Inhibition equivalency factors for microcystin variants in recombinant and wild-type protein phosphatase 1 and 2A assays

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Abstract

In this work, protein phosphatase inhibition assays (PPIAs) have been used to evaluate the performance of recombinant and wild-type PP1 and PP2A. The active enzymes were compared using MC-LR as a model cyanotoxin. $PP1_{Rec}$ and $PP2A_{Wild}$ provide limits of detection (LODs) of 0.6 and 0.5 µg/L respectively, lower than the guideline value proposed by WHO (1 µg/L). The inhibitory potencies of seven MCs variants (-LR, -RR, -dmLR, -YR, -LY, -LW and -LF) have been evaluated, resulting on IC_{50} values ranging from 1.4 to 359.3 µg/L depending on the MC variant and the PP. The PPIAs have been applied to the determination of MC equivalent contents in a natural cyanobacterial bloom and an artificially-contaminated sample, with multi-MC profiles. Results have been compared to those obtained by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis after application of the corresponding inhibition equivalency factors (IEFs). PPIAs have demonstrated to be applicable as MC screening tools with environmental applications and to protect human and animal health.

Keywords: Microcystins (MCs); protein phosphatase 1 (PP1); protein phosphatase 2A (PP2A); protein phosphatase inhibition assay (PPIA); inhibition equivalency factors (IEFs); liquid chromatography-tandem mass spectrometry (LC-MS/MS).

1. Introduction

Microcystins (MCs) and nodularins are potent hepatotoxic cyclic peptides produced by toxicogenic cyanobacteria (e.g. *Microcystis aeruginosa*) normally found in fresh and brackish water with low turbidity flow regimes (Carmichael, 1994). The general structure of MCs is cyclo-(D-Ala-X-D-MeAsp-Y-Adda-D-Glu-Mdha), X and Y being two variable L-amino acids. One of the main characteristic of MCs is the presence of the Adda chain (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) (Rinehart et al., 1988). There are more than 80 MC variants, varying by degree of methylation, hydroxylation, epimerization, peptide sequence and toxicity (Welker and Von Dohre, 2006). Among them, MC-LR is the most commonly frequent and toxic congener. MCs inhibit serine/threonine protein phosphatases type 2A (PP2A) and type 1 (PP1), increasing the phosphorylation levels in the cells and inducing morphology changes on their cytoskeleton (Mackintosh et al., 1990; Yoshizawa et al., 1990; Dawson, 1998; Kuiper-Goodman et al., 1999).

The presence of MCs in water supplies poses a threat to animal and human health because of the possible harmful effects such as gastrointestinal disturbances and dermatitis, but also more severe chronic toxicity effects (neurotoxicity, hepatotoxicity and liver cancer promotion) and even death (Nishiwaki-Matsuchima et al., 1992; Pouria et al., 1998; Jochimsen et al., 1998; De Figueiredo et al., 2004). Due to the possible serious implications in health, the World Health Organization (WHO) proposed a guideline value of 1 μg/L for MC-LR in drinking water (WHO, 1998). Although in Europe there is no legislation, several countries (e.g. Spain, France, Poland, Czech Republic, Norway, Brazil, Japan, Korea, New Zealand) have adopted the WHO recommended value in their regulations (Burch, 2008). This value, however, only covers MC-LR; thus, it was stated as “provisional” until more toxicity data for other cyanobacterial toxins are generated. In order to assure water quality and protect

human and animal health, the regulation should not be restricted to MC-LR, but it should consider the toxicity of the different variants. Consequently, the development of analysis methods that give an indication of the global toxicity of a sample is an evident necessity.

