



**Evaluation of the transfer and the accumulation of microcystins in tomato (*Solanum lycopersicum* cultivar MicroTom) tissues using a cyanobacterial extract containing microcystins and the radiolabeled microcystin-LR (14C-MC-LR)**

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Evaluation of the transfer and the accumulation of microcystins in tomato (*Solanum lycopersicum* cultivar MicroTom) tissues using a cyanobacterial extract containing microcystins and the radiolabeled microcystin-LR ( $^{14}\text{C}$ -MC-LR)

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## Highlights

Transfer of MCs from soil to tomato tissues is observed

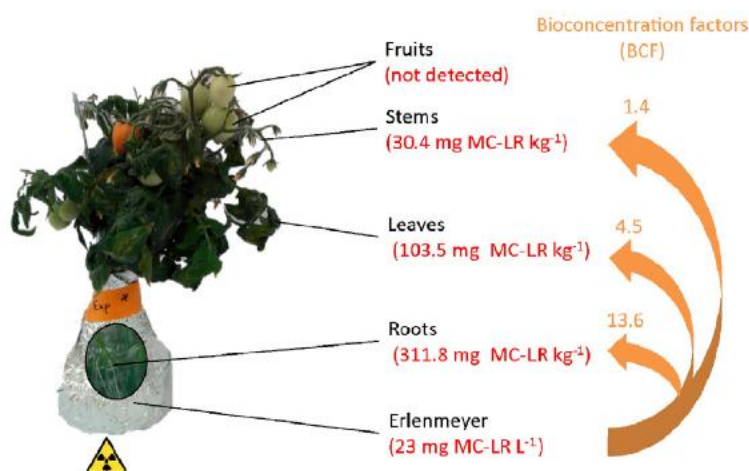
MCs are present in all tissues of the tomato except in the fruit

The bioconcentration factor of the MC-LR is much more important in the tomato roots

Bound fraction of MC-LR was higher than that of free fraction in roots and leave

## Graphical abstract

Bioconcentration factors in organs of the tomato *Solanum lycopersicum* cv. MicroTom, maintained 48 h in hydroponic growing system with  $^{14}\text{C}$ -MC-LR at  $23 \text{ mg L}^{-1}$ .



## Abstract

Microcystins are the most common cyanotoxins and may be expected wherever blooms of cyanobacteria occur in surface waters. Their persistence both in the irrigation water and in the soil can lead to their transfer and bioaccumulation into agricultural plants. The aim of this work was to investigate microcystin accumulation in *Solanum lycopersicum* cultivar MicroTom. The plant was exposed to either *Microcystis aeruginosa* crude extracts containing up to 100 µg eq. MC-LR L<sup>-1</sup> in a soil–plant system for 90 days or pure radiolabeled <sup>14</sup>C-MC-LR in a hydroponic condition for 48 h. Toxin bioaccumulation in the soil and different plant tissues was assessed both by the PP2A inhibition assay and by liquid chromatography-mass spectrometry (LC/MS/MS). After 90 days of exposure, microcystins persisted in the soil and their free extractable concentrations accumulated were very low varying between 1.6 and 3.9 µg eq. MC-LR kg<sup>-1</sup> DW. Free MC-LR was detected only in roots and leaves with concentrations varying between 4.5 and 8.1 µg kg<sup>-1</sup> DW and between 0.29 and 0.55 µg kg<sup>-1</sup> DW, respectively. By using radioactivity (<sup>14</sup>C-MC-LR), the results have reported a growing accumulation of toxins within the organs roots N leaves N stems and allowed them to confirm the absence of MC-LR in fruits after 48 h of exposure. The bioconcentration factor (BCF) was 13.6 in roots, 4.5 in leaves, and 1.4 in stems. On the other hand, the results highlight the presence of two radioactive fractions in different plant tissues. The non-extractable fraction of radioactivity, corresponding to the covalently bound MC-LR, was higher than that of the extractable fraction only in roots and leaves reaching 56% and 71% of the total accumulated toxin, respectively. Therefore, results raise that monitoring programs must monitor the presence of MCs in the irrigation water to avoid the transfer and accumulation of these toxins in crops.

Keywords:

Microcystins

## Introduction

Cyanobacteria harmful algal blooms represent one of the most emergent waterborne microbial hazards to human and agricultural water supplies, fisheries production, and freshwater and marine ecosystems (Ettoumi et al., 2011). Freshwater cyanobacteria are known to produce a variety of toxins such as hepatotoxins, neurotoxins, cytotoxins and lipopolysaccharides, which have adverse effects on animals, humans and plants (Corbel et al., 2014a). Hepatotoxins type microcystin are among the most widely investigated cyanotoxins because of their frequency in nature and their adverse health effects on humans, as well as fish, birds, zooplankton, aquatic and terrestrial plants and mammals (Ettoumi et al., 2011). Most of these microcystins are water soluble, which aids in their dispersal in water and results in their accumulation in the food web where they disrupt the key eukaryotic protein phosphatases PP1 and PPA2 which leads to the observed toxic effects of these compounds (MacKintosh et al., 1990; Hitzfeld et al., 2000). To date, there have been at least 100 different microcystin congeners characterized (Niedermeyer et al., 2014). The congener MC-LR is generally recognized as being the most studied due to its high toxicity, frequent detection and its concentrations in surface waters often exceed the World Health Organization advisory level of  $1 \mu\text{g L}^{-1}$  (Chorus and Bartram, 1999) and can reach  $100 \mu\text{g eq. MC-LR L}^{-1}$  (Corbel et al., 2014a). On the other hand, the raw water used in irrigation often comes from a plan of natural water or a pond constructed for agricultural purposes and is not subject to any treatment or control and monitoring for MCs. Furthermore, the cyclic structure of these toxins provides them a high chemical stability with a half-life time in an agricultural soil recently estimated to approximately 56 days (Corbel et al., 2014b). Consequently, the persistence of these toxins both in the irrigation water and in the soil can have a considerable impact on soil organisms as well as on the growth and development of cultivated plants (reviewed in Corbel et al., 2014a). In addition, questions arise about their transfer and bioaccumulation in agricultural plants and hazards associated as much for the herbivorous animals and humans. Consequently, the provision of additional data in the understanding of the bioconcentration and detoxification of MCs in the plants could not only assist in the understanding of their mechanisms of phytotoxicity but allow also both the assessment of

