °C until analysis by ELISA test.

ELISA analyses were performed in duplicate using the Microcystins Plate EnviroGard Kit (Strategic Diagnostics Inc., Newark, DE, USA). The EnviroGard Kit is a direct competitive ELISA test for quantitative detection of MCs and NOD (linear range 0.1, 0.2, 0.4, 0.56, 0.8, 1.6 ng mL^{-1}). It does not differentiate between microcystin variants but it detects them as a summation; therefore, validation tests were conducted by adding a standard of a microcystin isomer, with known concentration, on a blank sample. The concentrations at 50 % inhibition (50 % Bo) for these compounds are MC-LR 0.31 ppb, MC-RR 0.32 ppb, and MC-YR 0.38 ppb. The final absorbances in the microplate of the kit were measured at 450 nm with an Anthos 2010 spectrophotometer (Anthos-Labtech, Salzburg, Austria). ELISA antibodies were successfully used to detect MCs in organic matrices. In this study, validation tests were performed in order to prevent false positive/negative results, so vegetable blank samples were spiked with MC-LR standard at 4, 8, and 16 ng g^{-1} to evaluate specificity and precision as described in decision 2002/657/CEE.

Water samples analysis 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 the lake water samples was previously validated according to the decision 2002/657/CEE [22].

HPLC-ESI-MS/MS method

Crop samples processing and analysis A similar analytical procedure was applied for the determination of microcystins LR, LY, LA, YR, RR, LF, LW, and nodularin in crop samples using the instrumental HPLC-MS/MS method previously developed by Gambaro et al. Briefly, an Agilent 1100 Series HPLC System (Agilent, Waldbronn, Germany) was coupled with an ESI electrospray ion source and an API 4000 Triple Quadrupole Mass Spectrometer (Applied Biosystem, Concord, Ontario, Canada). Chromatographic performance was performed using a 4.6 × 150 mm Zorbax Extend-C18 column with mobile phase gradient elution consisting of water with 19 mM NH_4OH as eluent A and MeOH/acetonitrile

Table 1 *T* test for paired medium data in the first session

	Variable 1	Variable 2
Average	0.633986928	0.328808446
Variance	0.003552582	0.000388708
Remarks	10	10
Pearson correlation	−0.127627751	
Hypothesized difference medium	0	
gdl	9	
STAT *t*	14.81859716	
P (*T*≤*t*) one-tail	6.26892E-08	
t Critical one-tail	1.833112933	
P (*T*≤*t*) two-tail	1.25378E-07	
t Critical two-tail	2.262157163	

(80:20) as eluent B. A binary elution program at a flow rate of 0.5 mL min^{-1} was used as follows: 0–1 min, 0 % eluent B; 1–3 min, 45 % eluent B; 7 min, 90 % eluent B; 9–15 min, 100 % eluent B; 17–27 min, equilibration with 0 % eluent B; 80 μL of sample was injected for analysis. The ESI ion source was operated in negative polarity, and the data were acquired in multiple reaction mode (MRM) enabling highly selective and sensitive detection of selected fragments. The quantification was performed using ENK as internal standard in order to correct instrumental fluctuation. By adding ENK at the beginning of the extraction procedure, we could also correct analytes losses during the sample preparation steps. Then, 100 mg of the homogenized sample were spiked with 50 ng of ENK into a polyethylene tube before being ultrasonically extracted with 5 mL of MeOH for 10 min at ambient temperature. The extract was centrifuged at 3000 rpm for 5 min and then filtrated into a 15-mL polyethylene tube through a 0.45-μm PTFE filter. The pellet was extracted again with another 5 mL of methanol, centrifuged and filtrated into the same tube in order together with two extracts. The sample was then diluted 1:5 with ultrapure water in an amber vial by auto sampler and analyzed by HPLC-MS/MS system.