Several MCs analysis methods have been developed. The simplest detection method is the mouse bioassay, but it is widely known its low specificity and controversial ethical implications (Falconer, 1993; Campbell et al., 1994). Chromatographic techniques coupled to UV (Edwards et al., 1993; Lawton et al., 1994; Tsuji et al., 1994) or mass spectrometry detection (Spoof et al., 2001; Ortelli et al., 2008; Yang et al., 2009) are routinely used, providing high sensitivity and structural information on the different variants, but requiring expensive equipment and trained personnel. Also structural information is provided by enzyme-linked immunosorbent assays (ELISAs), which use monoclonal (Ueno et al., 1996; Zeck et al., 2001; Campàs and Marty, 2007; Pyo et al., 2004; Sheng et al., 2007) or polyclonal antibodies (Brooks and Codd, 1988; Chu et al., 1990; An and Carmichael, 1994) with high specificity towards several MCs and nodularin variants. The antibody cross-reactivity makes impossible the discrimination of individual toxins, but it can be considered as an advantage since all the toxins within the same structural family could be detected. Nevertheless, ELISAs do not distinguish between toxic and non-toxic variants. The inhibition of PPs by MCs and nodularins has also been exploited to develop assays for the determination of these toxins (An and Carmichael, 1994; Heresztyn and Nicholson, 2001; Bouaïcha et al., 2002; Campàs et al., 2005; Ikehara et al., 2008). Unlike ELISAs, the protein phosphatase inhibition assays (PPIAs) do not provide structural information but a functional response, indicating the inhibitory potency of a toxin or mixture of toxins. Thus, although the PPIA cannot be used as an identification technique, it can be applied to detect the toxicity of a sample. Nowadays, colorimetric PPIAs are widely used because of their simplicity, low cost and sufficient sensitivity. However, more inhibitory data from different MC variants, and

their inhibition equivalency factors (IEFs) in relation to MC-LR, are necessary to better understand the toxicity of samples and to provide useful screening/detection tools.

In this work, the enzyme activity of four PPs (PP1 and PP2A, recombinant and wild-type) has been firstly tested. Then, colorimetric PPIAs have been developed, using the appropriate enzymes and MC-LR as a model cyanotoxin, and the sensitivities and limits of detection (LODs) have been compared. Afterwards, the inhibitory potencies of seven MCs variants (-LR, -RR, -dmLR, -YR, -LY, -LW and -LF) on the PPs have been determined, and the corresponding IEFs have been established. Finally, PPIAs have been applied to the determination of MC equivalent contents in a natural cyanobacterial bloom and an artificially-contaminated sample. Results obtained with the functional assays have been compared with those obtained by LC-MS/MS analysis after application of the corresponding IEFs.

2. Materials and Methods

2.1 Reagents and materials

Microcystin-LR (MC-LR), MC-RR, MC-YR, MC-LY, MC-LW and MC-L, were purchased from Alexis Biochemicals (San Diego, USA) and dissolved in 100% methanol (the precise MC content was quantified by LC-MS/MS analysis). 3-Desmethylmicrocystin-LR (MC-dmLR, 7,072 µg/L in 100% methanol) was purchased from DHI (Hørsholm, Denmark).

A recombinant protein phosphatase 1 catalytic subunit was purchased from New England BioLabs (Ipswich, MA, USA). $PP1_{Rec}$ consists of a 330 amino-acid catalytic subunit of the α-isoform, and it was isolated from a strain of *E. coli*. A recombinant protein phosphatase 2A catalytic subunit was purchased from GTP Technology (Toulouse, France). $PP2A_{Rec}$ consists of a 39 kDa human catalytic (C) subunit of the α-isoform with a hexa-His tail at the C-terminus, and it was isolated from SF9 insect cells infected by baculovirus. Wild-type PP1

and PP2A were purchased from Upstate Biotechnology (NewYork, USA). $PP1_{Wild}$ consists of a 375 amino-acid 42 kDa, and it was purified from rabbit skeletal muscle, $PP2A_{wild}$ consists of a heterodimer of 60 kDa and 36 kDa subunits, and it was purified from human red blood cells. Components of buffers and *p*-nitrophenyl phosphate (*p*-NPP) were purchased from Sigma (St. Quentin Fallavier, France). All solutions were prepared using Milli-Q water.

In the LC-MS/MS analysis, all reagents used were of analytical grade or high-performance liquid chromatographic (HPLC) grade. Acetonitrile and methanol were obtained from Merck (Darmstadt, Germany) and formic acid from Panreac (Montcada i Reixac, Barcelona, Spain). Solutions were prepared using Milli-Q water.

2.2 Cyanobacterial bloom sample and MCs extraction

A sample of water with biomass (50 mL) was collected from a cyanobacteria bloom that occurred in the Alcántara reservoir of the Tajo River (Cáceres, Spain) in summer 2009. The sample was filtered through a Whatman GF/F filter (0.7 µm). The filter was then extracted three times by sonication for 15 min in acidified methanol (0.16% formic acid) according to Barco and co-workers (Barco et al., 2005). The extract was evaporated to dryness, reconstituted in 8 mL of methanol, and directly injected into the LC-MS/MS system. For the extract to be tested with the PPIA, methanol was evaporated in a Speed VAC concentrator (Organomation Associates, Inc., Berlin, USA) under nitrogen at room temperature, and the residue was resuspended in the corresponding buffer.