exposure to these toxins via the plants and the health risks associated. Therefore, in this study, we report for the first time that chronic irrigation (during 90 days) of the tomato plant *Solanum lycopersicum* cv. MicroTom growing on an agricultural soil with water containing a cyanobacterial extract at realistic environmental concentrations of MCs resulted in the transfer of these toxins only in roots and leaves but not detected in edible parts of the plant (tomato fruits). The use in the chronic study of a cyanobacterial extract with different congeners of microcystins instead of pure microcystins was for the purpose of approaching the real conditions; given that the irrigation water are usually used in a raw state without treatment and they often contain several congeners of microcystins. The detection of MCs in the different plant organs was done by using the PP2A protein inhibition assay and identified by using an ultrahigh-performance liquid chromatography combined with tandem mass spectrometry (UHPLC–MS/MS). The results were then confirmed by using the radiolabeled MC-LR ( $^{14}\text{C}$ -MC-LR), showing in addition that a part of MC-LR is covalently bound at the plant level, especially in leaves where the majority of the radiolabel carbon was not extractable.

## Materials and methods

Culture of *Microcystis aeruginosa* strain PCC7820, preparation of the unlabeled microcystins fraction and biosynthesis and purification of the radiolabeled MC-LR

### Preparation of the unlabeled microcystins fraction

The *M. aeruginosa* strain PCC7820 was supplied from the culture collection of the Pasteur Institute (Paris, France). This monoclonal strain was axenically grown in batch culture in 6 L flasks containing 3 L of BG11 liquid medium supplemented with  $\text{NaNO}_3$  (2 mM) and  $\text{NaHCO}_3$  (10 mM), at 25 °C, with an incident light intensity of 5-10  $\mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$  provided by cool white fluorescent tubes (16 h:8 h light/dark cycle). The cultures were vigorously bubbled with filtered (0.22  $\mu\text{m}$  Millipore, France) air at a flow rate of 0.23  $\text{L min}^{-1}$ . After three weeks of culture, samples of biomass were collected by centrifugation (25,000 g, 5 min) and lyophilized for further extraction of MCs as described by Corbel et al. (2015). Briefly, cyanobacterial cells were homogenized with 75% (v/v) aqueous methanol (MeOH). The homogenate was introduced for 3 min in an ultrasonic bath (Ultrasonic 300), followed by centrifugation at 4000 g at 4 °C for 10 min. The supernatant was

retained and the residue re-extracted two times as before. Combined supernatants were stored at -20 °C prior to be used for the chronic exposure of the tomato plant. Total MCs concentration, in this aqueous methanol extract determined by the PP2A inhibition assay was 21.6 mg eq. MC-LR L<sup>-1</sup> corresponding to 6.77 mg eq. MC-LR g<sup>-1</sup> dried cells as recently been shown by Corbel et al. (2015). The main congener present in this extract was MC-LR (68.4%). Five other major characterized congeners of MCs were also present: MC-LF (8.3%), demethylated [Asp3]-LR (6.9%), MC-LW (6.5%), MC-LY (2.9%) and MC-YR (2.4%). More details for the identification of all microcystin congeners on this extract are available in Corbel et al. (2015).

#### Biosynthesis and purification of the radiolabeled MC-LR (<sup>14</sup>C-MC-LR)

The same strain PCC7820 was used to synthesize the <sup>14</sup>C radiolabeled MC-LR as previously reported by Corbel et al. (2014b). Briefly, the strain was axenically grown in 1-L sealed flask containing 500 mL of BG11 liquid medium supplemented with 2 mM NaNO<sub>3</sub> and 5 mM unlabeled NaHCO<sub>3</sub>. The culture was continuously bubbled with sterile air at a flow rate of 0.23 L min<sup>-1</sup>, and the outlet of the flask was connected to a CO<sub>2</sub> trap containing 100 mL of 1 N NaOH solution that freshly changed every 2–3 days. Three additions of an aqueous solution of 0.62 MBq <sup>14</sup>C-NaHCO<sub>3</sub> (specific activity 310 MBq mmol<sup>-1</sup>, supplied from American Radiolabeled Chemicals, Isobio, Fleurus, Belgium) were performed after 7, 10 and 13 days of culture. The culture was stopped 4 days later, corresponding to a 10-day period of growth in presence of labeled bicarbonate. The labeled microcystin leucine–arginine (<sup>14</sup>C-MC-LR) was purified as described by Corbel et al. (2014b). The concentration of <sup>14</sup>C-MC-LR was estimated with the unlabeled MC-LR standard purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) and the radioactivity of the solution was determined by liquid scintillation counting. The concentration of <sup>14</sup>C-MC-LR in the purified extract obtained by semi-preparative HPLC was 26.6 mg L<sup>-1</sup> corresponding to a specific activity of 577 kBq mmol<sup>-1</sup>.

