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Sylvain Corbel, Christian Mougin, Fabrice Martin-Laurent, Olivier Crouzet, David Bru, et al.. Evaluation of phytotoxicity and ecotoxicity potentials of a cyanobacterial extract containing microcystins under realistic environmental concentrations and in a soil-plant system. Chemosphere, 2015, 128, pp.332-340. 10.1016/j.chemosphere.2015.02.008. hal-01535215

HAL Id: hal-01535215 https://hal.science/hal-01535215

Submitted on 5 Feb 2023

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Evaluation of phytotoxicity and ecotoxicity potentials of a cyanobacterial extract containing microcystins under realistic environmental concentrations and in a soil–plant system

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Highlights

We compared the impact of a cyanobacterial extract containing microcystins on seed germination of some plants.

Effects of a cyanobacterial extract containing microcystins on growth of terrestrial plants are described.

We report the impact of a cyanobacterial extract containing microcystins on some soil microbial parameters.

Realistic environmental concentrations of microcystins increased the nitrification potential.

Ammonia-oxidizing bacterial abundances was consistently impacted by a cyanobacterial extract containing microcystins.

Keywords: Microcystins, Seed germination, Plant growth, Soil enzymatic activities, Nitrification, Ammonia-oxidizing bacteria

Abstract

The impact of a crude extract of *Microcystis aeruginosa* (PCC7820) containing 14 microcystin variants was investigated on seeds germination and radicles development of four agricultural plants: two tomato varieties Solanum lycopersicum (MicroTom and Saint-Pierre), the wheat Triticum aestivum and the lettuce Lactuca sativa. In addition, the effect of 14 d-exposure to irrigation water containing realistic concentrations of microcystins (0–0.1 mg eq. microcystin-LR L⁻¹) on the tomato MicroTom seedling growth was further evaluated on roots and aerial part biomasses. Impacts on soil bacterial parameters, as such extra- cellular enzymatic activities, nitrification activity and abundances of ammonia-oxidizing microorganisms were also investigated. In germination-test, the cyanobacterial extract inhibited only the germination of the wheat seeds, with an EC^{50} of 11 mg eq. microcystin-LR L⁻¹; which is 13 times lower than that of the cadmium chloride (EC50 of 145 mg L⁻ ¹). Moreover, the cyanobacterial extract containing low concentrations of microcystins increased the growth of primary roots; however, high concentrations decreased it for all plants except for the wheat. In the soil-plant approach, only aerial part biomass of the tomato MicroTom was enhanced significantly. In addition, only soil nitrification potential and ammonia-oxidizing bacterial abundances were consistently impacted. A significant positive correlation (r = 0.56) was found between the increase of nitrification potential and abundances of ammonia-oxidizing bacteria. This work suggested, that exposure to a cyanobacterial extract containing realistic environmental microcystins concentrations could affect seed germination, depending plant species. It was also highlighted, for the first time, disturbances in soil bacteria functioning, evidences on soil nitrification process.

Introduction

The increased incidence of toxic cyanobacterial blooms in aquatic ecosystems represents a hazard for human and animal health (Ettoumi et al., 2011). Their toxicity is due to the presence of a wide range of toxins: microcystins (MCs) and nodularins (hepatotoxins), saxitoxins, anatoxins and β methylamino-L-alanine (neurotoxins), and cylindrospermopsins (cytotoxins) (Edwards and Lawton, 2009). MCs are the most common cyanotoxins and may be expected wherever blooms of cyanobacteria occur in surface water (Sivonen and Jones, 1999). MCs are cyclic heptapeptides, presenting high structural variability as more than 80 variants have been characterized to date (del Campo and Ouahid, 2010). Microcystin-LR (MC-LR) is generally recognized as being the most