Table 2 *T* test for paired medium data in the second session

	Variable 1	Variable 2
Average	0.634489693	0.325791855
Variance	0.003526462	0.000442633
Remarks	10	10
Pearson correlation	0.045859193	
Hypothesized difference medium	0	
gdl	9	
STAT *t*	15.72350167	
P (*T*≤*t*) one-tail	3.74416E-08	
t Critical one-tail	1.833112933	
P (*T*≤*t*) two-tail	7.48832E-08	
t Critical two-tail	2.262157163	

Water samples processing and analysis Lake samples, previously analyzed by ELISA test, were also analyzed using HPLC-MS/MS instrumental method developed by Gambaro et al. [23]. The pre-analytical procedure for lake water samples is quite similar than the protocol for a neurotoxin domoic acid in water samples developed by Barbaro et al. [24]. An aliquot of lake water was acidified with 2 % of formic acid and was then spiked with 50 ng 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 5 mL of MeOH followed by 5 mL of acidified ultrapure 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 5 mL of ultrapure water as a rinse of the sample tube and the SPE column. Cyanotoxins, adsorbed on the cartridge, were eluted dropwise with 5 mL of MeOH into a glass vial. The sample was then diluted 1:5 with ultrapure water in an amber vial by autosampler and analyzed using the same HPLC-MS/MS previously described.

Results and discussion

ELISA validation results of crop samples

Twenty blank samples were analyzed in order to assess the specificity. All the tests on the blank samples showed concentrations of microcystins less than the ELISA detection limit (0.1 ng mL^{-1}). Once performed the tests in two sessions, one on blank and one on additive samples at one level (8 ng/g), it is verified that they are comparable and that if, for the two tests, error β occurred (≤5 %). For both sessions, the error β was observed. Later, it was confirmed the comparability between sessions through the evaluation of the comparison between standard deviations (test *f*). The test was successful as the critical value *f* (tabulated value) was greater than the experimental *f* value (calculated value). The *t* test was also carried for paired medium data in the two sessions separately, checking that the difference between the blank samples and the corresponding additive is significantly different from zero. As the experimental STAT *t* is greater than *t* critical tabulated, the test occurred (Tables 1 and 2). Finally, an ANOVA test was performed between groups of blank samples and between groups of additives to the level of additivation chosen. ANOVA test confirmed that the value of *f* experimental is less than the *f* critical (Tables 3 and 4). The accuracy tests were performed at two levels, by performing a series of six tests at each level of interest, in restricted repeatability condition

Table 3 ANOVA test blank samples

Summary						
Groups	Count	Sum	Medium	Variance		
Column 1	10	6.339869281	0.633986928	0.003552582		
Column 2	10	6.344896933	0.634489693	0.003526462		
Variance analysis						
Origin of the variation	SQ	gdl	MQ	*F*	Values of significance	*F* crit
Inter-groups	1.26386E-06	1	1.26386E-06	0.000357072	0.98513168	4.413873
In-groups	0.063711398	18	0.003539522			
Total	0.063712662	19				

(Table 5). The robustness of the method was evaluated using the approach of Youden at the same level of concentration of the analyte to which the error β check has been carried out. Therefore, the variables have been identified during the analysis of the sample could theoretically affect the test results (Table 6).

Quality control of the determination of cyanotoxins in crop samples using HPLC-MS/MS method

Due to the lack of certified material for the quantification of MCs in plant matrices, the assessment of trueness, precision, and effect matrix was conducted using a microcystins free-matrix spiked with a know amount of the analytical standards of cyanotoxins (50 ng absolute for each microcystins and nodularin) and 50 ng of internal standard ENK. Trueness refers to the degree of closeness of the determined value to the known "true" value. It was calculated as $(Q\text{-}T)/T \times 100$ where Q is the quantified value and T is the "true" value; the obtained value is called percent error. The percent errors for each cyanotoxin were always within ±10 %, except to the quantification of MC RR in spinach and basil where the error was higher to an accurate quantification. The precision of pre-analytical protocol was evaluate as repeatability (RSD%) for the three replicates.

The method detection limits (MDLs) and the method quantification limits (MQL) for the analytical procedure were respectively quantified as three and ten times the standard deviation of the average values of the cyanotoxins free matrices ($n = 3$). The MDL is always less than 8 ng g^{-1}, despite the highest values are obtained with the spinach as a matrix.

