Absence of negative allelopathic effects of cylindrospermopsin and microcystin-LR on selected marine and freshwater phytoplankton species

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Abstract Cyanobacterial toxins have been regarded by some researchers as allelopathic substances that could modulate the growth of competitors. Nevertheless, often the concentrations of toxins used are too high to be considered ecologically relevant. In this work we tested the hypothesis that microcystin-LR (MC-LR) and cylindrospermopsin (CYN) at ecologically relevant concentrations have no allelopathic effects on some species of phytoplankton. Extracts containing the toxins as well as pure MC-LR and CYN toxins were used to assess their effects on the growth rates of *Nannochloropsis* sp., *Chlamydomonas reinhardtii*, and *Chlorella vulgaris*. Cyanobacterial crude extracts induced more pronounced effects on growth rates than pure toxins. *Microcystis aeruginosa* and *Aphanizomenon ovalisporum* crude extracts containing MC-LR and CYN at 0.025–2.5 mg l^{-1} stimulated growth rates of microalgae, whereas *A. ovalisporum* crude extracts containing 2.5 mg l^{-1} of CYN strongly inhibited growth rates of microalgae after 4 and 7 days of exposure. MC-LR and CYN at environmentally occurring concentrations were unable to affect negatively the growth of microalgae, and therefore these molecules may play roles other than allelopathy in natural ecosystems.

Keywords Cyanobacteria · Microalgae · *Chlamydomonas reinhardtii* · *Chlorella vulgaris* · *Nannochloropsis* sp. · Microcystin-LR · Cylindrospermopsin · Allelopathy

Introduction

The increasing eutrophication of aquatic ecosystems due to anthropogenic action, along with specific and favorable environmental conditions such as high temperature and high input of nutrients (particularly phosphorus and nitrogen), elevated light intensity, and low turbulence, may lead to high cyanobacterial cell densities. This overgrowth often gives rise to cyanobacterial blooms which can have serious adverse

effects on water quality and esthetics and consequently on aquatic communities worldwide (Lindholm et al., 1989; Kneale & Howard, 1997; Sukenik et al., 1998; Zurawell et al., 2005; Naselli-Flores et al., 2007). Negative impacts of these blooms include an increase of turbidity, nonpotability, and death of fish populations due to oxygen depletion and ammonia release as the cyanobacteria decay (Zurawell et al., 2005).

Cyanobacteria are able to produce a wide range of different types of toxins including hepatotoxins, neurotoxins, and dermatotoxins which may affect human and animal health and cause ecological and esthetic concerns (Zurawell et al., 2005; van Apeldoorn et al., 2007). Globally, the most frequently detected toxins in cyanobacterial blooms in fresh and brackish waters are the hepatotoxic microcystins (MCs) and the cytotoxic cylindrospermopsin (CYN) (Codd et al., 2005; Zurawell et al., 2005; Prasanna et al., 2010). MCs are produced by a larger number of cyanobacterial species such as *Microcystis aeruginosa* and *Planktothrix agardhii* and belong to a family of cyclic heptapeptides that potently inhibit protein phosphatases 1 and 2A (PPP1 and PPP2A, respectively) from both animals and higher plants (Mackintosh et al., 1990; Gulledge et al., 2002; Mezhoud et al., 2008). To date, more than 75 different structural variants have been identified, with MC-LR being the most frequent and studied variant to date (Amado & Monserrat, 2010). CYN is a tricyclic alkaloid produced by some species such as *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*. CYN may irreversibly inhibit glutathione and protein synthesis (Terao et al., 1994; Runnegar et al., 1995; Froscio et al., 2001, 2008), even though cytochrome P-450 (CYP450)-derived metabolites may likely be involved in its acute toxicity (Humpage et al., 2005).

Despite the increasing investigation on MC-LR and CYN toxicity, the biological role of these molecules in the aquatic ecosystem is still under discussion. Besides biological functions previously discussed by Babica et al. (2006), one of the recent hypotheses suggests that MCs are implicated in the development and persistence of bloom-forming *Microcystis* colonies through a mechanism that may involve activation of polysaccharide biosynthesis (overexpression of the genes *cap*D, *csa*B, *tag*H, and *eps*L) and the production of extracellular polysaccharides (Gan et al., 2011). The biological role and function of CYN were also recently clarified, showing it to be an allelopathic compound which increases the availability of organic phosphorus by inducing other phytoplanktonic organisms to overproduce and excrete large amounts of extracellular alkaline phosphatases to the water column, even when they are not phosphorus deficient (Bar-Yosef et al., 2010). In this way, CYN-producing species such as *A. ovalisporum* might apparently save energy by CYN production and control the sources of organic phosphorus.