#### Chronic exposure of the tomato MicroTom with unlabeled cyanobacterial extract containing microcystins in a plant–soil system

The topsoil from the experimental meadow site of INRA, Pierre-Plate (Versailles, France), collected in August 2012 was used for the culture of the tomato *S. lycopersicum* cultivar MicroTom. It was a silty sand soil (Luvic Cambisol/Cambisol, IUSS Working Group, 2006) comprising 76%

sand, 13% silt and 11% clay. Clay consisted mainly in illite/smectite. Its content in organic matter was 37.8‰ and its pH water was 5.6. Its water holding capacity was 35.5%. The soil was homogenized, air dried, sieved at 2 mm and stored at 20 °C. In each pot, 350 g of this soil was disposed and moistened at 50% of water holding capacity during 14 days. After this delay, one tomato seed, previously sterilized by washing with sodium hypochlorite solution (1.6% active chloride) during 1 min and rinsed three times with Milli-Q water, was sown in each pot. After the seed were sown, the seedlings (five replicates) were daily exposed to different cyanobacterial extract concentrations: 0, 5, 20, 50 and 100 µg eq. MC-LR L<sup>-1</sup>, diluted in nutritive solution NPK (15–10–30) purchased from Fertil society (Boulogne-Billancourt, France) with a final concentration of 0.75 g L<sup>-1</sup>. Milli-Q water containing methanol (MeOH) with the highest concentration (0.35% final concentration) was used to irrigate the control group. At this concentration, MeOH has no effect on the plant growth. The plant samples were disposed in a greenhouse chamber with an incident light intensity of 160 µmol of photons m<sup>-2</sup> s<sup>-1</sup> provided by cool white fluorescent tubes (Osram, Cool DayLight 865) during 16 h:8 h light/dark cycle. The temperature was maintained at 26 °C/21 °C (Day/Night) and the humidity of the soil for each treatment group was maintained at 60–70% of the water holding capacity, during 90 days, by daily irrigation. After the period of exposure, the soil was mixed and a subsample (approximately 20 g of fresh soil) was recovered. Tomato plants were harvested and roots were washed with Milli-Q water. Freshly harvested samples of soil and all plant organs (root, stem, leave and fruit) were frozen, lyophilized and then stored at -20 °C until further microcystins extraction and analysis.

#### Extraction and analysis of unlabeled microcystins from the soil

An aliquot of 8 g of each soil samples was extracted in a 50 mL tube with 15 mL of a mixture containing 0.1 M sodium pyrophosphate, 0.1 M ethylenediaminetetraacetic acid (EDTA) and 0.002 M trifluoroacetic acid (TFA) as described by Chen et al. (2006a) and Corbel et al. (2014b). The samples were agitated for 5 min on an orbital shaker at 300 rpm (KS501 digital, Ika), sonicated for 10 min at a temperature below 30 °C and centrifuged at 4000 g and 4 °C. The supernatant was collected and the residue re-extracted two times as before. The pooled supernatants were then semi-purified on C18 cartridges Bond Elut C18 (0.5 g) (Agilent Technologies, CA, USA) conditioned with 5 mL pure MeOH and 5 mL Milli-Q water. MCs were eluted with 3 mL MeOH acidified with 0.1% (v/v) TFA and the eluate was then divided in two equal aliquots, each being dried under

nitrogen flux. The residues were dissolved in 100  $\mu\text{L}$  of 50% (v/v) acetonitrile/water and 100  $\mu\text{L}$  50% (v/v) MeOH/water, respectively. The quasi-quantitative efficiency of this extraction method has been demonstrated through soil spiking at 5  $\mu\text{g MC-LR kg}^{-1}$  and extraction after 24 h of contact, i.e. without aging processes. The result showed that the observed concentration of MC-LR was underestimated by a factor of approximately 2 (global yield  $55.7 \pm 8.5\%$ ,  $n = 3$ ). The sample dissolved in 50% (v/v) MeOH was analyzed by the protein phosphatase type 2A (PP2A) inhibition assay according to Bouaiïcha et al. (2001) for quantification of total free MCs concentrations using the MC-LR as standard. However, the sample dissolved in 50% (v/v) acetonitrile was analyzed by the Ultra- High-Performance Liquid Chromatography tandem mass spectrometry (UHPLC–MS/MS) using a Waters Acquity UPLC system coupled to a triple-quadrupole mass spectrometer (TQD, Waters, France) via an electrospray interface. The two methods are complementary, the PP2A is more sensitive and it allows the quantification of all congeners of microcystin without making a distinction between them; the result is, therefore, expressed in equivalent MC-LR used as standard. However, the technique UHPLC–MS/MS is less sensitive, but it is much more specific. It allows at the same time to quantify the MC-LR, the major congener in the cyanobacterial extract used for the contamination of the irrigation water, and to confirm the presence of this toxin in the different samples tested. The electrospray source operated in positive mode with the following settings: capillary 3 kV, cone voltage 60 V, source and desolvation gas temperature 120 °C and 300 °C respectively, flow of desolvation gas and cone gas (nitrogen) 800 L h<sup>-1</sup> and 20 L h<sup>-1</sup>, respectively. Column conditions consisted of an Acquity UPLC BEH C18 (100  $\times$  2.1 mm i.d., 1.7  $\mu\text{m}$ ) maintained at 30 °C with a linear gradient (300  $\mu\text{L min}^{-1}$ ). The mobile phase was composed of Milli-Q water and acetonitrile at a flow of 0.3 mL min<sup>-1</sup>, both containing 0.1% of acetic acid. The acetonitrile phase linearly increased from 5% to 55% in 7.5 min, then increased to 100% in 1 min and was held for 4.5 min. The injection volume was 10  $\mu\text{L}$ . Analyses in MS–MS were performed in Multiple Reaction Monitoring (MRM) mode using argon for collision induced dissociation (50 eV, 3.4 mbar in collision cell). The detection was achieved using three specific transitions suggested by Schmidt et al. (2013): the major transition 995.3  $\rightarrow$  135 (m/z) was used for quantification of MC-LR, two others: 995.3  $\rightarrow$  213 (m/z) and 995.3  $\rightarrow$  107 (m/z) allowed confirming the identity of MC-LR. External calibration was performed using a standard MC-LR purchased from Sigma-Aldrich. The limit of quantification was approximately 0.12  $\mu\text{g kg}^{-1}$  in soil extract. Direct spiking of soil extract just prior to its LC–MS/MS analysis allowed evaluating the matrix effect.