2.3 PP activity

The PP enzyme activity was measured spectrophotometrically in a 1-mL cuvette by adding 10 µL of enzyme to 890 µL of 30 mM tris-HCl buffer solution, pH 8.4, with 20 mM $MgCl_2$, 2 mM EDTA, 2 mM DTT and 0.2 mg/mL BSA. After 5-min incubation, 100 µL of *p*-NPP

solution at 100 mM (prepared in the same buffer) were added to the cuvette and the absorbance at 405 nm was measured during 1 min with a U-2001 UV/Vis spectrophotometer from Hitachi High-Tech (Krefeld, Germany). When using $PP1_{Rec}$, 1mM $MnCl_2$ was also added to the enzyme and substrate solutions. Assays were performed at 22 °C in the dark in triplicate.

2.4 Colorimetric PPIA

The colorimetric PPIA was performed as follows: 50 µL of MC variants standard solutions (for the calibration curves, the study of the inhibitory potencies and the study of multi-MC profiles) at different concentrations or sample at different dilutions were added into microtiter wells containing 100 µL of enzyme solution at 1 U/mL for $PP1_{Rec}$ and $PP2A_{Wild}$ and 1.5 U/mL for $PP2A_{Rec}$ and incubated for 30 min. Then, 50 µL of 25 mM *p*-NPP solution were added, and after 1-hour incubation, the absorbance at 405 nm was measured with a KC4 automated microplate reader from Bio-Tek Instruments, Inc. (Bad Friedrichshall, Germany). MCs standard solutions were prepared in 30 mM tris-HCl buffer solution, pH 8.4, with 20 mM $MgCl_2$ and 2 mM EDTA. Enzyme and substrate solutions were prepared in the same buffer solution also containing 2 mM DTT and 0.2 mg/mL BSA. When using $PP1_{Rec}$, 1mM $MnCl_2$ was also added to the enzyme and substrate solutions. Assays were performed at 22 °C in the dark and in triplicate.

2.5 LC-MS/MS analysis

A triple quadrupole mass spectrometer (TSQ Quantum, Thermo Fisher Scientific, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source, a Surveyor MS plus pump and a HTC PAL autosampler were used for the LC-MS/MS analysis. The chromatographic separation was performed on a reversed-phase Kromasil C18 column (100 x 2.1 mm, 3.5 µm, Tracer, Teknokroma, Sant Cugat del Vallès, Spain). The mobile phase was composed of

Milli-Q water as solvent A and acetonitrile as solvent B, both containing 0.08% (v/v) formic acid at a flow rate of 200 µL/min. The linear gradient elution program was: 10-30% B 10 min, 30-35% B 20 min, 35-55% B 15 min, 55% B 5 min, 55-90% B 2 min, 90% B 3 min and return to initial conditions for re-equilibration (10% B 5 min). The injection volume was 10 µL.

The analyses were carried out in positive ion mode with the spray voltage at 4.5 KV and the optimum tube lens voltage (TL) for each m/z. The ion transfer tube temperature was set at 250 °C. Nitrogen (purity > 99.98%) was used as sheath gas, ion sweep gas and auxiliary gas at flow rates of 30 psi, 0 and 5 au (arbitrary units), respectively. Data were acquired in full scan mode (400-1200 m/z, 1.2 sec/scan) for screening of MC variants. MS/MS experiments were performed for confirmation of MC identity if commercial standards were not available. Its fragmentation pattern in positive ionization gives a majority ion of 135 m/z characteristic of the amino acid Adda residue. High purity argon (Ar_1) was used as collision-induced gas with a pressure of 1.5 mtorr and the optimum collision energy (CE) for each fragmentation was selected. Data acquisition was performed with Xcalibur 2.0.7 software (Thermo Fisher Scientific).

2.6 Statistical analysis

The paired t-test was used to compare the IC_{20} and IC_{50} values for MC-LR and the different PPs (N = 3), and the MCs equivalent contents in the natural cyanobacterial bloom obtained by PPIAs (N = 3). Differences in the results were considered statistically significant at the 0.05 level. SigmaStat 3.1 software was used. Principal Component Analysis (PCA) was used to compare the IC_{50} values for all MC variants as a whole and the different PPs. MATLAB 7.1 software was used for chemometric processing.