During a cyanobacterial bloom, unlike CYN, which can be continuously liberated into the water body due to its hydrophilic properties, cyanotoxins such as MCs and nodularins are largely accumulated within live cyanobacterial cells (Pflugmacher, 2002; Wiegand & Pflugmacher, 2005). Nevertheless, after application of algicides (Jones & Orr, 1994) and when chemical and physical conditions are unfavorable, high amounts of cyanotoxins are released into the water body upon senescence and/or cell lysis (Pflugmacher, 2002; Wiegand & Pflugmacher, 2005; Babica et al., 2006). In the environment, the concentration of dissolved MCs may reach values as high as 1.8 mg l^{-1} (Jones & Orr, 1994), while the reported CYN concentrations may attain 0.8 mg l^{-1} (Griffiths & Saker, 2003). In some cases, however, extremely high concentrations (up to 25 mg l^{-1}) can occur in the water after collapse of cyanobacterial blooms (Nagata et al., 1997; Kemp & John, 2006; Máthé et al., 2007). Once in the water column, cyanotoxins could come into direct contact with a range of aquatic organisms including algae, aquatic plants, and phytoplankton grazers (Pflugmacher et al., 1999; Vasconcelos, 2001; Pflugmacher, 2004; Cazenave et al., 2006; Cerbin et al., 2010).

Several studies have shown that MCs and CYN can induce genotoxicity and cytotoxicity (Humpage et al., 2005) and are potential carcinogens (Falconer & Humpage, 2001). Moreover, they can affect reproduction (Kinnear et al., 2007), induce oxidative stress (Li et al., 2003), alter detoxification enzyme activity and antioxidant system (Pietsch et al., 2001; Wiegand et al., 2002; Li et al., 2003), and induce growth inhibition (McElhiney et al., 2001; Beyer et al., 2009). Furthermore, changes in photosynthesis (Pietsch et al., 2001; Wiegand et al., 2002) and chlorophyll content (McElhiney et al., 2001; Wiegand et al., 2002), and induction of abnormal mitosis and alterations in microtubule organization in exposed macrophytes (Beyer et al., 2009) may support the hypothesis of allelopathic functions of CYN in higher plants.

However, not much is known about the potential effects of cyanotoxins on microalgae, an ecologically important group which plays an essential primary producer role in aquatic food chains. Moreover, besides the known toxins, other cyanobacterial compounds that could contribute to the increased toxicity of cyanobacterial blooms may be present in the water. This hypothesis should be taken into consideration, since several studies have already evidenced that cyanobacterial cell extracts display increased toxicity in comparison with pure toxins (Pietsch et al., 2001; Mohamed, 2008; Beyer et al., 2009).

The aim of this study is to test the hypothesis that MC-LR and CYN may act as allelopathic compounds in both marine and freshwater phytoplankton species. For this, we evaluated the potential toxic effects of MC-LR and CYN on growth of microalgae using *Chlamydomonas reinhardtii* and *Chlorella vulgaris* (freshwater algae), and *Nannochloropsis* sp. (marine alga species). These phytoplankton species were exposed to *M. aeruginosa* and *A. ovalisporum* cell crude extracts containing the cyanotoxins MC-LR and CYN, respectively, and also to the purified toxins. Toxicity was evaluated by determining the growth rate of microalgae during 4 and 7 days of exposure.

Materials and methods

Culture of cyanobacteria and green microalgae

Microcystis aeruginosa LEGE 91094, *A. ovalisporum* LEGE X-001, *C. reinhardtii* CCAP 11/45, and *C. vulgaris* LEGE Z-001 were maintained in Z8 medium (Kotai, 1972) and *Nannochloropsis* sp. LEGE Z-004 in Z8 medium containing 20 g l^{-1} of NaCl ($Z8_{20}$). The strain LEGE 91094 was verified to produce mainly the MC-LR variant (approximately 95% of total MCs) in the laboratory (Pereira et al., 2009). The freshwater microalga *C. reinhardtii* was described as being sensitive to low concentrations of MCs (0.01 mg l^{-1} concentrations of pure MC-LR and semipurified extract containing MCs) (Kearns & Hunter, 2000, 2001). The freshwater green alga *C. vulgaris* is normally used as a model organism in algal growth inhibition tests. Since the two microalga species are from freshwater environment, they could be found together with MC-LR- and CYN-producing cyanobacteria. Both species can therefore provide further insights into the effects of toxins on freshwater phytoplankton and regarding the putative changes in primary production in ecosystems where the species occur. Despite being commonly found in the marine environment, the marine algae *Nannochloropsis* sp. has recently been found in fresh and brackish waters (including estuaries) (Fawley & Fawley, 2007) and could also co-occur with MC-LR- and CYN-producing cyanobacteria. Some strains of this genus are also important food organisms for aquaculture, and therefore more knowledge is required regarding the putative effects of cyanotoxins on the growth of this microalga species and the possible function in toxin transfer in the trophic chain (Fawley & Fawley, 2007). All species were grown for 3–4 weeks at 25 ± 2°C under light intensity of 10 µmol m^{-2} s^{-1} photon irradiance provided by cool white with a photoperiod of 14 h light and 10 h dark. Cultures were aerated with ambient air filtered through a 0.22-µm membrane. The species were grown as unialgal/unicyanobacterial and nonaxenic cultures. Microbial contamination was regularly monitored by optical microscopy (100× magnification objective) and before performing subcultures. Microalgal cultures were clear of bacteria.