In order to assure the linearity of the instrumental response with each crop matrix, a series of standard solutions containing cyanotoxins at the concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 ng mL^{-1} with a constant concentration of internal standard ENK (1 ng mL^{-1}) were prepared using the matrices obtained with the extraction procedure without the addition of the internal standard. The solutions were injected three times in order to also evaluate the instrumental precision, and RSD% values below of 10 % were obtained at three concentration levels (0.1, 1, and 10 ng mL^{-1}). The linearity of instrumental signal for each matrix was always $R^2 > 0.9$ (see Electronic Supplementary Material (ESM), Table S1). A quantitative study of the matrix effects was realized as suggested by Matuszewski et al. [25]. The matrix effect (ME) is obtained by dividing the signal response of a standard present in the sample extract with the response of a standard prepared

Table 4 ANOVA test additive samples

Summary						
Groups	Count	Sum	Medium	Variance		
Column 1	10	3.288084465	0.328808446	0.000388708		
Column 2	10	3.257918552	0.325791855	0.000442633		
Variance analysis						
Origin of the variation	SQ	gdl	MQ	*F*	Values of significance	*F* crit
Inter-groups	4,54991E-05	1	4.54991E-05	0.109459459	0.744580592	4.413873419
In-groups	0,007482077	18	0.000415671			
Total	0,007527576	19				

Table 5 Accuracy test data: additive replicates (Spk), medium, recovery medium (R%), standard deviation (RSD), and coefficient of variation (CV)

	4 ng g^{-1}	16 ng g^{-1}
Spk1	3.2	14.8
Spk2	2.8	16.2
Spk3	3.4	16.7
Spk4	3.2	16.9
Spk5	3	16.9
Spk6	3	18.8
Medium	3.1	16.7
R% medium	77.5	104.5
RSD	0.2	1.3
CV	0.07	0.08

in a pure solvent and expressing the result as a percentage. This effect is absent if the ME % value is equal to 100 %; it leads to a suppression of the signal if the values are below 100 %, while an increase of the signal is obtained if the value exceeds 100 %.

We have verified that the matrix effect leads to a suppression of the signal (ME < 100 %) for the majority of the matrices, with the exception of the leaves of tomato and the low-concentration cabbage where instead there is an increase in signal intensity (ESM Fig. S1). The matrix less involved in this effect is the lettuce. These results show that the use of the method of measurement matrix-matched calibration is critical to ensure the reliability of the results. The internal standard, although eliminating problems arising from instrumental errors and causal operator, fails to eliminate matrix effects. In order to ensure an accurate method, the quantification was carried out using a calibration curve constructed by diluting the matrix in standard.

Quality control of the determination of cyanotoxins in water samples using HPLC-MS/MS method

Due to the lack of certified material for the quantification of cyanotoxins in water samples, we assessed the trueness, precision, and yields by analyzed mineral water ($n = 3$) spiked with a know amount of the analytical standards of cyanotoxins (25 ng absolute for each MC and NOD) and 50 ng of internal standard ENK. Table 7 shows that the pre-analytical protocol allow to accurately quantify each analytes, expect the MC-LW. In fact, percent errors ranged between −13 % (MC YR) and +1 % (MC RR) but MC-LW was underestimated with error percent of −29 %. Low extraction yield was determined for MC-LW, while other cyanotoxins had yields always above 80 %.

Table 6 Variables tested in the approach of Youden

Variables	Variable chosen	Level 1	Level 2
1	Extraction	Methanol 95 %	Methanol 100 %
2	Agitation	10 min	15 min
3	Prepared fresh/day before	Day before	Same day
4	Centrifugation	5 min	10 min
5	Washings	3	4
6	Final volume	1-2 mL	3–4 mL
7	Filter	Whatman	Tissue paper

Table 7 Validation parameters of protocol to determine cyanotoxins in water samples. Accuracy is expressed as percent error (E%) and precision as RSD%. Extraction yield, method detection (MDL), quantification limits (MQL), and matrix effect (ME%) are also reported

	LA	LY	YR	NOD	LR	LW	LF	RR
E %	−9	−5	−13	−7	−9	−29	−6	1
Yield (%)	90	91	84	96	87	72	90	95
RSD%	3	1	7	4	11	11	8	6
MDL (pg mL^{-1})	0.2	0.4	0.3	0.3	0.6	0.1	0.1	0.3
MQL (pg mL^{-1})	0.6	1.2	0.8	1.1	2.1	0.2	0.3	0.9
ME%	46	60	39	151	26	48	301	55

The method precision in terms of repeatability was obtained by calculating the relative deviation standard (RSD%) by consecutive measurements of spiked samples ($n = 5$). Repeatability was always below 11 %. Table 7 also repots method detection limit (MDL) and quantification limits, calculated using three procedural blank (mineral water) spiked with internal standard ENK (50 ng). We obtained values of MDLs ranged between 0.1 (MC-LF) and 0.6 pg mL^{-1} (MC-LR). To the best of our knowledge, our MDLs were the lowest values than the best MDL reported in literature by Wang et al. [26]. They used an UPLC-MS/MS and a SPE preconcentration system, obtaining MDLs ranged between 1.3 and 6 pg mL^{-1}, one order of magnitude higher than our values.