3. Results and discussion

3.1 MC-LR calibration curves by PPIAs

The enzyme activity of the stock solutions were 655 U/mL for $PP1_{Rec}$, 2 U/mL for $PP2A_{Wild}$, 1140 U/mL for $PP2A_{Rec}$, and 8935 U/mL for $PP2A_{Wild}$, 1 U being defined as the amount of enzyme required to hydrolyse of 1 nmol of *p*-NPP in 1 min at 22 °C. Since the activity of the $PP1_{Wild}$ stock solution was so low, it was not economically viable to perform the corresponding PPIA and thus $PP1_{Wild}$ was put aside.

Colorimetric PPIAs were developed using $PP1_{Rec}$, $PP2A_{Rec}$ and $PP2A_{Wild}$, and MC-LR as model toxin. The PPIAs demonstrated the inhibitory effect of MC-LR on the enzyme activity. The calibration curves were described by the sigmoidal logistic four-parameter equation (SigmaPlot software package 10.0):

$$y = y_0 + \frac{a}{1+(x/x_0)^b}$$

where a and y_0 are the asymptotic maximum and minimum values, respectively, x_0 is the x value at the inflection point and b is the slope at the inflection point.

In Table 1, the 50% inhibition coefficient (IC_{50}) values and the working ranges (defined between IC_{20} and IC_{80}) are presented together with the equation and the corresponding R values. To evaluate the PPs performance, the IC_{50} and IC_{20} values were compared. The calibration curves may differ not only in LOD (x displacement) but also in sensitivity (curve shape and slope at the linear range), and these two values may provide different information. Comparing the IC_{50} values, $PP1_{Rec}$ and $PP2A_{Wild}$ provided similar sensitivities (t = 4.221, p = 0.052), in both cases different to $PP2A_{Rec}$ ($PP2A_{Rec}$ *vs.* $PP1_{Rec}$: t = 33.325, $p < 0.001$; $PP2A_{Rec}$ *vs.* $PP2A_{Wild}$: t = 31.059, p = 0.001). Regarding the IC_{20} values (considered as the LOD), $PP1_{Rec}$ and $PP2A_{Wild}$ provided similar LODs (t = 0.896, p = 0.465), both enzymes being able to detect the regulated value of 1 µg/L of MC-LR in drinking water. The $PP2A_{Rec}$

provided a significantly higher LOD compared to the other PPs ($PP2A_{Rec}$ *vs.* $PP1_{Rec}$: t = 54.202, $p < 0.001$; $PP2A_{Rec}$ *vs.* $PP2A_{Wild}$: t = 15.878, p = 0.004). Although $PP2A_{Rec}$ would not detect 1 µg/L of MC-LR in drinking water, it could provide additional and/or complementary information to the other PPs. Consequently, it was still considered in next experiments.

3.2 Inhibition equivalency factors (IEFs)

Calibration curves of MC-LR, -RR, -dmLR, -YR, -LY, -LW and -LF were performed to evaluate the inhibitory potencies of different MC variants on the activity of $PP1_{Rec}$, $PP2A_{Rec}$ and $PP2A_{Wild}$. It is important to mention that stock MC concentrations were previously quantified by LC-MS/MS. The IEFs were calculated as the ratios of the IC_{50} for MC-LR to the IC_{50} for the corresponding variant, for each enzyme. Table 2 shows the IC_{50} values as well as the IEFs. In general terms, $PP2A_{Wild}$ provided the lowest IC_{50} values and $PP2A_{Rec}$ the highest ones. The regulatory subunit of the $PP2A_{Wild}$ may be mediating the MC binding and thus promoting inhibition compared to the $PP1_{Rec}$ and $PP2A_{Rec}$ catalytic subunits.

As expected, MC-LR was the most potent variant for all enzymes. In all cases the variants with arginine in position 4 (-LR, -RR, -dmLR and -YR) inhibit more than the rest. Whereas the trend for the recombinant enzymes was MC-LR > -RR > -dmLR > -YR > -LY > -LW > -LF, the trend for the $PP2A_{Wild}$ was different, i.e. MC-LR > -RR > -YR > -dmLR > -LY > -LW > -LF. As it can be observed, the discrepancy appears between -dmLR and -LY. It is difficult to explain this effect, since multiple factors, could be playing a role. Nevertheless, it is important to note that the demethylation of leucine in position 2 decreases the inhibitory potency of MC-dmLR compared to MC-LR. In fact, it is known that demethylation of an amino acid can alter the solubility, membrane permeability, and ionic or hydrogen bond formation at the binding site of MCs (Ikehara et al., 2009).