Harvesting of cyanobacterial strains

After 3–4 weeks of growth, *M. aeruginosa* cells were harvested by centrifugation at 4,495 × *g* and 4°C for 15 min while *A. ovalisporum* cells were harvested from the culture medium by filtration through a 20-µm pore plankton net. Samples were then frozen at −80°C and freeze-dried. Lyophilized material was then stored at room temperature and in the dark until toxin extraction and analysis.

Cyanobacterial crude extracts

Microcystis aeruginosa cell crude extract was prepared according to Ramanan et al. (2000) with some modifications. This method was chosen to allow high recovery of MC-LR from the cyanobacterial cells. Besides MC-LR, other hydrophobic metabolites can be extracted and concentrated using the organic solvent methanol. Therefore, further variations in growth of microalgae should be linked to this class of metabolites, which can be overrepresented in the extracts compared with aqueous media or environmental waters. Briefly, lyophilized *M. aeruginosa*

biomass (0.4 g) was extracted with 10 ml MeOH 50% (v/v) by continuous stirring for 20 min at room temperature. The sample was then sonicated in a bath for 15 min at room temperature and ultrasonicated on ice at 60 Hz with five cycles of 1 min (Vibra-Cell 50; Sonics & Material Inc., Danbury, CT, USA). The resulting slurry was centrifuged at $10{,}000 \times g$ for 15 min to remove cell debris. The supernatant was then collected and stored at −4°C. The pellet was re-extracted with an equal volume of solvent following the same approach. The supernatants resulting from both steps of extraction were combined and dried by rotary evaporation at 35°C. The resulting residue was then resuspended in 40 ml distilled water, and the extract was stored at −20°C until use. After quantification (described below) the concentration of MC-LR obtained from lyophilized cells was 8.77 mg l^{-1}. The extract was diluted to the desired concentrations in the culture medium of each microalga for use in the experiments.

Aphanizomenon ovalisporum cell crude extract was obtained based on a modified version of the method described by Welker et al. (2002). Briefly, freeze-dried cells (0.7 g) were extracted with 5 ml distilled water acidified with trifluoroacetic acid (TFA) 0.1% (v/v) similarly to as described above, but the residue resulting from the rotary evaporation was resuspended in 5 ml distilled water. CYN is a hydrophilic molecule and therefore is efficiently extracted using aqueous solution. The extract was stored at −20°C until use. After quantification (described below) the concentration of CYN obtained from lyophilized cells was 51 mg l^{-1}. The extract was diluted to the desired concentrations in the culture medium of each microalga for use in the experiments. *A. ovalisporum* cell extracts may provide a better representation of natural contamination by release of cellular contents, being prepared using water instead of methanol (as for, e.g., *M. aeruginosa* crude extracts).

Toxin extraction and purification

MC-LR was extracted and purified according to Ramanan et al. (2000), with some modifications. Briefly, lyophilized *M. aeruginosa* cells were extracted with 15 ml MeOH 75% (v/v) according to the procedure described above. The concentrate from the extraction step was subjected to solid-phase extraction (SPE) at 1 ml min^{-1} using a Water Sep-Pak® Vac 6 ml C_{18} cartridge that had been preconditioned with MeOH 100% and distilled water. The loaded column was washed with MeOH 20% (v/v), and then the toxin MC-LR was eluted using MeOH 80% (v/v). The MC-LR fraction was evaporated by rotary evaporation at 35°C to remove the entire MeOH portion. Thereafter, the concentrated MC-LR fraction was purified by a Waters Alliance e2695 high-performance liquid chromatography (HPLC) system coupled with a photodiode array 2998 (PDA). The MC-LR semipreparative assay was performed using a reversed-phase column (Phenomenex Luna RP-18, 25 cm × 10 mm, 10 µm) kept at 35°C. The gradient elution was with MeOH and water, both acidified with TFA 0.1%, at flow rate of 2.5 ml min^{-1}. The injected volume was 500 µl. Peak purity and percentage of purified MC-LR were calculated at 214 and 238 nm. The fraction with purified MC-LR was then evaporated with air nitrogen for 1 day until all solvent was removed. The residue was resuspended in distilled water to final concentration of 106.4 mg l^{-1}. The chromatographic purity of MC-LR was 93%. Purified MC-LR was then diluted to the desired concentrations in the culture medium of each microalga for use in the experiments.