## Extraction, identification and quantification of unlabeled MC-LR from tomato tissues

The lyophilized tomato tissues (root, stem, leave and fruit at different stages of maturity) were ground with mortar to fine powders. An aliquot of 0.05 g of root, 0.5 g of stem/leave, and 2 g of fruit were homogenized with 2, 7 and 20 mL of 75% (v/v) aqueous MeOH, respectively and then centrifuged at 4000 g for 10 min at 4 °C. The supernatant was collected and the residue re-extracted two times as before. The pooled supernatants were diluted to a final concentration 20% (v/v) aqueous MeOH and were then semi-purified on C18 cartridge Bond Elut C18 (0.5 g) (Agilent Technologies, CA, USA) as described above. MCs were eluted with 3 mL MeOH acidified with 0.1% (v/v) TFA and the eluate was then dried under nitrogen flux. The residue was dissolved in 50  $\mu$ L 50% (v/v) acetonitrile and stored at -20 °C before analysis by UHPLC–MS/MS as described above for confirmation and quantification of the major MC-LR in each plant tissue sample. Limits of quantification were 2 and 0.2  $\mu$ g MC-LR kg<sup>-1</sup> in roots and leaves, respectively. The detection of total MCs by the PP2A assay has not been possible because the matrix of the different extracts of each tissue of the plant is too loaded and the analysis did not allow giving reliable results.

## Exposure of the tomato MicroTom with the radiolabeled <sup>14</sup>C-MC-LR in hydroponic conditions

The sterilized tomato seeds, were sown in sterilized sand (2 mm) contained in a plastic box and saturated in water. The box was disposed in an incubator at 25 °C with incident light of 160  $\mu$ mol of photons m<sup>-2</sup> s<sup>-1</sup> provided from two fluorescent tubes (55 W/864) during 16 h/8 h light/dark cycle. After 14 days, each seedling was disposed in an Erlenmeyer containing 100 mL of nutritive solution (NPK 15–10–30). One replicate of each exposed and non-exposed (control) seedlings was used. The two Erlenmeyer flasks were enveloped by aluminum paper to maintain the roots and nutritive medium in the dark and then was placed in an incubator with the same conditions described above where the nutritive solution was continuously bubbled with air ( $Q_{\text{air}} = 0.2 \text{ L min}^{-1}$ ). Every 3 days, the level of nutritive solution was readjusted with a new input of nutritive solution. After 82 days, the nutritive solution of one Erlenmeyer was replaced by 36 mL of nutritive solution containing the labeled <sup>14</sup>C-MC-LR (470 Bq) corresponding to a final concentration of 23 mg L<sup>-1</sup> of MC-LR. The tomato plant was exposed during 24 h and then 20 mL of nutritive solution without labeled MC-LR was added for 24 h supplementary. As indicated in the Section 2.1.2, the specific activity of the radiolabeled MC-LR biosynthesized was low, therefore, in order to have a quantity of radioactivity

sufficient to be quantified in the different tissues of the tomato plant, we were forced to use a high concentration of toxin ( $23 \text{ mg L}^{-1}$ ) but for a duration of contact more short in order to avoid a strong phytotoxicity. The second Erlenmeyer with nutritive solution containing methanol (vehicle to dissolve MC-LR) at 0.35% final concentration was used for the control plant. After exposure, the roots of the plants were rinsed three times with Milli-Q water and the tissues (root, stem, leave and fruit at different stages of maturity) of each plant were harvested and dried at  $80^\circ\text{C}$  for 24 h.

#### Extraction and analysis of $^{14}\text{C}$ -MC-LR from plant tissues

Dry tissues (root, stem, leave and fruit) of the exposed and non- exposed (control) plants were ground with mortar to fine powder. An aliquot (0.1-0.2 g) of each tissue powder was used to the determination of the total radioactivity by combustion with a Biological Oxidizer OX 700 (Zinsser Analytic, Frankfurt, Germany). The  $^{14}\text{CO}_2$  produced, reflecting the total radioactivity quantity accumulated in each tissue of the exposed plant, was trapped and measured with Oxysolve C400 (Zinsser analytic) as liquid scintillation cocktail. Other aliquots of each plant tissue (0.05 to 1 g) were homogenized with 75% (v/v) aqueous MeOH for 3 successive extractions as described above. The radioactivity in the liquid extract of each plant tissue sample was obtained by liquid scintillation and corresponded to the free extractable fraction of the radiolabeled  $^{14}\text{C}$ -MC-LR. Furthermore, the remaining pellets were dried and burnt to obtain the non-extractable covalently bound fraction of this toxin.

#### Statistical analysis

Data concerning the concentrations of MC-LR in plant organs were represented by the means  $\pm$  the standard deviation (SD). The normality and homogeneity of variance were checked with Kolmogorov-Smirnov and Bartlett tests with a significance level of 5%. The data, that didn't verify these conditions, were compared by a non-parametric comparison using a Kruskal-Wallis test at a probability level of 0.05. If there is a difference, “nparcomp” post-hoc assay was used.