studied due to its high toxicity, frequent detection, and its concentration in surface waters often exceed the World Health Organization advisory level of 0.001 mg L⁻¹ (Corbel et al., 2014). The phytotoxic effects of cyanotoxins on higher plants were firstly focused on macrophytes that are naturally exposed to cyanotoxins (Ikawa et al., 2001; Mitrovic et al., 2004; Ha and Pflugmacher, 2013). Since few years, some studies were also focused on terrestrial plants sensitivity, because irrigation practices with contaminated water may lead to the spreading of MCs mixtures in soils (Järvenpää et al., 2007). Data on their ability to affect other soil microorganisms such as bacteria, fungi and protozoa and macro organisms such as arthropods, mollusks, and earth- worms are particularly scarce. This interest has been aroused by the fact that irrigation waters from sources containing cyanobacterial blooms and toxins are generally used without treatment for spraying agricultural crops and plants and (2) the potential that these toxins can be accumulated in the terrestrial food chains implying a health risk and potential economic losses. Several works previously investigated deleterious effects on terrestrial plants (germination, growth, photosynthesis, and oxidative stress) and some others reported accumulation of MCs in crops and plants (see in Corbel et al., 2014). Since most studies focus on the phytotoxic effects produced by cyanobacterial crude extracts containing high concentrations of MCs in soil-free systems (Sagrane et al., 2008; Peuthert et al., 2010) or with culture on an inert substrate as sand (Crush et al., 2008; El Khalloufi et al., 2012). However, in an earlier study, Järvenpää et al. (2007) assessed the effects of MC exposure on broccoli (Brassica oleracea var. Italica) and mustard (Sinapis alba) by watering the seedlings with water containing environmentally relevant MCs concentrations (0, 0.001 or 0.01 mg MCs L⁻¹). Morphological characteristics (leaf size and mass, stem length and mass, and root mass), chlorophyll concentrations and chlorophyll fluorescence were investigated, but the only distinct difference compared to control plants was a slight (<10%) growth inhibition seen in broccoli. In the present work we attempt to determine also whether by cyanobacterial crude extracts containing environmentally relevant MCs concentrations can affect tomato plants and soil bacterial activities. Indeed, any impact of MCs on bacterial communities could potentially affect bacterial mediated nutrient and trace-element cycling, therefore disturbs plant seedling growth. Our first objective was to examine the acute effects of Microcystis aeruginosa PCC7820 extracts at high concentrations of MCs (0–20 mg L⁻¹) on seed germination and radicles development of the tomato Solanum lycopersicum cultivars: MicroTom and Saint-Pierre, the wheat Triticum aestivum and the lettuce Lactuca sativa var. Capitata. Our second objective was to assess effects, in a soilplant approach, both on the seedling growth of the tomato plant S. lycopersicum var. MicroTom and on soil bacterial functioning, following a repeated exposure to irrigation water containing realistic concentrations of MCs. The ecotoxicity of MCs on soil microorganisms was assessed by different assays: the Microtox assay, soil extracellular enzyme activities, nitrification potential and abundance of nitrifiers' microorganisms.

Materials and methods

Culture of M. 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 NaNO₃ (2 mM) and NaHCO₃ (10 mM), at 25 °C, with an incident light intensity of 5–10 μ mol of photons m 2 s⁻¹ provided by cool white fluorescent tubes (16 h:8 h light/dark cycle). The cultures were vigorously bubbled with filtered (0.22 lm Millipore, France) air at a flow rate of 0.23 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 microcytins.

Extraction, analysis and identification of microcystin variants

Freeze-dried cyanobacterial cells (1.43 g DW) were homogenized with 150 mL of 75% (v/v) aqueous methanol. The homogenate was introduced for 3 min in an ultrasonic bath (Ultrasonic 300), followed by centrifugation at 3,000 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 analysis by the protein phosphatase type 2A (PP2A) inhibition assay according to Bouaïcha et al. (2001). For confirming the identities of some MC-LR variants, the cyanobacterial cells extract was analyzed by 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 ionization (ESI) interface. The MC variants were identified according to the literature by comparison of their relative LC-retention times as well as pseudo-molecular and fragment ions. The evaluation of MC percentages was per- formed using the ion current of the sum of major ions (i.e. exceeding 10% of base peak) in the MS data acquired with 60 V cone voltage.

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Four different seed samples: S. lycopersicum cultivars MicroTom (INRA Avignon, France) and Saint-Pierre (Vilmorin, La Ménitré, France), T. aestivum BIO (Attlass) (Semences de France, La Chapelle d'Armentières, France), and L. sativa var. Capitata (Vilmorin, La Ménitré, France) were used to evaluate the effect of a cyanobacterial crude extract containing environmentally relevant MCs concentrations on seeds germination. After sterilization by washing with sodium hypochlorite solution (1.6% active chloride) during 1 min, seeds (20) were then deposited in Petri dishes and covered with filter paper. Seeds were moistened with solutions of MCs prepared from the aqueous methanol (75%, v/v) extract of the cyanobacterial cells at 0–20 mg eq. MC-LR L⁻¹. A sample of MilliQ water containing methanol at 1.35% final concentrations, corresponding to the upper concentration of MeOH during the higher treatment was used for the control. At this concentration, MeOH has no effect on seeds germination. The cadmium chloride (0.050 mg to 1 g L⁻¹) was used as positive control following the AFNOR X31-201 guidance (AFNOR, 1986). Three replicates were used for each condition. The Petri dishes were maintained in the dark during 1 week at 25 °C. After this period, the germination rate was calculated and the length of radicles of seedlings exposed to microcytin extracts was measured with ImageJ 1.46 (2012) software (National Institutes of Health, USA). For the cadmium chloride, the germination rate was calculated for all plants, however, only the length of radicles of the tomato S. lycopersicum cultivars: MicroTom and the length of coleoptiles of the wheat plant were measured.