Pure CYN was kindly provided by the Laboratory of Prof. Kevin James, Cork University, Ireland. CYN (chromatographic purity 100%) was diluted to the desired concentrations in the culture medium of each microalga for use in experiments.

Toxin quantification

MC-LR quantification in the *M. aeruginosa* cell crude extract was carried out using the same HPLC–PDA system on a Merck Lichrospher RP-18 endcapped (25 cm × 4.6 mm i.d., 5 µm) equipped with a guard column (4 × 4 mm, 5 µm), both kept at 45°C. The linear gradient elution consisted of (A) MeOH + TFA 0.1% and (B) H_2O + TFA 0.1% (55% A and 45% at 0 min, 65% A and 35% B at 5 min, 80% A and 20% B at 10 min, 100% A at 15 min, 55% A and 45% B at 15.1 and 20 min) at flow rate of 0.9 ml min^{-1}. The injected volume was 20 µl. The PDA range was 210–400 nm, with a fixed wavelength at 238 nm. The MC-LR was identified by comparison of spectra and retention time against a MC-LR standard (batch no. 018K1209, 10.025 mg l^{-1} in MeOH, 98% purity; Cyano Biotech GmbH, Berlin). The system was

calibrated by using a set of dilutions of MC-LR standard (0.5–20 mg l^{-1}) in MeOH 50%. Each vial was injected in duplicate, and every HPLC run series of ten samples was constituted with a blank and two different standard concentrations. Empower 2 chromatography data software was used for calculations and reporting peak information. The minimum amount of MC-LR that can be detected in water is 0.2 mg l^{-1}, based on a signal-to-noise ratio of 3. The retention time of the MC-LR peak was 10.44 min (data from method validation not presented).

CYN quantification in *A. ovalisporum* cell crude extract was carried out using the same HPLC system on an Atlantis® HILLIC phase column (250 mm × 4.6 mm i.d., 5 µm) from Waters kept at 40°C. The isocratic elution was a solution of MeOH 5% (v/v) containing 2 mM sodium 1-heptanesulfonate monohydrate (99%) with flow of 0.9 ml min^{-1} and injection volume of 10 µl. The PDA range was 210–400 nm with a fixed wavelength of 262 nm. The system was calibrated by using a set of seven dilutions of CYN standard (0.5–25 mg l^{-1}) in ultrapure water. Each vial was injected in duplicate, and every HPLC run series of ten samples was constituted with a blank and two different standard concentrations. Empower 2 chromatography data software was used for calculations and reporting peak information. The minimum amount of CYN that can be detected in water is 0.3 mg l^{-1}, based on a signal-to-noise ratio of 3. The retention time of the CYN peak was 7.35 min (data from method validation not presented).

All HPLC solvents were filtered (Pall GH Polypro 47 mm, 0.2 µm) and degassed by ultrasonic bath.

Algal growth inhibition assays

Algal growth inhibition tests adapted from Gantar et al. (2008) were performed in 96-well polystyrene microplates because of the need to reduce the test volume to restrict the amount of toxins necessary for the concentrations chosen for the tests. The final test volume was 200 µl per well. One hundred microliters of each log-phase growing microalga stock culture was added to each well containing 100 µl of the desired concentration (prepared in culture media) of each cell crude extract and pure toxin to achieve a final cell density of 5×10^5 cells ml^{-1}. Final concentrations of each cell crude extract were 0.005, 0.025, 0.05, 0.25, 0.5, and 2.5 mg l^{-1}. In addition, cultures without toxin were performed as negative control and for comparison of growth variations. For the growth inhibition tests with crude cell extracts, a second control, consisting of 100 µl of culture media and 100 µl of each concentration of each cell crude extract, was used to evaluate the interference of the extracts in the absorbance at 750 nm. Taking into account that pure toxins usually have lower toxic effects than extracts (Pietsch et al., 2001; Mohamed, 2008) and that one of the aims of this study is to determine 50 % inhibition concentration (IC_{50}) values, we decided to increase the toxin concentrations when used in pure solutions. Final effective concentrations of MC-LR were 0.04, 0.2, 0.4, 0.8, and 1.6 mg l^{-1} in the assays with *C. reinhardtii* and *Nannochloropsis* sp. and 1.2, 6.5, 11.2, 23.9, and 37.3 mg l^{-1} in the assay with *C. vulgaris*, including a negative control. Final effective concentrations of CYN were 0.4, 2.2, 4.4, 8.5, and 16.7 mg l^{-1} plus a negative control. We have also performed a validation of our algal growth inhibition assay using the reference substance potassium dichromate (5, 10, and 20 mg l^{-1}). Three replicates were performed for each cyanotoxin concentration. Cell crude extract experiments were repeated three times, while one experiment was performed for purified toxins. Microplates were sealed with perforated Parafilm M (Pechiney Plastic Packaging, Chicago, USA) to reduce evaporation and allow gas exchange, and then incubated under the same conditions as described above for cultures of microalgae. The duration of the assays was initially established as 4 days. However, the duration was extended to 7 days of exposure since previous studies showed that toxins cause weaker effect in short-term exposures (Babica et al., 2007). The pH was evaluated at the beginning (pH between 7.3 and 7.4) and at the end of each experiment (pH between 9.1 and 9.4). Algal growth was measured indirectly by optical density (OD) at 750 nm using a microplate reader (PowerWave; Biotek, VT, USA). The first reading was taken at the beginning of each experiment with subsequent readings at 4 and 7 days, being converted to cell density (cells ml^{-1}). Cell density was calculated by using a calibration curve for each microalga species ($R^2 = 0.99$ for all curves, data not shown). Before measuring OD, the microalgal cells in each well were resuspended using a pipette. The stability of MC-LR and CYN was monitored by HPLC-PAD using solutions with the same concentrations and conditions as the inhibition assays. It was concluded