Compared to other works, differences in the IC_{50} values and trends can be observed. The source and nature of the enzyme as well as the concentrations of the MC variants stock solutions, some of them not certified, may be responsible for the different observed inhibitory potencies. Nevertheless, in general terms the MC-LR > -RR > -YR inhibitory trend is also observed in other works (Heresztyn and Nicholson, 2001; Ikehara et al., 2008 and 2009).

PCA was applied to the IC_{50} values (triplicates) in order to evaluate the differences among MC variants and the capability of the PPs to distinguish them. PCA allows the projection of the information carried by the original variables onto a smaller number of underlying ("latent") variables called principal components (PCs) with new coordinates called scores, obtained after data transformation. Consequently, by plotting the PCs, it is possible to obtain information about the interrelationships between different samples and variables, and detect and interpret sample patterns, groupings, similarities or differences.

Figure 1 shows an accumulated variance of 97.9% from the two first PCs, this high value indicating that nearly all the variance contained in the original information is represented by only these two new coordinates. As it can be observed, discrimination of the different MC variants can be achieved with this simple analysis of the scores, where PC1 seems to be related with the IC_{50} values and PC2 with the different inhibitory potencies towards PP1 and PP2As.

Figure 1 also shows the loadings of the three PPs. This representation allows detecting uninformative or meaningless variables by evaluating the proximity to the centre (0,0), a criterion that might be used to discard non-useful variables. In our case, the wide distance of the different PPs to the centre indicates that all PPs provide useful information. Besides, the proximity between the two PP2As denotes a similar response towards the different MCs compared to PP1. Moreover, these two PP2As present opposed coordinates values respect to

PP1, thus providing complementary information. Although this analysis could seem opposite to the information obtained from the MC-LR calibration curves, it is necessary to take into account that unlike the previous results, the PCA is considering the behaviour of all MC variants simultaneously. This is particularly important in the analysis of natural samples, where multi-MC profiles are almost always encountered.

3.3 Application of the PPIAs to multi-MC profiles and comparison with LC-MS/MS analysis

The PPIAs with $PP1_{Rec}$, $PP2A_{Rec}$ and $PP2A_{Wild}$ were applied to the determination of MC-LR equivalent contents in the natural cyanobacterial bloom from Alcántara (intracellular fraction). The Alcántara bloom sample was diluted until the dose-response curve provided the corresponding sigmoidal shape. MC-LR calibration curves were performed in parallel to the quantification analysis due to possible slight differences in the inhibition percentages between assays. Results were provided as MC-LR equivalent contents (μg/L), calculated from the IC_{50} values of the MC-LR calibration curves and the IC_{50} values of the sample dose-response curves, values determined from the sigmoidal logistic four-parameter equations. $PP1_{Rec}$ and $PP2A_{Wild}$ provided similar MC-LR equivalent contents, 11292 ± 1685 and 13646 ± 442 μg/L respectively (t = 1.941, p = 0.192), and different to $PP2A_{Rec}$, 16482 ± 849 μg/L ($PP2A_{Rec}$ *vs.* $PP1_{Rec}$: t = 5.221, p = 0.035; $PP2A_{Rec}$ *vs.* $PP2A_{Wild}$: t = 5.594, p = 0.030). LC-MS/MS analysis showed significantly lower MC contents, 7576 μg/L, the composition being 30% of MC-RR, 18% of MC-LR, 7% of MC-YR, 5% of MC-LW, 2% of MC-LY and 38% of minority and/or non-identified variants. The overestimation observed in the PPIAs respect to LC-MS/MS could be due to the presence of natural compounds from the cyanobacterial matrix, which would be inhibiting the PPs, since it is not expected that the minority and/or non-identified variants play an important role in the inhibitory potency of the mixture. The

cyanobacterial matrix compounds, which could be inhibiting the PPs in a different extent depending on the enzyme source and nature, could also be responsible for the different MC-LR equivalent contents reported by the three enzymes.