Plant growth inhibition assay

The plant model used to evaluate the effect of the crude cyanobacterial extract containing MCs on the growth of seedlings is the tomato *S. lycopersicum* variety MicroTom. Its culture and its life cycle are well controlled in our laboratory. The plant growth inhibition assay was conducted following the NF EN ISO 22030 guidance (ISO, 2011). A topsoil from a meadow (Pierre Plate, Versailles, France) sampled in August 2012 was used in this study. It was a silty sand soil comprising 76% sand, 13% silt and 11% clay. The content of organic matter in the soil was 37.8‰ and its pH was 5.6. It was air-dried, sieved (2 mm mesh) and stored at 20 °C for less than 3 months. A quantity of 350 g of this dried soil per pot was maintained at 50% of its water holding capacity

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(WHC) with MilliQ water during two weeks. Seeds of the tomato MicroTom were then planted (10 seeds per pot). A daily irrigation was carried out with MilliQ water containing different concentrations of MCs (0.005, 0.02, 0.05 and 0.1 mg eq. MC-LR L⁻¹). MilliQ water containing methanol with the highest concentration (0.35% final concentration) was used as the vehicle control. At this concentration MeOH has no effect on the plant growth. For each treatment, three replicates were placed in a greenhouse chamber with an incident light intensity of 160 μ mol of photons m 2 s⁻¹ 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). After 14 d of exposure, the fresh and dry (70 °C during 24 h) weights of both aerial parts and roots were obtained for each seedling. The whole soil collected from each pot was then homogenized and fresh aliquots were immediately used for enzymatic activities, potential nitrification determinations, and a subsample for nucleic acid extraction and analysis was stored at 80 °C.

Luminescent bacteria assay

The Microtox bioassay on the marine bacterial species, *Vibrio fischeri* NRRL B11177 (Microtox® test) was carried out according to the NF EN ISO 11348–3 guidance (ISO, 2007). The cyanobacterial extract containing MCs was diluted in a saline solutio (20 g NaCl L⁻¹) to reach the final working concentration (0.005, 0.02, 0.05 and 0.1 mg eq. MC-LR L⁻¹) and then the effect of each concentration on the bioluminescence of the marine bacterial species was evaluated. The 5-, 15-, and 30-min EC50 (concentrations of toxicant required to decrease by 50% the bacterial bioluminescence) were then calculated for each MC concentration relative to the blank control (20 g NaCl L⁻¹).

Soil enzymatic activities

Four soil extracellular enzyme activities: arylsulfatase, β -D-glucosidase, phosphatase and urease, were determined in the soil exposed for 14 d to the cyanobacterial crude extract containing MCs, as described by de Santiago-Martín et al. (2013). Briefly, an aliquot of 4 g of fresh soil samples was mixed with 25 mL of MilliQ water and incubated for 10 min at ambient temperature under continuous stirring (250 rpm). An aliquot of 0.125 mL (4 replicates) was then incubated in a 96-well microtitre plate with substrates: potassium-4-nitrophenylsulfate (0.025 M, 4 h at 37 °C) for arylsulfatase, 4-nitrophenyl- β -D-glucopyranoside (0.05 M, 1 h at 37 °C) for β -D-glucosidase, 4-

nitrophenylphosphate (0.05 M, 30 min at 37 °C) for phosphatase, and urea (0.4 M, 3 h at 37 °C) for urease. After the incubation period, reactions were stopped with a CaCl₂ solution (0.5 M) and Tris–HCl (0.1 M, pH 12). The microplate was then centrifuged at 3000 g for 5 min and an aliquot of 0.200 mL of each well was used to evaluate the enzymatic activity. For phosphatase, b-D-glucosidase, and arylsulfatase the para-nitrophenol released was measured at 405 nm with a microplate reader (SAFAS, Monaco) and the concentration of the product released was compared with para-nitrophenol standards (Sigma). For urease activity, the ammonium ion NH₄⁺ released was determined at 610 nm with an HACH reagent (Loveland, CO, USA) and the concentration of the product released in mU g^{-1} dry soil.

Nitrification potential in soils

The nitrification potential activity (NPA) was assessed after 14 d of the soil exposure to cyanobacterial extracts. NPA was determined for each microcosms (n = 3), in accordance to Petersen et al. (2012), with some modifications. Moist soils equivalent to 15 g of dry soils were mixed, in Erlenmeyer flasks, with 60 mL of 1 mM phosphate buffer (pH 7.2) and 0.6 mM of the ammonium ion (NH⁺₄-N), and aerobically incubated in a rotary shaker at 150 rpm, at 25 °C. After 3 h and 46 h of incubation, a 2 mL aliquot was transferred in Eppendorf vials, centrifuged for 5 min at 13,000 g. Then supernatants were filtered (0.45 lm) and stored at 20 °C until analyses of the ions nitrate (NO₃⁻-N) + nitrite (NO₂⁻-N) by capillary electrophoresis. NPA was calculated as the rate of production of N-NO₃⁻ + N-NO₂⁻ between 18 h and 2 h, following the Eq. (1). The result was expressed in μ g NO₃⁻ g 1 fresh soil h⁻¹.