## Results

### Quantification of MC-LR equivalent and MC-LR congener in the soil

The agricultural soil was daily irrigated for a period of 90 days with several concentrations of a semi-purified *M. aeruginosa* cyanobacterial extract containing environmentally relevant concentrations (0 to 100  $\mu\text{g eq. MC-LR L}^{-1}$ ) of microcystins (Corbel et al., 2014a). Subsamples of the exposed and the control soil were extracted and analyzed using two methods: the inhibition of protein phosphatase type 2A (PP2A) assay and with UHPLC–MS/MS for the quantification of total free extractable MCs expressed as MC-LR equivalent and the major MC-LR congener, respectively. After the chronic exposure for 90 days of the plant–soil system with different concentrations of MCs (5, 20, 50 and 100  $\mu\text{g eq. MC-LR L}^{-1}$ ), the free extractable concentrations of MCs accumulated in the soil are very low and varied between 1.6 and 3.9  $\mu\text{g eq. MC-LR kg}^{-1}$  DW according to PP2A inhibition assay (Table 1). These free extractable fractions of MCs represent no more than 3.47% of the total amount of MCs added to the soil during the entire period of the experiment of 90 days. The analysis by UHPLC–MS/MS was focused only on the congener MC-LR, the more preponderant in the cyanobacterial extract. Its identity was ascertained by the signal collected through all MRM transitions. As through the enzymatic method, the concentrations found in the soil are weak, varying between 0.26 and 0.36  $\mu\text{g kg}^{-1}$  DW (Table 1). As described in the Section 2.2.1, soil spiking by MC-LR 24 h before extraction showed that these concentrations were underestimated by a factor of approximately 2. It was potentially due both to extraction yield and to matrix effect through ion suppression during electrospray ionization. Direct spiking of soil extract just prior to its LC–MS/MS analysis allowed evidencing that underestimation was only due to matrix effect ( $54.3 \pm 7.4\%$ ). However, and similarly as for all microcystin congeners detected by the PP2A assay, the accumulated quantity of the congener MC-LR in the soil after 90 days of exposure never exceeded 1% of the total delivered quantity (Table 1).

### Quantification of unlabeled MC-LR congener in tomato tissues

Quantification of the free extractable MC-LR congener in plant tissues was accomplished by UHPLC–MS/MS analysis. As in soil extracts, the most sensitive MRM transition, from  $m/z$  995.3 to 135, allowed detecting MC-LR and quantifying it through external calibration. The microcystin

identity was ascertained by the signal also observed at the same retention time for the two confirmation transitions ( $m/z$  995.3  $\rightarrow$  107 and  $m/z$  995.3  $\rightarrow$  213). The extraction yields of the MC-LR from plant tissues could not be determined as spiking the dry tissues could not mimic potential inclusion in the vegetal. Spiking the extracts of roots and leaves (with 0.2 and 0.4 ng MC-LR, respectively) allowed evidencing a reduced but positive matrix effect, the signal enhancement being evaluated as  $15.1 \pm 5.6\%$  and  $27.3 \pm 1.4\%$ , respectively. MC-LR concentrations in leaves from tomato plant exposed to cyanobacterial extracts (20 to 100  $\mu\text{g eq. MC-LR L}^{-1}$ ) varied between 0.29 and 0.55  $\mu\text{g kg}^{-1}$  DW, while in roots from tomato plant exposed to all cyanobacterial extracts (5 to 100  $\mu\text{g eq. MC-LR L}^{-1}$ ) the concentrations reported were between 4.5 and 8.1  $\mu\text{g kg}^{-1}$  DW (Table 1). These MC-LR concentrations at the end of the exposure experiment (90 days) are very low and never exceeded 0.001% of the total MCs quantity exposed to the plant during this period (Table 1).

#### Transfer and bioconcentration of $^{14}\text{C}$ -MC-LR in tomato plant tissues

The quantification of the total radioactivity transferred in the tomato plant that was grown during 48 h in hydroponic conditions with nutritive solution containing the labeled  $^{14}\text{C}$ -MC-LR (470 Bq) at a final concentration of 23  $\text{mg L}^{-1}$  was determined by combustion of an aliquot of each plant tissue (root, leave, stem, and fruit). For the determination of the free extractable fraction of the radioactivity, each plant tissue was extracted with 75% (v/v) aqueous MeOH and the remaining pellet was further combusted for the determining of the non-extractable fraction. After exposure to the labeled  $^{14}\text{C}$ -MC-LR, 52% of the initial total radioactivity was remained in the plant nutritive medium contained in the Erlenmeyer. However, the percentage of the total radioactivity accumulated in the plant tissues was only 48%. This radioactivity accumulated in the plant was distributed differently in the plant tissues with 56.1% in leaves, 37.5% in roots, and 6.3% in stems (Fig. 1). However, no radioactivity was detected in the fruit. Therefore, the bioconcentration factor (BCF) was 13.6 in roots, 4.5 in leaves, and 1.4 in stems. The percentage of the extractable and non-extractable labeled carbon was 5.1 and 3.8 in the stems, 12.9 and 16.2 in the roots, and 18.0 and 44.0 in the leaves, respectively (Fig. 1). Therefore, the non-extractable fraction of radioactivity, corresponding to the covalently bound MC-LR, was higher than that of the extractable fraction only in roots and leaves reaching 56% and 71% of total accumulated of the toxin, respectively. Taking into account the specific activity of the labeling of the MC-LR, the concentration of the toxin accumulated in each tissue of the plant varies between 311.8  $\text{mg MC-LR kg}^{-1}$  DW for the roots,

103.6 mg MC-LR kg<sup>-1</sup> DW for the leaves, and 30.4 mg MC-LR kg<sup>-1</sup> DW for the stems.