A quantitative study of the matrix effect demonstrated that a signal suppression occurred for each cyanotoxins, except the MC-LW and NOD. These observations suggest that matrix-matched calibration was necessary to correct matrix effect and to obtain an accurate quantification.

Results of water and crop samples

The water and crop samples examined by the ELISA method, with very low concentrations and high, were processed for the confirmatory analysis by HPLC/ESI-MS/MS with triple quadrupole. For an analysis of the analytical results, it must be considered that for drinking water, there is a limit on concentrations of MCs equal to 1 μg L^{-1} dictated by the World Health Organization (WHO) and the guidelines of the EPA

Table 8 Concentrations of microcystins by ELISA and HPLC-MS/MS in water samples collected

Description of water sample	ELISA results (ng mL^{-1})	HPLC-MS/MS results (ng mL^{-1})
Tavoliere Tank 16/11/2015	0.2	0.022
Superficial Center Lake Occhito 16/11/2015	0.2	0.022
Pozzilli Tank 15/11/2015	0.2	0.026
Superficial Tributary Lake Occhito 16/11/2015	0.2	0.017
Centrer Lake Occhito 10 m in depth 16/11/2015	0.2	0.027
Lake Occhito Junction 15 m in depth 16/11/2015	0.3	0.021
Superficial Lake Occhito Junction 16/11/2015	0.2	0.022
Finocchito Tank 16/11/2015	0.3	0.18
Finocchito Tank 7/7/2015	0.2	0.024
Superficial Tributary Lake Occhito 16/6/2015	0.1	0.007
Lake Occhito Junction 15 m in depth 7/7/2015	0.5	0.022
Tavoliere Tank 7/7/2015	0.3	0.013
Superficial Center Lake Occhito 7/7/2015	0.6	0.020
Pozzilli Tank 7/7/2015	0.3	0.024
Tributary Lake Occhito 10 m in depth 7/7/2015	2.1	0.018
Finocchito Tank 26/8/2015	0.2	0.010
Superficial Lake Occhito Junction 24/08/2015	0.2	0.008
Centrer Lake Occhito 10 m in depth 24/08/2015	0.3	0.007
Tavoliere Tank 24/08/2015	0.2	0.021
Pozzilli Tank 25/09/2015	0.1	0.022
Superficial Tributary Lake Occhito 19/10/2015	0.2	0.1
Superficial Lake Occhito Junction 19/10/2015	0.1	0.009
Finocchito Tank 19/10/2015	0.1	0.019
Tavoliere Tank 19/10/2015	0.1	0.014
Pozzilli Tank 19/10/2015	0.1	0.019
Tributary Lake Occhito 10 m in depth 21/12/2015	0.1	0.018
Superficial Lake Occhito Junction 21/12/2015	0.1	0.025
Superficial Tributary Lake Occhito 21/12/2015	0.1	0.012
Lake Occhito Junction 15 m in depth 21/12/2015	0.1	0.022
Superficial Center Lake Occhito 21/12/2015	0.1	0.021
Centrer Lake Occhito 10 m in depth 21/12/2015	0.1	0.016
Superficial Center Lake Occhito 13/01/2016	0.1	<MQL
Superficial Tributary Lake Occhito 13/01/2016	0.1	<MQL
Tributary Lake Occhito 10 m in depth 13/01/2016	0.1	<MQL
Centrer Lake Occhito 10 m in depth 02/03/2016	0.2	0.022
Superficial Lake Occhito Junction 02/03/2016	0.1	0.013
Finocchito Tank 02/03/2016	0.1	0.020
Pozzilli Tank 17/05/2016	0.1	<MQL
Lake Occhito Junction 15 m in depth 17/05/2016	0.1	0.018
Tavoliere Tank 16/02/2016	0.1	0.010
Tributary Lake Occhito 10 m in depth 30/03/2016	0.1	0.008

(Environmental Protection Agency USA 2006), who they have established for MCs tolerable daily intake (TDI) limit for the acute and chronic risk in man pointing, respectively, 0.006 mg kg^{-1} bw day^{-1} for acute injury and 0.003 mg kg^{-1}

