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# Soil irrigation with toxic cyanobacterial microcystins increases soil nitrification potential

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## Abstract

Microcystins are cyclic heptapeptide hepatotoxins produced by aquatic cyanobacteria such as *Microcystis aeruginosa*. The wide occurrence of toxic microcystins in freshwater is a threat to the quality of water, agriculture, and human and animal health. There is actually little knowledge on the impact of microcystins on soil biomass. Here, an agricultural soil was daily irrigated with a cyanobacterial extract diluted at environmental concentrations of microcystin–leucine–arginine, from 0.005 to 0.1 mg equivalent MC-LR L<sup>-1</sup>, for 90 days. We measured soil enzymatic activities, nitrification potential activity of the soil microbial community, abundances of ammonia-oxidizing bacteria, and ammonia-oxidizing archaea amoA genes. Our results show an increase in potential nitrification for microcystin levels ranging between 0.005 and 0.02 mg eq. MC-LR L<sup>-1</sup>. Global enzymatic activities were unchanged. Abundances of total bacteria, archaea, and ammonia-oxidizing functional groups were not modified and could not explain the increase in nitrification.

## Keywords

Microcystins, Irrigation, Soil, Nitrification, Enzymatic activities, Microbial ecotoxicology

## Introduction

In light of global climate change, the development of cyanobacteria in freshwater, as bloom, will go on world-wide. One main associated concern is the production and release in the environment of different cyanotoxins that are proved toxic for humans. They affect the nervous system (neurotoxins), the skin (dermatotoxins), and the liver (hepatotoxins). Among them, the hepatotoxic microcystins are commonly found in 40–75 % of the cyanobacterial blooms (Sivonen and Jones 1999). Microcystins are cyclic heptapeptides, which exhibit high structural variability because of (1) the identity of amino acids, mainly in positions 2 and 4 of the ring; (2) side-chain modifications, such as desmethylation; and (3) variations in the Adda moiety (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid). The MC-LR (microcystin with leucine and arginine in positions 2 and 4, respectively) is the most studied due to its high frequent detection in freshwater, but at least 90 congeners have been identified to date (Puddick et al. 2013). After the Caruaru accident in 1996 (Brazil) where 60 patients died in a hemodialysis center (Pouria et al. 1998), the World Health Organization established a concentration limit of  $1 \mu\text{g MC-LR L}^{-1}$  in drinking water. In the aquatic ecosystems, the concentrations of microcystins often exceed this limit and induce deleterious effects on aquatic plants and animals (Corbel et al. 2014b). Irrigation is necessary for agricultural production and may use waters contaminated by microcystins and without a prior treatment. Consequently, recent studies focused on the assessment of phytotoxic effects of microcystins on terrestrial plants (Chen et al. 2004; Saqrane et al. 2008; El Khalloufi et al. 2012; Corbel et al. 2015). Although we reported that microcystins could be persistent in soils, little is known about their potential effects on soil biological life (Corbel et al. 2014a, b). Concerning microorganisms, we recently highlighted that irrigation for 15 days with water containing microcystins impacted soil nitrification processes (Corbel et al. 2015).

In the present study, we aim at determining the effects on a 90-day irrigation with water containing microcystins at realistic environmental concentrations on the activity of soil microbial communities, considering global enzymatic activities, nitrification potential activity, and the abundances of ammonia-oxidizing bacteria and ammonia-oxidizing archaea amoA genes.

## Materials and methods

### Culture of *Microcystis aeruginosa* strain PCC7820

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 NaNO<sub>3</sub> (2 mM) and NaHCO<sub>3</sub> (10 mM), at 25 °C, with an incident light intensity of 5-10 μmol of photons m<sup>-2</sup> s<sup>-1</sup> provided by cool white fluorescent tubes (16:8 h light/dark cycle), as previously reported by Gupta et al. (2001) and Robillot and Hennion (2001). The cultures were vigorously bubbled with filtered (0.22 μm Millipore, France) air at a flow rate of 0.23 L min<sup>-1</sup>. After 3 weeks of culture, samples of biomass were collected by centrifugation (25,000g, 5 min) and lyophilized for further extraction of microcystins.