that, during the 7 days of exposure, no toxin degradation was observed. Moreover, no significant changes in the pH of the cultures were observed, indicating that this parameter did not interfere with the response of microalgae to the toxin treatments.

The average specific growth rate was calculated as the logarithmic increase in cell density after 4 and 7 days according to the following equation:

$$\mu_{i-j} = \frac{\ln B_j - \ln B_i}{t_j - t_i},$$

where μ_{i-j} is the average specific growth rate from time i to j, t_i is the time for the start of the exposure period, t_j is the time for the end of the exposure period, B_i is the cell density at time i, and B_j is the cell density at time j.

The inhibition of algal growth was estimated as the percentage reduction of growth rate with respect to the control as

$$\%I = \frac{\mu_c - \mu_t}{\mu_c} \times 100,$$

where $\%I$ is the mean percentage inhibition of specific growth rate, μ_c is the mean value for the growth rate of the control, and μ_t is the mean value for the growth rate in the treatment.

Statistical analysis

All of the experiments with cell crude extracts were repeated three times. Regarding pure toxins, only one experiment was performed. The results were considered valid when reproducible in at least two independent experiments.

Significant differences in growth rate between the negative control and the treatments were analyzed using parametric one-way analysis of variance (ANOVA). A post hoc multiple-comparison Dunnett's method was applied when differences were obtained in data that followed a normal distribution with homogeneity of variances (Zar, 1996). Results were considered significant at the $P < 0.05$ level. IC_{50} values were calculated, when possible, using a sigmoidal function (logistic three-parameter). The statistical analyses and IC_{50} calculations were performed using the SigmaPlot software package (SPSS, 2002).

Results

Algal growth inhibition assays with cyanobacterial extracts

The results obtained for the growth of microalgae following exposure to the cyanobacterial extracts are presented in Figs. 1 and 2. A significant increase in growth rates was registered upon exposure to the MC-LR-containing *M. aeruginosa* cell extract (Fig. 1), though with different trends. The growth rates of *C. reinhardtii* and *C. vulgaris* were significantly stimulated only in the extracts with the highest MC-LR concentrations (0.5 and 2.5 mg l^{-1}) after 4 days of exposure to *M. aeruginosa* cell extract (Dunnett's method, $P < 0.05$), while the *Nannochloropsis* sp. growth rate was significantly increased when exposed to *M. aeruginosa* cell extract containing 0.025 mg l^{-1} or higher concentrations of MC-LR after the same exposure period (one-way ANOVA, $F_{6,14} = 14.86$,

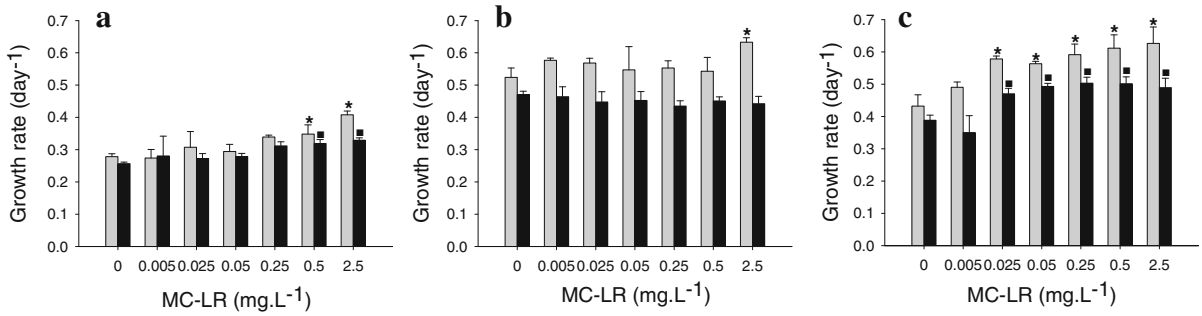

Fig. 1 Growth rates of the microalgae *Chlamydomonas reinhardtii* (**a**), *Chlorella vulgaris* (**b**), and *Nannochloropsis* sp. (**c**) after 4 (*grey bars*) and 7 (*black bars*) days of exposure to MC-LR-containing *M. aeruginosa* crude extract. Results are expressed as average ± standard error. Data significantly different from controls at the fourth (*asterisks*) and seventh (*filled squares*) day of growth (Dunnett's test, $P < 0.05$) are indicated