Potential nitrification
$$= \frac{[NO_3^-]_{final} - [NO_3^-]_{initial}}{T_{final} - T_{initial}} \times Vs \div W$$
(1)

Vs volume of solution, W weight of fresh soil (approximately 15 g), T time of incubation

DNA purification and real-time PCR quantification of AOB and AOA amoA genes

For each soil sample, exposed for 14 d to the cyanobacterial extract containing 0-0.1 mg eq. MC-

LR L⁻¹, the DNA was extracted and purified in duplicate from an aliquot of 0.5 g dry weight frozen samples (80 °C) using the FastDNA SPIN Soil Kit (MP Biomedicals, Illikrich, France) following the manufacturer's instructions. The soil DNA was quantified by absorption at 260 nm using a Smart Spec3000 (Biorad, Hercules, CA, USA). Purified 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 quantify the abundance of the total bacterial and archaeal communities by targeting 16S rRNA sequences according to Bru et al. (2008) and Ochsenreiter et al. (2003). The ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) abundances were estimated by qPCR assays targeting the amoA gene according to Wessén et al. (2011).

Statistical analysis

Data were represented by the means \pm the standard deviation (SD). The normality and homogeneity of variance were checked with Kolmogorov–Smirnov and Bartlett assays with a significance level of 5%. If these conditions were obtained, we used an ANOVA and a Tukey post hoc. In contrast, when these assumption were not satisfied, a non parametric Kruskal-Wallis test (P < 0.05) was done by "nparcomp" post-hoc multiple comparison analysis. A Pearson correlation was used between potential nitrification and the abundance of ammonia-oxidizing bacteria and archaea (AOB and AOA) communities, obtained by molecular analysis. All statistical analyses were performed using "R" software (R development Core Team, 2012). The EC50 of MCs or cadmium chloride on germination rate was obtained with Vindimian Macro (RegTox) with Microsoft Excel Software.

Results

Quantification and identification of MC variants in the cyanobacterial biomass extract

The total MCs concentration, in the aqueous methanol extract of the cultivated cyanobacterial strain *M. aeruginosa* PCC7820 determined by the PP2A inhibition assay was 21.6 mg eq. MC-LR L⁻¹, corresponding to 6.77 mg eq. MC-LR g⁻¹ dried cells. As has previously been shown by other authors (Bateman et al., 1995; Lawton et al., 1994, 1995; Robillot et al., 2000), this strain mainly produced the variant MC-LR, observed at the retention time 6.38 min (Fig. 1). As already described for the same strain, the variants MC-LY, -LW and -LF were also observed at 7.65, 8.35 and 8.51 min, respectively. Furthermore, we also detected the variant MC-YR (5.62 min) and trace of the

variant MC-LM (8.01 min), already described in crude extracts of other strains of Microcystis (Lawton et al., 1995; Yuan et al., 1999). Two desmethylated forms of MC-LR were observed at 5.39 and 6.45 min. The first one did not show in its MS and MS/MS spectra [M+H-134]⁺ neither m/z 135, allowing suspecting DMAddaMC-LR. The second one, almost co-eluted with MC-LR, presents in its MS/MS spectrum the ions m/z 272, 383, 539, 556 and 682 described by Yuan et al. (1999) as characteristics of the [DMAsp3]MC-LR form. The methylated variant [Me-Ser7]-LR was also detected at 6.19 min ([M+H]⁺ 1013) and its identity confirmed on the basis of the MS/MS fragment ions 393, 571, 862, 884 and 996 as described by Diehnelt et al. (2006). Traces of desmethylated MC-LF (7.37 and 8.40 min, [M+H]⁺ 972) and -LW (6.32, 7.24 and 8.21 min, [M+H]⁺ 1011) were also suspected. The absence of [M+H-134]⁺ and m/z 135 ions in the 7.37 and 7.24 min spectra suggests that those variants correspond to the DMAddaMC-LF and the DMAddaMC-LW, respectively.

Thus, the cyanobacterial extract of the *M. aeruginosa* strain PCC7820 cultivated in the present study presents 6 major characterized variants of MCs, with the variant MC-LR being the most abundant (68.4% of the total MCs), followed by the variants MC-LF (8.3%), desmethylated [Asp3]-LR (6.9%), MC-LW (6.5%), MC-LY (2.9%), and MC-YR (2.4%). However, the 8 minor variants represent less than 4.6% of the total MCs with an abundance ranging from 0.3% to 1.3%.