To study in depth the effect from the sample matrix and/or the multi-MC profile, a spiking experiment was performed using approximately the same MC composition found in the natural cyanobacterial bloom. Since, as previously mentioned, the sample contained 38% of minority and/or non-identified variants, two extreme scenarios were established: considering that this 38% did not inhibit the PPs (minimum contents), and considering that it inhibited them at the same potency than MC-LR (maximum contents). Table 3 shows the MC-LR equivalent contents obtained by the PPIAs as well as the IE values calculated by the application of the corresponding IEFs to the individual MCs quantifications reported by LC-MS/MS analysis. The highest MC-LR equivalent contents are those provided by $PP2A_{Rec}$, followed by $PP2A_{Wild}$ and finally $PP1_{Rec}$, following the same trend than in the natural bloom quantification. Minimum and maximum MC-LR equivalent contents determined by PPIAs also follow the appropriate trend. However, the contents determined by PPIAs slightly differ from the IE values, but still they are in the same order of magnitude. Unexpectedly, the MC contents are lower than those reported in the quantification of the natural bloom. This result reinforces the role of the matrix compounds in the PPs inhibition.

Although the presence of non-identified MC variants could be in part responsible for the MC equivalent contents overestimations by PPIAs in natural samples, their usually smaller abundance percentage and the probably lower inhibitory potency would not justify it. It is fairer to think about the presence of matrix compounds that interfere on the PPIAs, despite the high dilutions used to fit the inhibition sigmoidal shape. Still, the developed PPIAs can be

used as tools to screen the presence of MCs in natural cyanobacterial blooms. As a future work, an exhaustive evaluation of the possible matrix effects would be convenient.

4. Conclusions

In this work, colorimetric PPIAs using $PP1_{Rec}$, $PP2A_{Rec}$ and $PP2A_{Wild}$ have been developed for the determination of MCs. Although our purpose was to compare four different PPs, $PP1_{Wild}$ was put aside because of the low enzyme activity of the stock solution. The three PPIAs provided low LODs for MC-LR, two of them attaining values even below 1 µg/L. But when analysing natural blooms, extracts rarely contain a single MC variant. Consequently, the inhibitory potencies of seven MC variants were determined. The establishment of IEFs contributes to better understand the PPIAs performance and the correlation with LC-MS/MS analysis.

Despite the exhaustive PPs characterisation performed in this work as regards LODs and IEFs, the interpretation of the results provided in the analysis of samples with multi-MC profiles is not straightforward. In this work, a single natural sample and its corresponding spiked sample with a MC-profile have been analysed in detail as a proof of concept. In the spiked sample, obtained (from PPIAs) and expected (from theoretical calculation based on empirical LC-MS/MS data and IEFs) MC contents were slightly different, but still followed appropriate trends. When analysing the cyanobacterial bloom, PPIAs overestimated the MC-LR equivalent contents. It is crucial to understand the complexity of natural samples, not only because of the multi-toxin profiles, but also because of the cellular compounds that may be present in the extracts. A more complete study may not be always possible due to the lack of some MCs standards. Moreover, the variety of natural samples and the presence of still non-identified MC variants make difficult to fully explain the results obtained with a functional assay. Of course, an exhaustive evaluation of the matrix effects could help to better explain

the results, and the possible removal of interfering compounds by the application of purification protocols could simplify the assays. In any case, the PPIAs developed in this work do not provide false negatives; only false positives could occur, which would not be of concern. Consequently, the PPIAs could be used as tools to screen the presence of MCs and, in case of a positive or suspicious result, to address towards confirmatory analytical methods such as LC-MS/MS analysis.

Summarising, the simultaneous application of PPIAs has resulted to be an appropriate analysis tool for screening MCs in the environment in a simple, fast and cost-effective way. It requires little training and allows the analysis of multiple samples simultaneously. The assays measure the total inhibition activity providing an indication of the global toxicity of the sample, guaranteeing public health protection.

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Figure 1. Scores and loadings plot of the first two components obtained after PCA analysis of the IC_{50} values (triplicates) from the inhibition of $PP1_{Rec}$, $PP2A_{Rec}$ and $PP2A_{Wild}$ by MC-LR, -RR, -YR, -dmLR, -LY, -LW and -LF.