Table 9 Concentrations of microcystins by ELISA and HPLC-MS/MS in crop samples collected

Description of crop samples	ELISA results (ng mL^{-1})	HPLC-MS/MS results (ng mL^{-1})
Savoy cabbage 23/10/15	1.3	<MQL
Cabbage 23/10/15	1.3	<MQL
Broccoli 18/11/15	0.4	<MQL
Savoy cabbage 18/11/15	0.2	<MQL
Fennel 23/10/15	1.1	<MQL
Tomato 17/6/15	0.4	<MQL
Tomato leaves 9/7/15	1.0	<MQL
Tomato leaves 17/6/15	1.0	<MQL
Tomato 9/7/15	0.8	<MQL
Tomato leaves 17/6/15	0.4	<MQL
Tomato leaves 15/6/15	1.0	<MQL
Tomato leaves 17/6/15	0.6	<MQL
Lettuce 16/02/2016	1.0	<MQL
Spinach 16/02/2016	0.3	<MQL
Broccoli 16/02/2016	1.5	<MQL
Fennel 11/04/2016	1.3	<MQL
Fennel 13/01/2016	1.2	<MQL
Cabbage 11/04/2016	0.8	<MQL
Lettuce 11/04/2016	0.6	<MQL
Spinach 11/04/2016	0.5	<MQL
Broccoli 13/01/2016	1.1	<MQL
Yellow pepper 13/01/2016	1.0	<MQL
Spinach 13/01/2016	1.2	<MQL
Yellow pepper 02/03/2016	0.8	<MQL
Broccoli 02/03/2016	0.6	<MQL
Lettuce 02/03/2016	0.5	<MQL
Cabbage 02/03/2016	1.0	<MQL
Cabbage 17/05/2016	1.3	<MQL
Broccoli 17/05/2016	0.7	<MQL
Yellow pepper 17/05/2016	1.1	<MQL
Fennel 17/05/2016	0.3	<MQL

bw day^{-1} for chronic damage. In the case of crops, on the other hand, currently, there are no legal limits regarding the accumulation of microcystins, but only a TDI value of 0.04 mg kg^{-1} bw day^{-1} for chronic risk established by WHO. The results of the two methods, refer to samples taken during the 15 months between March 2015 and May 2016, have shown that with regard to the results of ELISA screening of water samples, concentrations of MCs have been detected over the limit of detection in 41 samples, with a concentration range between 0.1 to 2.1 ng mL^{-1}. Concentrations above the limit of quantification for the only MC-LR were confirmed by HPLC-MS/MS technique for 37 water samples, with concentrations ranging between 0.007 and 0.18 ng mL^{-1}. The results of ELISA screening of crop samples showed values of microcystin concentration above the limit of detection in 31 samples, with concentration ranges between 0.2 and 1.5 ng mL^{-1}. However, concentrations were revealed not exceeding the detection limit after being processed by HPLC-MS/MS technique. Tables 8 and 9 report results about concentrations of microcystins by ELISA and HPLC-MS/MS methods, respectively, in water and crop samples collected. The tables mentioned above reports only concentrations over the ELISA detection limit (0.1 ng mL^{-1}) and the possible confirmation by HPLC-MS/MS. Given the concentration of MCs values obtained with the ELISA test, both in the water samples that of crops, and comparing them with the values obtained by HPLC-MS/MS, it can be deduced that with the ELISA technique, we detect higher concentrations as the sum of free and bound MCs. The chromatography coupled with mass spectrometry, however, quantifies separately the various isomers of MCs present in the

samples. Therefore, for this reason, very low values detected with ELISA technique in most cases were not confirmed by HPLC-MS/MS technique.

Conclusion

The method here proposed for the detection and quantification of MCs in water and crop samples by screening ELISA and HPLC-MS/MS as a confirmatory method is very satisfactory. The study of the parameters for the validation of both methods allowed not only the quantification with high precision and accuracy but also gave the opportunity to discriminate the different isomers of MCs. Both the processing samples analysis, obtained according to the optimized procedure, are simple and sensitive. As regards the results of the monitoring, it was found a situation in which the values of MCs concentrations, both in the water samples that of crop samples, are below the limits dictated by the WHO and also below the suggested values in the TDI guidelines of EPA. However, it is still recommended the continuation of monitoring activities of concentration levels of MCs, both in lakes and in crops irrigated with water coming from these lakes, so as to provide a greater number of available data.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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