Microtox® bioassay results

No inhibitory effect on the bioluminescence of the marine bacterial species *V. fisheri* was observed for all concentrations (0.005, 0.02, 0.05, 0.1 mg eq. MC-LR L⁻¹) of crude extract of cyanobacteria tested.

Effects on seeds germination and radicles growth

Fig. 2 shows the effect of the cyanobacterial crude extract containing different concentrations of MCs (0–20 mg eq. MC-LR L⁻¹) and different concentrations of the pure cadmium chloride (0.05 mg L⁻¹ to 1 g L⁻¹) on seeds germination of the two tomato varieties: MicroTom and Saint-Pierre, the wheat species *T. aestivum*, and the lettuce species *L. sativa* var. Capitata. After 7 d of exposure, the germination rate of all plant seeds was significantly affected by the cadmium chloride at 100 mg L⁻¹ and higher concentrations (Fig. 2A–D). The EC50 was 145 mg L⁻¹ for the wheat, 390 mg L⁻¹ for the

lettuce, and more than 1 g L^{-1} for the two tomato varieties. However, the cyanobacterial crude extract in the range of 0–20 mg eq. MC-LR L^{-1} inhibited only the germination of the wheat seeds, with an EC50 of 11 mg eq. MC-LR L^{-1} (Fig. 2C).

In addition, the exposure of seeds of the two tomato varieties and the lettuce species to the cyanobacterial crude extract at various MC concentrations up to 20 mg eq. MC-LR L⁻¹ caused a significant effect on growth and development of the radicles (Fig. 3A). After 7 d of exposure to the low concentration of MCs (0.05 mg eq. MC-LR L⁻¹), the length of the radicles of the tomato Saint-Pierre and the lettuce increased significantly by 125% and 109%, respectively when compared to the control. In the concentration range of 0.1–5 mg eq. MC-LR L⁻¹, the length of radicles of the-se two plants decreased but remains significantly higher than the control value to reach it at 10 mg eq. MC-LR L⁻¹. However, at the highest cyanobacterial crude extract containing 20 mg eq. MC-LR L⁻¹ only the radicle length of the tomato Saint-Pierre decreased significantly under the control value (Fig. 3A). A similar effect was observed for the tomato MicroTom radicles (Fig. 3A and B) but less pronounced than that observed with the variety Saint-Pierre (Fig. 3A). However, the significant decrease of the radicle length of this variety under the control value appeared from 5 mg eq. MC-LR L 1. In contrast, no significant differences compared to the control were observed on the emergence either of the radicle system or the coleoptile of the sprouted wheat (data not shown). As for the cadmium chloride, a significant decrease of the radicle length of the tomato MicroTom was observed from the concentration of 10 mg L 1 (data not shown). However, for the wheat, the cadmium chloride affects only the coleoptile lengths with a significant decrease from the concentration of 100 mg L 1 (data not shown).

Effects on the growth of S. lycopersicum var. MicroTom seedlings

During this experiment where seeds of MicroTom were planted in the soil and exposed for 14 d to the cyanobacterial crude extract containing different concentrations of MCs, no significant delay or inhibition of the germination of seeds has been observed in comparison with the control. However, a significant increase in dry weight of the aerial part of the tomato seedling was registered upon plant exposure (Fig. 4). This effect was observed from the lowest concentration of the cyanobacterial crude extract containing 0.005 mg eq. MC-LR L⁻¹.

But, the cyanobacterial crude extract tested at different MC concentrations has no significant effect on the dry weight of roots (Fig. 4).

Soil extracellular enzymatic activities and potential nitrification

The enzymatic activities of arylsulfatase, phosphatase, urease, and β -D-glucosidase were subsequently determined to assess potential alterations in sulfur, phosphorus, and nitrogen mineralization and cellulose degradation in soil, respectively. All enzymatic activities were not significantly affected by 14 d of irrigation with water containing realistic environmental MC concentrations up to 0.1 mg eq. MC-LR L⁻¹ (Table 1). However, the lowest MC concentrations (0.005, 0.02, and 0.05 mg eq. MC-LR L⁻¹) increased significantly the nitrification potential in the soil with a maximum activity reaching approximately 5 times the control value (Fig. 5). The highest concentration also stimulated the nitrification potential, but in a lesser extend compared to those observed for other MC doses.

Effects on the abundances of total bacteria and archaea and ammonia-oxidizing bacteria (AOB) and archaea (AOA)

The bacterial and archaeal 16S rRNA gene abundance, reflecting the size of the total bacterial and archaeal communities, showed no significant variations between the control group and treated soils with the cyanobacterial crude extract containing different concentrations of MCs (Fig. 6). The amoA–AOA gene abundance was also in the range of the soil control and relatively unaffected by treatments with these concentrations. However, the amoA–AOB gene abundance, corresponding to the AOB community, was significant- ly increased in all treatments with the cyanobacterial crude extract containing of MCs (Fig. 6), having an AOB/total bacterial 16S rRNA ratio approximately 13 times higher than that of the control value. A significant positive correlation was observed in the soil exposed to extract of MCs between the nitrification potential increases and AOB gene abundance increase (r = 0.56, P < 0.05).