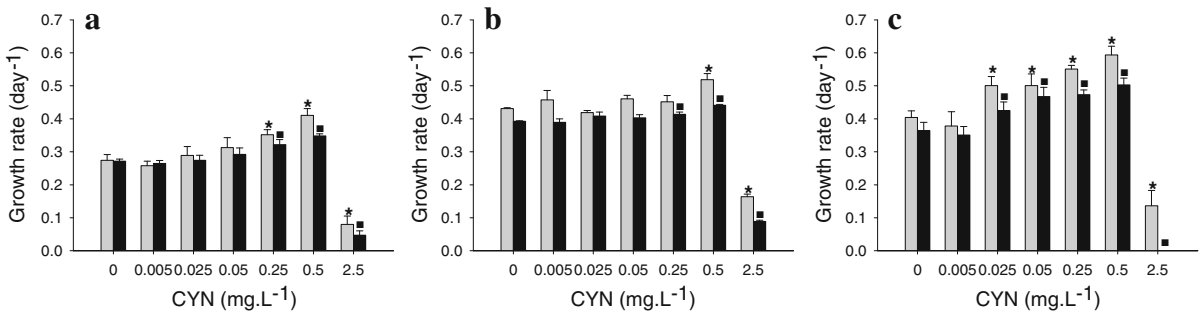

Fig. 2 Growth rates of the microalgae *Chlamydomonas reinhardtii* (**a**), *Chlorella vulgaris* (**b**), and *Nannochloropsis* sp. (**c**) after 4 (*grey bars*) and 7 (*black bars*) days of exposure to CYN-containing *A. ovalisporum* crude extract. Results are expressed as average ± standard error. Data significantly different from controls at the fourth (*asterisks*) and seventh (*filled squares*) day of growth (Dunnett's test, $P < 0.05$) are indicated

$P < 0.001$). Although the growth pattern of *C. reinhardtii* and *Nannochloropsis* sp. was maintained after 7 days of exposure to the MC-LR-containing *M. aeruginosa* cell extract, the increase of growth rates was less pronounced compared with the control (Table 1). On the other hand, no significant differences were observed in the growth rate of *C. vulgaris* compared with control after 7 days of exposure (one-way ANOVA, $F_{6,14} = 0.83$, $P = 0.567$). In general, the marine microalga *Nannochloropsis* sp. was the species which showed the highest increases in growth rate after 4 and 7 days of exposure, with percentages of growth rate stimulation above 30% compared with control (Table 1).

Contrary to what was verified with MC-LR-containing *M. aeruginosa* cell extract, the growth rates of the studied microalga species were dramatically affected by *A. ovalisporum* cell extract containing 2.5 mg l^{-1} CYN after 4 and 7 days of exposure (Dunnett's method, $P < 0.05$, Fig. 2) leading to percentages of growth rate inhibition higher than 60% (Table 1). This inhibition was more pronounced (>70%) after 7 days of exposure. However, *Nannochloropsis* sp. was the microalga most affected by the CYN-containing *A. ovalisporum* cell extract, with a percentage of growth rate inhibition of 100% after 7 days of exposure (Table 1). Lower concentrations of CYN (<2.5 mg l^{-1}) in the *A. ovalisporum* cell extract led to an increase of microalgal growth rates (Fig. 2). A significant increase of algal growth rates was observed in *C. vulgaris* and *C. reinhardtii* after 4 and 7 days of exposure to *A. ovalisporum* cell extract containing 0.25 and 0.5 mg l^{-1} of CYN (Dunnett's method, $P < 0.05$). However, the growth rate of *Nannochloropsis* sp. exhibited a different trend, as a significant increase in the growth rate was observed also for lower CYN concentrations, between 0.025 and 0.5 mg l^{-1}, after 4 and 7 days of exposure (one-way ANOVA, $F_{6,14} = 67.44$, $P < 0.001$ and one-way ANOVA, $F_{6,14} = 183.22$, $P < 0.001$, respectively). The highest increases in growth rates were registered for *Nannochloropsis* sp., with a percentage of growth stimulation above 28% for both monitoring days (Table 1).

The IC_{50} values obtained for exposure of microalga species to CYN-containing *A. ovalisporum* cell extract varied between 1.43 and 2.44 mg l^{-1} during the exposure period (Table 2). *Nannochloropsis* sp. displayed lower IC_{50} (1.43 mg l^{-1}) in comparison with *C. reinhardtii* and *C. vulgaris* (2.22 and 2.28 mg l^{-1}, respectively) after 7 days of exposure.