## Discussion

The persistence of MCs both in the irrigation water and in the soil can have a considerable impact on soil organisms as well as on the growth and development of cultivated plants. In addition, for risk assessment purposes it is important to know if MCs can be transferred and accumulated in edible tissues of plants when chronically irrigated with water containing realistic environmental concentrations of these toxins. In the present study we evaluated the transfer and the bioaccumulation of MCs in different tomato plant *S. lycopersicum* cv. MicroTom tissues after daily irrigation for 90 days with water containing a cyanobacterial extract with different environmentally relevant concentrations of MCs between 5 and 100 µg eq. MC-LR L<sup>-1</sup>. The detection of MC-LR in tomato tissues was accomplished by solvent extraction, which is limited only to the free extractable fraction (i.e., non-covalently bound) of toxin. This fraction may be the most important for monitoring and for food safety assessment since it is readily available and bioactive. Before dosing MC-LR in the different tissues of the plant, we have first studied the persistence of total MCs in the soil. In this regard, free extractable total MCs and MC-LR congener were detected by the PP2A enzymatic inhibition assay and UHPLC–MS/MS analysis, respectively. After the period of treatment, the detection of total MCs and the MC-LR congener was evidenced in the soil extract and their observed concentrations were very low varying between 1.3 and 3.9 µg eq. MC-LR kg<sup>-1</sup> DW and between 0.34 and 0.36 µg MC-LR kg<sup>-1</sup> DW, respectively. Consequently, these concentrations represent no more than 4% (Table 1) of the total amount of toxins added to the soil during the entire period of the experiment of 90 days. This could be explained either by their degradation in the soil, or their covalent binding to the clay or organic matters of the soil, or their transfer and accumulation in the tissues of the plant grown on this soil.

MCs are water-soluble molecules, and their cyclic structure provides them a high chemical stability. Nevertheless, numerous studies reported that during soil irrigation practice, one part of MCs may be degraded rapidly by sunlight or by some species of soil bacteria (Bourne et al., 2001; Manage et al., 2009) but another part could persist. Their half-life in soils was estimated between 6 and 17.8 days (Chen et al., 2006b). Miller et al. (2001) and Morris et al. (2000) showed that the clay and organic matter content in soils played a key role in the MCs adsorption. In support of this hypothesis, different studies have shown a decrease of MCs concentrations in soils with increasing

carbon and clay content (Miller and Fallowfield, 2001; Morris et al., 2000). On the other hand, it seems that the adsorption of MCs onto the soil was low. In a recent study, based on the use of radiolabeled MC-LR ( $^{14}\text{C}$ -MC-LR), Corbel et al. (2014b) observed that this heptapeptide cyanotoxin when it introduced in a silty sand soil remains quantified in high amounts (74-80%) in aqueous extracts from the soil and, therefore, less than 14% of  $^{14}\text{C}$ -MC-LR was adsorbed on soil particles. In addition, the authors reported that the  $^{14}\text{CO}_2$  from the microbial mineralization under aerobic conditions of the  $^{14}\text{C}$ -MC-LR amounted to only 11% of the total  $^{14}\text{C}$  initial input. Consequently, the very low concentrations of total MCs and MC-LR congener detected in the soil after chronic exposure to a *M. aeruginosa* extract containing MCs during 90 days can be explained by the translocation of these toxins on the tomato plant tissues grown on this soil. In fact, Mohamed and Al Shehri (2009) reported that different crops growing in the field and irrigated with contaminated groundwater ( $0.3\text{--}1.8\ \mu\text{g L}^{-1}$  MCs) accumulated MCs between  $50$  and  $1200\ \mu\text{g kg}^{-1}$  FW (fresh weight). In addition, laboratory studies have shown that clover, rape, and lettuce plants accumulated between  $200$  and  $1450\ \mu\text{g kg}^{-1}$  DW MCs when exposed to  $2100\ \mu\text{g L}^{-1}$  MCs for 15 days (Crush et al., 2008). However, in the present work, when we evaluated the accumulation of MC-LR in different tissues of the tomato *S. lycopersicum* cv. MicroTom cultivated in a soil-plant system, we observed at the end of the exposure experiment (90 days) that concentrations of free extractable MC-LR accumulated in roots and leaves of the plant are very low ( $0.29\text{--}8.1\ \mu\text{g kg}^{-1}$  DW) and never exceeded 0.001% of the total MCs quantity exposed to the plant during this period. However, with similar realistic environmental concentrations ( $5\ \mu\text{g L}^{-1}$  MC-LR or MC-LF) used for spray irrigation for 24 h, Peuthert et al. (2007) reported the accumulation of MCs in roots and shoots of seedlings of 11 agricultural crops with concentrations varying between 12 and 125; and 4 and  $44\ \mu\text{g kg}^{-1}$  FW, respectively. On the other hand, Mohamed and Al Shehri (2009) reported that when different crops growing in the field and irrigated with contaminated groundwater ( $0.3\text{--}1.8\ \mu\text{g L}^{-1}$  MCs) accumulated MCs with concentrations varying between 70 and  $1200\ \mu\text{g kg}^{-1}$  FW. When high concentrations of MCs were used, Crush et al. (2008) reported that exposure via roots of clover, rape, and lettuce plants, with lake water containing  $2100\ \mu\text{g L}^{-1}$  total MCs during 15 days, led to the accumulation of  $200\text{--}1450\ \mu\text{g kg}^{-1}$  root DW. Moreover, Chen et al. (2010) reported that *Malus pumila* shoot cultures accumulated MCs up to  $510\ \mu\text{g kg}^{-1}$  FW after 14 days of exposure to  $3000\ \mu\text{g L}^{-1}$  total MCs. In general, as reported in the present work for the tomato plant, vegetables accumulated more toxins in the roots than in the other tissues. However, in a recent study Gutiérrez-Praena et al. (2014) reported for the first time that the tomato species *Lycopersicon esculentum*, exposed for 2 weeks to either pure MC-LR ( $100\ \mu\text{g L}^{-1}$ ) or *M. aeruginosa* crude extracts containing