Discussion

The analysis of the cultured *M. aeruginosa* PCC7820 extract by the UPLC–MS/MS technique highlighted the presence of 6 characterized microcystin variants: MC-LR (68% of the total MCs), MC-LF, [DMAsp3] MC-LR, MC-LW, MC-LY, and MC-YR, and 8 minor other variants representing less than 4.6% of the total MCs. Therefore, the toxic potential and the microcystin

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patterns of this strain are in accordance with those reported by several previous studies on the same strain (Akin-Oriola and Lawton, 2005; Ríos et al., 2013), where 4 major MC variants (MC-LR, -LF, -LW, and -LY) and 2–6 minor variants were identified.

The first aim of this study was to examine the effect of this crude cyanobacterial extract with different concentrations of MCs, in comparison to the CdCl₂ on seeds germination carried on filter paper within Petri dishes of 4 different plants: two tomato varieties: MicroTom and Saint-Pierre, the wheat T. aestivum, and the lettuce L. sativa var. Capitata. After 7 d of exposure, the CdCl₂ harmfully impacted the germination of all plant seeds from 100 mg L⁻¹ with greater effects on lettuce and wheat (EC50 of 145 mg L⁻¹ and 390 mg L⁻¹, respectively) than the tomato varieties (EC50 of 1 g L⁻¹), while the crude cyanobacterial extract only inhibited the wheat seed germination (EC50 of 11 mg eq. MC-LR L⁻¹). Although the cyanobacterial crude extract containing MCs was tested only up to 20 mg eq. MC-LR L⁻¹, its inhibition effect of the wheat seed germination was 13 times higher than that of the CdCl₂. Similar results were found by Saqrane et al. (2008), who showed that an aqueous cyanobacterial extract containing MC-LR as the most abundant congener showed a significant inhibition of the wheat germination (T durum) with an EC50 of 11 mg eq. MC-LR L⁻¹. However, contrary to our findings, El Khalloufi et al. (2012) reported a significant reduction (40%) in the tomato (Lycopersicon esculentum) seed germination after exposition to a cyanobacterial extract containing 16 mg eq. MC-LR L⁻¹. Therefore, we can conclude that cyanobacterial crude extract containing high concentrations of MCs may affect the metabolic activities of seeds during the germination process. However, the sensitivity to these cyanotoxins varies within plants. For example, rice seeds have been found to be more resistant than rape seeds (Chen et al., 2004). In addition, Sagrane et al. (2008) reported that the effect of a cyanobacterial extract at different concentrations of MCs (0–12 mg L⁻¹) on seed germination rates of the terrestrial plants such as Lens esculenta, Zea mays, Triticum durum and Pisum sativum was dose dependent and significantly different according to the sensitivity of these tested plants.

Although crude cyanobacterial extracts containing environmentally relevant MCs concentrations between 0 and 0.1 mg L⁻¹ had no significant effects on germination of several plant seeds, significant effects of these low concentrations were observed on the growth and morphology of radicles of these plants. For example, after 7 d of exposure to these environmental realistic concentrations of MCs, the radicle lengths of the two tomato varieties and the lettuce increased significantly in comparison to the control. However, the high concentration (20 mg eq. MC-LR L⁻¹) reduced significantly the length of radicles of the two tomato varieties. Similarly, Chen et al. (2012) have recently reported that a cyanobacterial extract containing high MC-LR concentrations (>2 mg L^{-1}) affects rice roots morphogenesis by inhibiting roots elongation, crown roots formation, and lateral roots development from primordia. By contrast, lower toxin concentrations (0–0.2 mg L⁻¹) did not affect rice roots growth (Chen et al., 2004). In the present study no significant differences compared to the control were observed either with low or high concentrations of MCs on the emergence of radicles of the sprouted wheat. However, Pflugmacher et al. (2007) reported that the root length of the germinated seed of the wheat *T. aestivum*, was inhibited by 44% using a crude cyanobacterial extract containing 5 10 4 mg eq. MC-LR L⁻¹. Bibo et al. (2008) demonstrated that the pure MC-RR at low concentrations (<0.01 mg L⁻¹) could accelerate the growth of plants, while high concentrations (>0.1 mg L⁻¹) significantly inhibited the growth of plants. The authors explained these results with the concept of hormesis, characterized by an inverted U-shaped dose response.