### Extraction and identification of microcystin variants of cyanobacterial extract

The method of extraction was described by Corbel et al. (2015). Briefly, freeze-dried cyanobacterial cells were homogenized with aqueous methanol and introduced for 3 min in an ultrasonic bath (Ultrasonic 300), followed by centrifugation at 3000g 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 analysis. Microcystin concentrations in the extract were determined using the protein phosphatase type 2A inhibition assay according to Bouaïcha et al. (2001). For confirming the identities of some microcystin–leucine–arginine (MC-LR) variants in the cyanobacterial cells extract, the extract was analyzed by liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) using a Waters Acquity ultra-high-pressure liquid chromatography–electrospray–triple quadrupole system as described by Corbel et al. (2015).

### Soil exposure experiments

The topsoil (0–20 cm) from a grassland (Pierre Plate, experimental site of INRA Versailles, France) was sampled in August 2012. It was air-dried, sieved (2-mm mesh), and stored at 20 °C for 3 month (Corbel et al. 2015). It was a silty sand soil (76 % sand, 13 % silt, and 11 % clay), with 37.8 % of organic matter and the pH = 5.6. Soil microcosms consisted in 350 g of dry soil per pot adjusted a

50 % of the maximum water-holding capacity with MilliQ water and incubated for stabilization for 2 weeks. Then, one seed of *Solanum lycopersicum* cultivar MicroTom from INRA (Avignon, France) was planted in each pot. We carried out daily irrigation, to adjust at 70 % of water-holding capacity, with aqueous extract of cyanobacteria prepared from aqueous methanol (75 %, v/v) diluted in nutritive solution of N–P–K (15–10–30), 0.75 g L<sup>-1</sup>, with final concentrations of 0.005, 0.02, 0.05, and 0.1 mg eq. MC-LR L<sup>-1</sup>. For controls, samples of nutritive solution containing methanol (3.5 % final concentrations) were used. There were five replicates for each treatment. Microcosms were incubated in greenhouse chamber: 16 h:8 h light/dark cycle, light intensity of 160  $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$  provided by cool white fluorescent tubes (Osram, Cool Day- Light 865), temperature of 26 °C/21 °C light/dark cycle. The exposition held 90 days, and the total quantities of microcystins brought after irrigation with 0.005, 0.02, 0.05, and 0.1 mg eq. MC-LR L<sup>-1</sup> were, respectively, 0.05, 0.18, 0.46, and 0.92  $\mu\text{g eq. MC-LR g}^{-1}$  of dry soil. Then, plants were harvested, weighed, and frozen (-20 °C) before being freeze-dried and toxin extraction. Then, the whole soil collected from each pot was homogenized. Fresh soil aliquots were used for microbial activity measurements, and a subsample was stored at -80 °C for DNA extraction and analysis.

#### Soil microbial parameters

#### Soil enzymatic activities

Three soil enzyme activities for arylsulfatase,  $\beta$ -D-glucosidase, and phosphatase were measured by the method of Santiago-Martin et al. (2013) as described in Corbel et al. (2015). Briefly, an aliquot of 4 g of fresh soil samples, exposed to cyanobacteria extract for 90 days, was used to determine these activities. After hydrolysis of nitrophenyl substrates, the fluorescence was measured at 405 nm with a microplate reader (SAFAS, Monaco). Results were then expressed in mU g<sup>-1</sup> of dried soil.

#### Nitrification potential activity

The nitrification potential activity was assessed after 90 days of soil exposure to cyanobacterial extracts as described by Petersen et al. (2012) and Corbel et al. (2015). Briefly, an aliquot of 15 g of fresh soil was placed in Erlenmeyer flasks with 60 mL of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> solution (pH 7.2) supplemented with KH<sub>2</sub>PO<sub>4</sub> (0.2 M) and K<sub>2</sub>HPO<sub>4</sub> (0.2 M). After 3 and 46 h of incubation, the

concentrations of ions nitrate (N-NO<sub>3</sub><sup>-</sup>-N) + nitrite (NO<sub>2</sub><sup>-</sup>-N) were measured by capillary electrophoresis technique. Nitrification potential activity was obtained using the Eq. (1) where the result was expressed in µg NO<sub>3</sub><sup>-</sup> g<sup>-1</sup> fresh soil h<sup>-1</sup>:

$$\text{Potential Nitrification} = \frac{[\text{NO}_3^-]_{\text{final}} - [\text{NO}_3^-]_{\text{initial}}}{T_{\text{final}} - T_{\text{initial}}} \times V_s \div W \quad (1)$$

V<sub>s</sub> is volume of solution, T is time of incubation, W is weight of fresh soil (approximately 15 g).