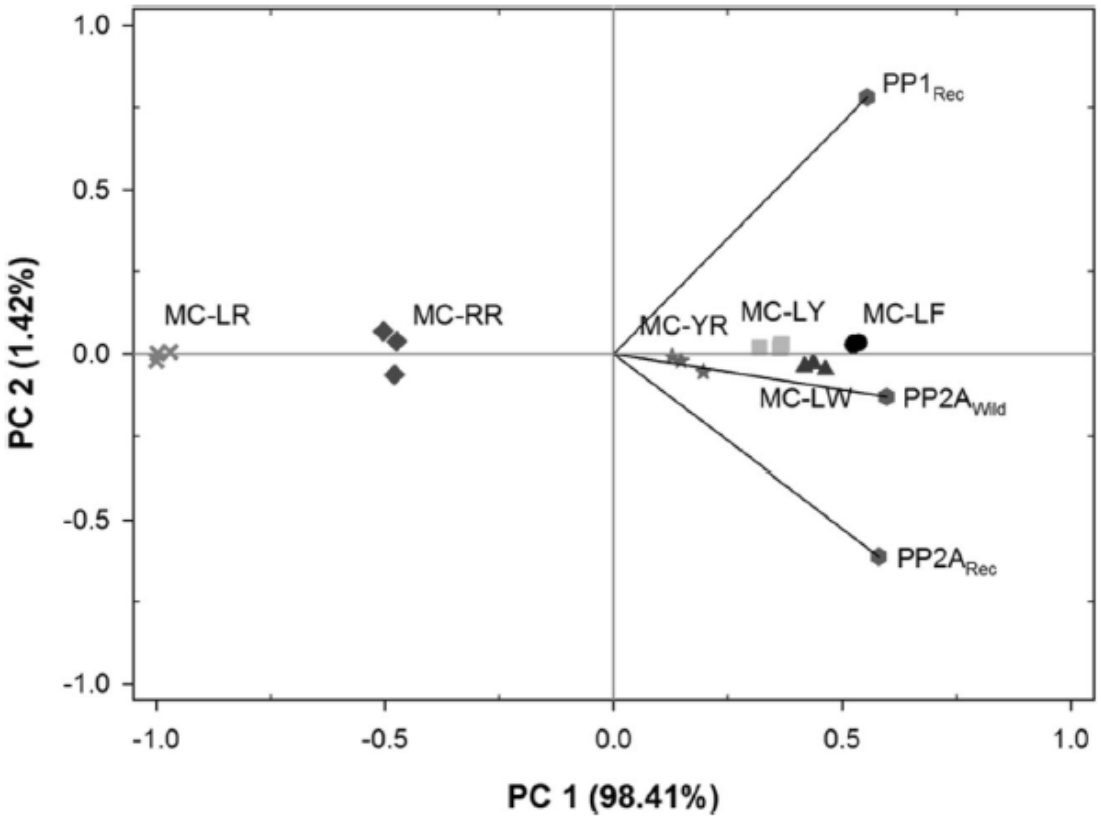

Table 1. Curve parameters derived from the sigmoidal logistic fourparameter fitting for the inhibition of the PPs by MC-LR

Enzyme	IC50 (µg/L)	Working range IC20-IC80 (µg/L) IC20 – IC80	Equation	R
PP1Rec	2.1	0.8–4.1	$y = 15.2 + \frac{80.8}{1 + (x/2.3)^{-2.5}}$	1.000
PP2ARec	7.3	3.1–18.7	$y = 1.6 + \frac{93.1}{1 + (x/7.0)^{-1.7}}$	1.000
PP2AWild	1.4	0.6–2.8	$y = 11.1 + \frac{85.4}{1 + (x/1.6)^{-2.4}}$	1.000

Table 2. IC50 (µg/L) values derived from the sigmoidal logistic fourparameter fitting for the inhibition of the PPs byMC-LR, -RR, -YR, -LY, -LWand -LF, and corresponding IEFs calculated with respect to MC-LR for each PP.

	PP1Rec		PP2ARec		PP2AWild	
	IC50	IEF	IC50	IEF	IC50	IEF
MC-LR	2.1	1.00	7.3	1.00	1.4	1.00
MC-RR	14.6	0.14	21.3	0.34	7.9	0.18
MC-YR	74.2	0.03	129.9	0.06	37.7	0.04
MC-LY	188.2	0.01	167.5	0.04	83.3	0.02
MC-LW	187.3	0.01	239.1	0.03	105.3	0.01
MC-LF	359.3	0.01	255.0	0.03	117.8	0.01