No significant effect on the dry weight of roots of the tomato MicroTom plant exposed for 14 d were registered. However, a significant increase in the dry weight of the aerial part of the tomato seedling was registered from the lowest MCs concentration (0.005 mg L⁻¹). In a recent study performed by Azevedo et al. (2014) in hydroponic conditions and at low concentrations of MCs (0–0.078 mg eq. MC-LR L⁻¹), no significant differences on the dry and the fresh weight of roots and leaf of 20-day-old rice plants exposed for 7 d were registered. By contrast, in a earlier study, Gehringer et al. (2003) observed a decrease in roots and leaf biomasses of Medicago sativa with low concentrations of MCs (0.005 and 0.01 mg eq. MC-LR L⁻¹). These contradictory results could be explained by differences in the MCs variant composition of the cyanobacterial extract used and/or experiment conditions such as hydroponic versus plant-soil systems. Therefore, cyanobacterial crude extracts containing a high quantity of the variant MC-LR, the more toxic, should had the high impact on seedling and root plants development. On the other hand, the use of an agricultural soil in our experiments which containing organic matters and clay could increase MCs adsorption and consequently decreases their bioavailability for living organisms, comprising microorganisms and plants. In support of this hypothesis, different studies have shown a decrease of MCs concentrations in soils with increasing carbon and clay content (Morris et al., 2000; Miller and Fallowfield, 2001). In addition, many of these researches are per- formed with cyanobacterial extracts, and they can content other compounds different to MCs that can contribute to the toxic effects observed. In fact, Palikova et al. (2007) have investigated embryolarval toxicity in carp after exposures to various cyanobacterial samples and their fractions differing by composition and the con- tent of MCs. They concluded that the most pronounced toxic effects on embryo-larval

development of carp were observed with the non-microcystins dominated samples and therefore, were independent of MCs content. Jungmann (1992) reported that the two microcystin variants, MC-LR and 3-desmethyl MC-LR, present in the crude water extract of the Microcystis strain PCC7806 are not the compounds which are toxic to *Daphnia pulicaria*. Nevertheless, in a recent study Puerto et al. (2010) compared the effects on the antioxidant status in the human colon carcinoma cell line Caco-2 of a pure MC-LR, and a microcystin-containing and non-containing cyanobacterial crude extracts. Their results indicated that the MC- containing cyanobacterial extract showed the higher toxic effects, followed by the pure MC-LR. However, the non-MC containing cyanobacterial extract showed limited toxic effects. The authors suggested that MC-LR is the responsible of the oxidative stress responses observed in this cell line, but other compounds contained in the cyanobacterial extract can contribute to the toxic effects.

Furthermore, a significant stimulation of soil nitrification potential was observed from the lowest concentration of the cyanobacterial crude extract containing 0.005 mg eq. MC-LR L⁻¹. However, we noticed that this increase of nitrification potential was not accompanied by a significant modification of total bacterial and archaeal communities. In addition, no inhibitory effect on the bioluminescence of the marine bacterial species V. fisheri was observed for all concentrations of crude extract of cyanobacteria tested. Nevertheless, our results showed for the first time that the ammonia-oxidizing bacterial community (AOB) was positively enhanced by this cyanobacterial crude extract containing different MC concentrations. This increase was positively correlated with the increase of the nitrification potential. For example, in aquatic ecosystems Giaramida et al. (2013) reported that the exposure to cyanobacterial extracts containing MCs drives changes in structure and physiology of bacterial communities and in turn those com- munities differentially perform degradation of MC-LR. Therefore, the induction of seedlings growth and the increase of the nitrification potential and of the AOB community abundance, at low concentrations of MCs, could be traced to the inhibition of protein phosphatases and/or the presence of other allelopathic secondary metabolites in the crude cyanobacterial extract. Protein phosphatases have been known to be involved in the regulation of a number of physiological processes by dephosphorylation of regulatory proteins (Corbel et al., 2014). Several studies reported that chemical signaling, inhibition/stimulation of growth activities along with toxicity to microorganisms are the most probable direct effect of MCs on bacterial communities (Christoffersen, 1996; Kearns and Hunter, 2001; Babica et al., 2006). We can, therefore, also suggested that the increase of the nitrification potential and the AOB community abundance, observed at low concentrations of the cyanobacterial extract containing MCs, may contribute to the induction of the seedling growth of the tomato

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MicroTom plant. However, more investigations seem, therefore, necessary to evidence and understand the effect of pure MCs on soil microbial communities, in particular, nitrifying bacteria.