Algal growth inhibition assays with pure toxins

The results obtained for the growth of microalgae following exposure to pure toxins are presented in Figs. 3 and 4. A significant increase in the growth rates was registered upon exposure of microalgae to the purified MC-LR at the highest tested concentrations (Fig. 3). Stimulation of the *C. vulgaris* growth rate was observed after 7 days of exposure to purified MC-LR at 37.3 mg l^{-1} (Dunnett's method, $P < 0.05$), with a growth rate increase of about 10% (Table 1). Growth rates of *C. reinhardtii* and *Nannochloropsis* sp. were significantly increased at concentrations between 0.4 and 1.6 mg l^{-1} after 4 days of exposure (one-way ANOVA, $F_{5,12} = 41.89$, $P < 0.001$ and

Table 1 Growth rate inhibition and stimulation (in percent, compared with control) of microalgae exposed to cyanobacterial crude extracts and pure toxins, on days 4 and 7

Toxin	Concentration (mg l^{-1})	*Chlamydomonas reinhardtii*		*Chlorella vulgaris*		*Nannochloropsis* sp.	
		% Growth rate inhibition[a]		% Growth rate inhibition[a]		% Growth rate inhibition[a]	
		Day 4	Day 7	Day 4	Day 7	Day 4	Day 7
MC-LR							
Crude extract	0.005	−1.4	9.3	10.1	−1.3	13.5	−9.8
	0.025	10.5	6.3	8.5	−4.8	33.8	21.2
	0.05	5.8	8.6	4.4	−3.8	30.4	27.1
	0.25	22.0	21.4	5.5	−7.5	36.9	29.6
	0.5	25.2	24.3	3.6	−4.1	41.6	29.2
	2.5	46.6	28.3	20.8	−5.9	44.9	26.0
Pure toxin	0.04	1.4	−3.2	–	–	12.6	−1.1
	0.2	8.7	−2.7	–	–	22.7	8.4
	0.4	25.5	6.8	–	–	22.1	9.5
	0.8	56.4[b]	19.7	–	–	27.9	10.1
	1.6	96.3[b]	39.2	–	–	36.4	16.8
	1.2	–	–	−3.1	−1.3	–	–
	6.5	–	–	−3.5	−0.1	–	–
	11.2	–	–	−8.0	0.1	–	–
	23.9	–	–	−2.2	6.0	–	–
	37.3	–	–	−3.9	10.4	–	–
CYN							
Crude extract	0.005	−5.3	−2.5	6.1	−0.8	−6.3	−3.9
	0.025	5.5	0.9	−2.8	4.1	24.0	16.7
	0.05	14.2	7.5	6.9	2.8	24.0	28.2
	0.25	28.5	18.5	4.9	5.4	36.4	29.9
	0.5	50.0[b]	28.1	20.3	12.4	47.0	38.0
	2.5	−71.0[b]	−82.7[b]	−62.0[b]	−77.4[b]	−66.3[b]	−100[b]
Pure toxin	0.4	−0.6	−8.1	2.4	1.0	−1.1	−8.6
	2.2	3.4	−3.6	−3.0	0.1	7.8	−4.5
	4.4	7.7	1.2	−3.2	2.2	12.5	−3.1
	8.5	15.8	4.0	−9.5	−0.4	14.1	−0.2
	16.7	21.8	12.8	−6.8	−2.0	17.1	−2.0

[a] Negative values indicate growth rate inhibition; positive values indicate growth rate stimulation

[b] Growth rate inhibition or stimulation higher than or similar to 50%

one-way ANOVA, $F_{5,12} = 4.39$, $P = 0.017$, respectively), with *C. reinhardtii* displaying the highest percentage increase in growth rate (>50% compared with control) (Table 1). After 7 days of exposure, these microalga species exhibited lower growth rates in comparison with day 4 (Fig. 3) and consequently lower percentages of growth stimulation when compared with control (approximately between 20 and 40%) (Table 1).

Pure CYN caused different effects on the growth rates of the three microalgae (Fig. 4). The growth rate of *C. vulgaris* was slightly inhibited at 8.5 and 16.7 mg l^{-1} (percentages of growth rate inhibition of approximately 10%) after 4 days of exposure (Dunnett's method, $P < 0.05$), but no significant differences were observed after 7 days of exposure (one-way ANOVA, $F_{5,12} = 1.43$, $P = 0.281$). The growth rates of *C. reinhardtii* and *Nannochloropsis*

Table 2 IC_{50} values obtained from growth rate data for different microalga species exposed to CYN-containing *A. ovalisporum* crude extract and pure CYN during 4 and 7 days

Test species	Toxin	Days of exposure	IC_{50} (mg CYN l^{-1})	SE
Chlamydomonas reinhardtii	Crude extract	4	2.31	n.d.
		7	2.22	n.d.
Chlorella vulgaris	Crude extract	4	2.44	0.02
		7	2.28	0.02
	Pure toxin	4	>16.7	n.d.
		7	>16.7	n.d.
Nannochloropsis sp.	Crude extract	4	2.33	n.d.
		7	1.43	n.d.