100  $\mu\text{g L}^{-1}$  MC-LR, accumulated more MCs in leaves (12,298  $\mu\text{g kg}^{-1}$  FW). The authors suggest for this exception plant a putative role of evapotranspiration at the leaf level that is the main motor of water movement from the soil across the plant and allowing the uptake and accumulation of soil pollutants in plant leaves.

Therefore, the low concentration of free extractable MC-LR detected both in the soil and plant tissues in the present work must be due either to its degradation or to its adsorption or both. In this regard, Miller et al. (2001) demonstrated that loss of MCs in different soils was controlled by a fast adsorption to soil particles and a slower microbial degradation. Bibo et al. (2008) were investigated under laboratory conditions the adsorption and biodegradation of MC-RR in cropland soils and they conclude that MC-RR at 6500  $\mu\text{g L}^{-1}$  could be completely degraded within 6 days with a lag phase of 1–2 days. In the presence of humic acid, the same amount of MC-RR could be degraded within 4 days without a lag phase.

For risk assessment purposes it is important to know if MC-LR can be transferred and accumulated in edible tissues (fruit) of the tomato plant that is chronically irrigated with water containing realistic concentrations of MCs. In this respect, accumulation of MCs in water chestnuts (average values of 7  $\mu\text{g kg}^{-1}$  DW) (Xiao et al., 2009), rice grains (between 1.01 and 3.19  $\mu\text{g kg}^{-1}$  MC-LR) (Chen et al., 2012), and *Capsicum annuum* fruits (1.03  $\mu\text{g kg}^{-1}$  DW total MCs) (Romero-Oliva et al., 2014) provide further evidence that MCs are accumulated in vegetable products destined for human consumption. More recently Romero-Oliva et al. (2014) reported 1.16  $\mu\text{g kg}^{-1}$  DW total MCs in fruits of *L. esculentum* field cultivated with lake water highly contaminated with MCs (intra- and extracellular 1931 and 90  $\mu\text{g L}^{-1}$ , respectively). In addition, Gutiérrez-Praena et al. (2014) showed that the tomato species *L. esculentum*, exposed for 2 weeks to either pure MC-LR (100  $\mu\text{g L}^{-1}$ ) or *M. aeruginosa* crude extracts containing 100  $\mu\text{g L}^{-1}$  MC-LR, accumulated MCs in green tomatoes (5.15–5.41  $\mu\text{g kg}^{-1}$  FW), and in mature tomatoes (10.52–10.83  $\mu\text{g kg}^{-1}$  FW). However, in the present study, we have observed that even in the high concentration of MCs (100  $\mu\text{g eq. MC-LR L}^{-1}$ ) used for the irrigation of the tomato *S. lycopersicum* cv. MicroTom cultivated in a soil–plant system, the accumulation of MC-LR was observed only in the leaves and the roots but not in the fruits. Compared to the results reported by Gutiérrez-Praena et al. (2014), the lack of detection of free MC-LR in the tomato fruits can be explained by the duration of exposure of 90 days in the present study versus 2 weeks in the study of Gutiérrez-Praena et al. (2014). In effect, in the last study MC-LR was detected in the tomato fruits only in the first week of exposure and concentrations in fruits decreased to below the limit of detection during the second week of the

exposure experiment. The authors suggest that the lack of detection of MC-LR during the second week is not just a consequence of dilution in the fruits due to an increase in the volume of the fruits and increase in water accumulation related to the fruit growth and maturation, from week 1 to week 2, it may also be metabolized and becomes inaccessible for quantification as a result of binding to other biomolecules, e.g., protein phosphatases or the peptide glutathione.

In order to confirm these results and make an overall assessment of the distribution of the MC-LR in the various tissues of the tomato plant, we have grown this plant during 48 h in hydroponic conditions with nutritive solution containing the labeled  $^{14}\text{C}$ -MC-LR (470 Bq) at a final concentration of  $23 \text{ mg L}^{-1}$ . This approach allowed a specific study of the transfer and accumulation of MCs in plants. As the results obtained after 90 days of irrigation with a *M. aeruginosa* extract containing MCs, we confirmed that the radioactivity was detected only in leaves and roots, and in addition in stems but not in fruits. The highest radioactivity reflecting MC-LR accumulation in the plant tissues was found in the order roots N leaves N stems. The average bioconcentration factor (BCF) in roots was 13.6, in leaves 4.5 and in stems 1.4. With the exception of the fruit tissue, all other *S. lycopersicum* cultivar MicroTom tissues presented toxin bioconcentration factor within the range of bioconcentration factors reported previously for 11 agricultural seedlings (Peuthert et al., 2007).