Conclusion

The results from this study demonstrate that, in hydroponic conditions, high concentrations of MCs affected only seeds germination of the wheat with an EC50 of 11 mg eq. MC-LR L⁻¹. However, low environmental concentrations of MCs enhance significantly the growth length of the primary roots of a two tomato varieties MicroTom and Saint-Pierre and the lettuce *L. sativa* var. Capitata. In a plant–soil system, we noticed that realistic concentrations of MCs (0–0.1 mg eq. MC-LR L⁻¹) have no significant effects on dry weights of roots of the tomato variety MicroTom; however, they increase significantly the dry weight of aerial part of the plant. In addition, it appears that activities of the different tested enzymes (arylsulfatase, phosphatase, urease, and β -D-glucosidase) in the soil were not significant induction of the nitrification potential in the soil from the lowest concentration of MCs tested (0.005 mg eq. MC-LR L⁻¹) accompanied by a significant positive correlation (r = 0.56, P < 0.05) with the increase of the amoA gene abundance corresponding to the ammonia-oxidizing bacterial community. In light of these results, more investigations seem necessary to check the effects of realistic concentrations of MCs during a longer chronical exposition.

Acknowledgements

Authors thank G. Delarue (UMR1402 ECOSYS, INRA, France), J. Le Golvan, V. Grondin, C. Marrault, F. Poiroux, A. Trouvé, N. Cheviron (Biochem-Env platform, UMR1402 EcoSys, INRA, France) and J-P. Meunier (INRA, IJPB, France) for their help and technical assistances. Biochem-Env is a service of the "Investment d'Avenir" infrastructure AnaEE-France, overseen by the French National Research Agency (ANR) (ANR-11-INBS-0001). They acknowledge Pascal Pandard (INERIS, Verneuil-en-Halatte, France) for performing the Luminescent bacteria assay. This work is part of the French Investments for the Future "Investment d'Avenir" Program overseen by the French National Research Agency (ANR) (LabEx BASC, ANR-11-LABX-0034). The departments PESSAC and ESE are members of the EcoBASC Network. The research was also supported by grants from Région Ile-de France for UHPLC–MS–MS equipment and for S. Corbel PhD work (DIM-ASTREA Program No ast11005).

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Table 1. Global enzymatic activities of the soil (mU g⁻¹ dry soil) exposed for 14 d to various MC concentrations. Results data are expressed as mean \pm SD (n = 4).

	Concentrations of cyanobacterial extract (mg eq. MC-LR L ⁻¹)				
	Control	0.005	0.02	0.05	0.1
Phosphatase	61.65 ± 10.09	65.84 ± 16.27	71.59 ± 7.41	76.19 ± 3.51	84.65 ± 18.69
β-D-Glucosidase	8.20 ± 2.36	9.36 ± 1.98	8.91 ± 1.50	10.89 ± 0.53	12.66 ± 2.80
Arylsulfatase	8.97 ± 1.59	10.66 ± 2.60	8.52 ± 1.51	8.61 ± 0.52	9.31 ± 1.35
Urease	0.036 ± 0.005	0.031 ± 0.003	0.030 ± 0.003	0.031 ± 0.005	0.034 ± 0.004



Fig. 1. UHPLC–MS ([M+H]⁺ ion currents) chromatograms of a crude extract of the strain *Microcystis aeruginosa* (PCC7820) showing the different variants of microcystins present.

Fig. 2. Germination rate (%) of *Solanum lycopersicum* var. MicroTom (A) and Saint-Pierre (B), *Triticum aestivum* (C) and *Lactuca sativa* var. Capitata (D) seeds exposed for 7 d to various concentrations of cadmium chloride (black) or MCs (white). Data are means \pm SD (n = 3). Different letters show significant differences at P < 0.05.



Fig. 3. (A) Evolution development of radicles length of the tomato varieties MicroTom and Saint-Pierre, and the lettuce *L. sativa* var. Capitata exposed for 7 d to various MC concentrations. Results data expressed as mean \pm SD (n = 3). Asterisks () indicate significant differences with the control group at P < 0.05. (B) Morphology of the radicle of the tomato MicroTom seedling exposed for 7 d to water (1), to water containing MCs 0.1 mg eq. MC-LR L⁻¹ (2), and 20 mg eq. MC-LR L⁻¹ (3). Arrows sign development of lateral roots.



Fig. 4. Evolution growth of aerial parts and roots biomass (dry weight) of the tomato MicroTom seedlings exposed for 14 d to various MC concentrations. Results data are expressed as mean \pm SD (n = 3). Different letters show significant differences at P < 0.05.



Fig. 5. Potential nitrification in fresh soil exposed for 14 d to various MC concentrations. Results data are expressed as mean \pm SD (n = 3). Different letters show significant differences at P < 0.05.



Fig. 6. Log gene copy numbers of total bacteria and archaea, and ammonia-oxidizing functional groups: archaeal amoA (AOA) and bacterial amoB (AOB) in soil exposed for 14 d to various MC concentrations. Results data are expressed as mean \pm SD (n = 3). Asterisks (/) indicate significant differences with the control group at P < 0.05.