DNA extraction and real-time PCR quantification of ammonia-oxidizing bacteria and ammonia-oxidizing archaea amoA genes

For each soil sample exposed for 90 days to cyanobacterial extract, the DNA was extracted in duplicate from 0.5 g soil (equivalent dry weight) stored at -80 °C frozen samples using the FastDNA SPIN Soil Kit (MP Biomedicals, Illkirsch, France) following the manufacturer's instructions. The soil DNA extracts were quantified by absorption at 260 nm using a Smart Spec3000 (Biorad, Hercules, CA, USA). Soil DNA samples were stored at -20 °C until their use. DNA extracts were then used as template for real-time quantitative PCR (qPCR) assays to estimate the abundances of ammonia-oxidizing archaea and ammonia-oxidizing bacteria by qPCR assays targeting the amoA gene according to Wessen et al. (2011). The abundances of the total bacterial and archaeal communities by targeting 16S rRNA sequences according to Bru et al. (2008) and Ochsenreiter et al. (2003) were also quantified and used to normalize the abundances of nitrifiers.

Statistical analysis

Data were represented by the mean ± standard deviation. The normality and homogeneity of variance were checked with Kolmogorov–Smirnov and Bartlett tests (p < 0.05). If these conditions were checked, an ANOVA with Tukey's post hoc comparison was performed. In contrast, data were compared with a nonparametric Kruskal-Wallis test and “nparcomp” post hoc comparisons. All statistical analyses were performed using “R” software, and significance level was 0.05.

## Results and discussion

The objective of the present work was to determine the potential effects of a 90-day irrigation with water containing realistic environmental concentrations of microcystins on global enzymatic activities, nitrification potential activity, and the abundances of ammonia-oxidizing bacteria and ammonia-oxidizing archaea *amoA* genes.

### Quantification and identification of microcystins in cyanobacterial extract

The total concentration of microcystins in the extract calculated with the protein phosphatase assay and expressed according to microcystin–leucine–arginine was 21.6 mg eq. MC-LR L<sup>-1</sup>. It corresponded to 6.8 mg eq. MC-LR g<sup>-1</sup> cyanobacteria's cell dry weight. The cyanobacterial extract of *M. aeruginosa* (PCC 7820) was characterized, and the different congeners identified were described in Corbel et al. (2015). The most abundant microcystin congener in this extract was the MC-LR (68.4 % of the total microcystins). This extract diluted at concentrations from 0 to 0.1 mg eq. MC-LR L<sup>-1</sup> was used daily for soil irrigation.

### Effects of microcystins on soil enzymatic activities

Soil enzymatic activities were measured in soils irrigated for 90 days with water containing microcystins. The results (not shown) indicated that the phosphatase activity was comprised between 41.1 and 54.1 mU g<sup>-1</sup> dry soil, the β-D-glucosidase activity varied from 7.9 to 11 mU g<sup>-1</sup> dry soil, and the arylsulfatase was comprised between 2.5 and 2.9 mU g<sup>-1</sup> dry soil. These soil activities did not significantly differ in soils irrigated with water containing microcystins, when compared to the control soil.

### Effects of microcystins on soil nitrification potential activity

The potential nitrification activity was significantly increased in soils irrigated with water containing the lowest concentrations of microcystins, 0.005 and 0.02 mg eq. MC-LR L<sup>-1</sup>, when compared to controls (Fig. 1). By contrast, for the highest concentrations of microcystins (0.05 and 0.1 mg eq. MC-LR L<sup>-1</sup>), activity values were similar to those of the controls.

## Effects of microcystins on the abundances of ammonia-oxidizing bacteria and ammonia-oxidizing archaea amoA genes

The bacterial and archaeal 16S rRNA gene abundances, reflecting the size of the total bacterial and archaeal communities, were not significantly different between the control and the soils irrigated with water containing microcystins (Fig. 2). In the later soils, the ammonia-oxidizing archaea and ammonia-oxidizing bacteria genes abundances were in the range measured in the control soil and did not explain the increase in nitrifying activity. In a previous work reporting the effects of a short-term exposure (14 days) to microcystins, the nitrification potential activity responses were partially explained by shifts in amoA ammonia-oxidizing bacteria abundances (Corbel et al. 2015). Conversely, here, after 90 days of irrigation with water containing cyanobacterial microcystins extract, the abundance of ammonia-oxidizing bacteria did not explain the shift of the soil nitrification. Effects on nitrifying activity could be explained by the presence of microcystins which could affect the metabolic activity of bacterial nitrifiers, without a toxic effect leading to the cell death affecting the abundances. These different results between short-term and long-term effects on microbial abundances could be explained by a resilience of nitrifiers. Indeed, with time increasing after the initial impact, the most tolerant nitrifiers replace the most sensitive ones. Such resilience has been often described in response to pesticides (Hernandez et al. 2011; Ruyters et al. 2013; Crouzet et al. accepted). Furthermore, the detection of microcystins in the soil, after 90 days of irrigation, revealed their presence with concentrations comprised between 1.3 and 3.9 ng MC-LR g<sup>-1</sup> dry soils (data not shown). These results were in accordance with the half time of <sup>14</sup>C-MC-LR, exceeding 60 days in this agricultural soil (Corbel et al. 2014a).