By using radioactive MC-LR, a large total amount (free and bound) of this cyanotoxin has been observed to accumulate in all tomato tissues except in the fruits, reaching  $311.8 \text{ mg MC-LR kg}^{-1} \text{ DW}$  for the roots,  $103.6 \text{ mg MC-LR kg}^{-1} \text{ DW}$  for the leaves, and  $30.4 \text{ mg MC-LR kg}^{-1} \text{ DW}$  for the stems. Therefore, after 48 h of exposure in hydroponic conditions, the concentrations of non-extractable radiolabeled carbon, reflecting the binding of MC-LR to some biomolecules, in roots and leaves were  $173.6$  and  $73.5 \text{ mg }^{14}\text{C-MC-LR kg}^{-1} \text{ DW}$ , respectively. Consequently, the non-extractable fraction of MC-LR, corresponding to the covalently bound MC-LR, was higher than that of the free fraction in roots and leaves reaching 56% and 71% of total accumulated toxin, respectively. However, labeled carbon was not detected in fruits even when we have combusted an aliquot of this plant tissue without solvent extraction. The observed differences in the absence of MC-LR detection in the tomato fruits in the present experiments compared to that reported by Gutiérrez-Praena et al. (2014) may be due either to exposure time shorter 48 h versus 1 week or to the concentration of MC-LR used  $23 \text{ mg L}^{-1}$  versus  $100 \text{ } \mu\text{g L}^{-1}$ . After 48 h exposure, the toxin may have not the necessary time for translocation to the fruit tissue. Furthermore, we speculate that the high concentration ( $23 \text{ mg L}^{-1}$ ) of MC-LR used in the current study may be inhibiting the mechanisms of its uptake and accumulation in the fruit tissue. Therefore, the necessity to obtain



more information regarding the dynamics of MCs uptake and accumulation, and regarding the mechanisms of their uptake, translocation, and metabolization become evident.

## Conclusion

In this work, tomato plants were exposed for 90 days in a soil-plant system to MC-LR in *M. aeruginosa* extracts or for 48 h in hydroponic conditions to pure radiolabeled MC-LR ( $^{14}\text{C}$ -MC-LR), followed by an investigation of the toxin accumulation in the different plant tissues. At the end of exposure (90 days), the extractable fraction of MC-LR was detected in the soil but at low concentrations, suggesting its degradation or adsorption on particles and/or organic matters of the soil. Moreover, the toxin was detected in roots and leaves, suggesting that it is taken up by the plant and is transported to the different organs except fruits. By using pure radiolabeled MC-LR ( $^{14}\text{C}$ -MC-LR), we observed similar results showing a growing accumulation of this toxin within the organs roots N leaves and in addition stems, but not in fruits. On the other hand, results highlight that the covalently bound fraction of MC-LR was higher than that of the free extractable fraction in roots and leaves reaching 56% and 71% of the total accumulated toxin, respectively.

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Table 1. Concentrations of the total extractable MCs ( $\mu\text{g eq. MC-LR kg}^{-1} \text{ DW}$ ) or the extractable congener MC-LR ( $\mu\text{g MC-LR kg}^{-1} \text{ DW}$ ) in soil and plant tissue (leaves and roots) samples after 90 days of irrigation with realistic environmental concentrations of a *M. aeruginosa* extract containing 5 to 100  $\mu\text{g eq. MC-LR L}^{-1}$ . Value in parentheses represents the percentage of extractable MCs or MC-LR found in soil and plant tissue samples by the report of the total amount of MCs exposed to the soil–plant system during 90 days. Results data are expressed as mean  $\pm$  SD ( $n = 5$ ). Different letters show significant differences at  $P < 0.05$ .

Sample	Treatments with <i>Microcystis aeruginosa</i> extract ( $\mu\text{g eq. MC-LR L}^{-1}$ )				
	Control	5	20	50	100
Soil					
$\mu\text{g eq. MC-LR kg}^{-1} \text{ DW}^*$	nd <sup>a</sup>	$1.6 \pm 0.2^b$ (3.47)	$1.7 \pm 0.2^b$ (0.93)	$1.3 \pm 0.1^c$ (0.28)	$3.9 \pm 0.9^d$ (0.42)
$\mu\text{g MC-LR kg}^{-1} \text{ DW}^a$	nd <sup>a</sup>	$0.34 \pm 0.10^b$ (0.75)	$0.26 \pm 0.12^b$ (0.14)	$0.32 \pm 0.07^b$ (0.06)	$0.36 \pm 0.17^b$ (0.04)
Plant tissues					
Leaves, $\mu\text{g MC-LR kg}^{-1} \text{ DW}^a$	nd <sup>a</sup>	nd <sup>a</sup>	$0.29 \pm 0.06^b$ ( $1.10^{-4}$ )	$0.33 \pm 0.01^b$ ( $5.10^{-5}$ )	$0.55 \pm 0.19^c$ ( $4.10^{-5}$ )
Roots, $\mu\text{g MC-LR kg}^{-1} \text{ DW}^a$	nd <sup>a</sup>	$4.5 \pm 1.1^b$ ( $7.10^{-4}$ )	$4.8 \pm 0.6^b$ ( $2.10^{-4}$ )	$5.7 \pm 0.8^b$ ( $9.10^{-5}$ )	$8.1 \pm 1.0^c$ ( $7.10^{-5}$ )

\* Concentration of MCs estimated with the PP2A inhibition assay.

<sup>a</sup> Concentration of MC-LR estimated with the UHPLC–MS/MS analysis.

Fig. 1. Percentage of the total (black), extractable fraction (gray), and non-extractable fraction (white) of radioactivity accumulated in the different organs (leave, stem and root) of the tomato *Solanum lycopersicum* cv. MicroTom aged 84 days and exposed to the  $^{14}\text{C}$ -MC- LR at  $23\text{ mg L}^{-1}$  (470 Bq) during 48 h in hydroponic growing system.