## Conclusion

We showed for the first time that the long-term irrigation of a soil-plant system with water containing low concentrations of microcystins (0.005 and 0.02 mg eq. MC-LR L<sup>-1</sup>) stimulated soil nitrification. That functional response was not explained by the abundances of ammonia-oxidizing archaea and ammonia-oxidizing bacteria. We conclude that a long exposition of soil microorganisms to low amounts of microcystins can modify their functional response, and that microcystins are not always associated with toxic effects. The mechanisms involved over a long exposure period should be determined as a way for future research, as well as the effects of these



compounds on soil (micro-)organisms.

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Fig. 1. Nitrification potential activity in soils irrigated for 90 days with water containing various concentrations of microcystins. Note that the nitrification activity was stimulated by the lowest concentrations of microcystins. Results are expressed as mean  $\pm$  standard deviation (n = 5). Asterisks (\*) indicate significant differences with the control group at P < 0.05

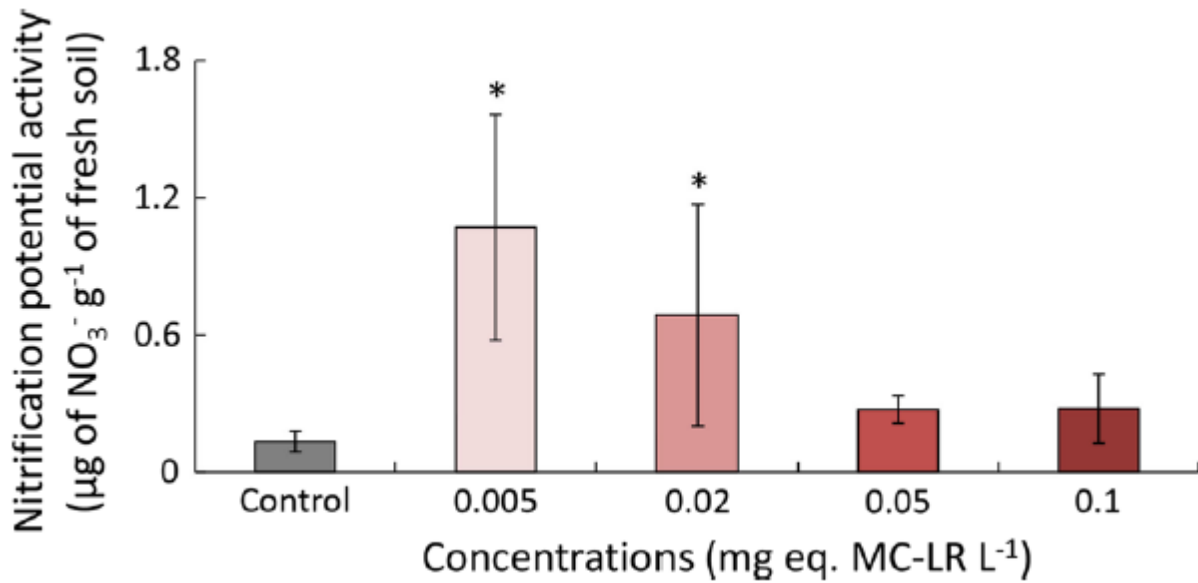


Fig. 2. Abundances of total bacteria, archaea, and ammonia-oxidizing functional groups, expressed as log of numbers of gene copies of archaeal amoA gene and bacterial amoA gene, in soil exposed for 90 days to various microcystin concentrations. Note that these abundances were not affected by the presence of microcystins in the soils. The increase in nitrification potential activity was not explained by the abundances of archaea and bacteria. Results data are expressed as mean  $\pm$  standard deviation (n = 5)