SE standard error, *n.d.* not determined

sp. increased slightly over the 4-day exposure period at the highest concentrations (one-way ANOVA, $F_{5,12} = 8.48$, $P = 0.001$; one-way ANOVA, $F_{5,12} = 5.34$, $P = 0.008$), with stimulation percentages not exceeding 21%. After 7 days of exposure, pure CYN slightly increased the growth rate of *C. reinhardtii* (percentage growth rate stimulation of approximately 13%) only at the concentration of 16.7 mg l^{-1} (Dunnett's method, $P < 0.05$), while in *Nannochloropsis* sp. no alteration in the growth rate was verified.

Discussion

The development of cyanobacterial blooms in eutrophic water is becoming a serious problem due to the production of potent cyanotoxins, which could have adverse effects on aquatic organisms, including microalgae (Wiegand & Pflugmacher, 2005; Babica et al., 2006). MCs were shown to induce both growth inhibition (Kearns & Hunter, 2000; Mahmoud, 2005; Ou et al., 2005; Babica et al., 2007; Valdor & Aboal, 2007) and stimulation (Sedmak & Kosi, 1998; Ou et al., 2005) in microalgae exposed to pure MCs and

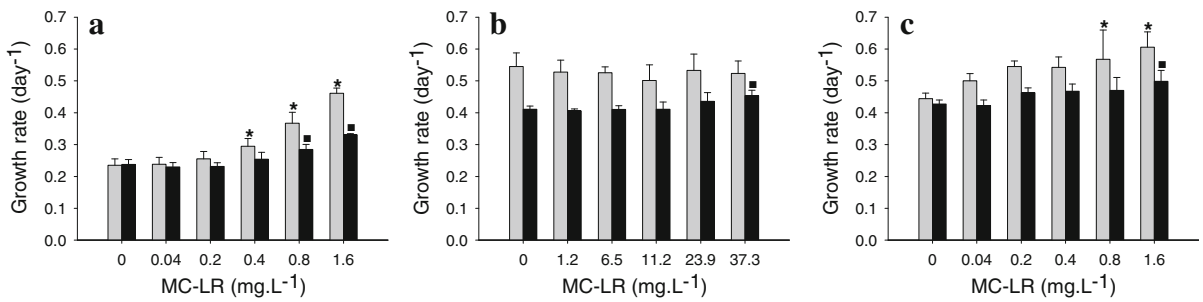

Fig. 3 Growth rates of the microalgae *Chlamydomonas reinhardtii* (**a**), *Chlorella vulgaris* (**b**), and *Nannochloropsis* sp. (**c**) after 4 (*grey bars*) and 7 (*black bars*) days of exposure to purified MC-LR. Results are expressed as average ± standard error. Data significantly different from controls at the fourth (*asterisks*) and seventh (*filled squares*) day of growth (Dunnett's test, $P < 0.05$) are indicated

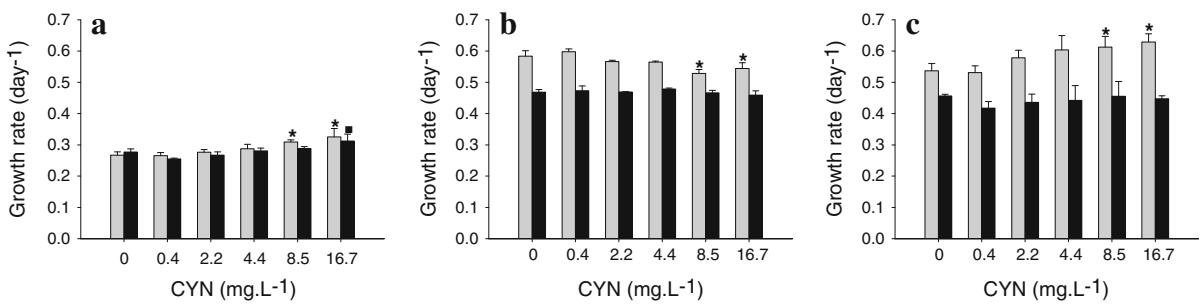

Fig. 4 Growth rates of the microalgae *Chlamydomonas reinhardtii* (**a**), *Chlorella vulgaris* (**b**), and *Nannochloropsis* sp. (**c**) after 4 (*grey bars*) and 7 (*black bars*) days of exposure to pure CYN. Results are expressed as average ± standard error. Data significantly different from controls at the fourth (*asterisks*) and seventh (*filled square*) day of growth (Dunnett's test, $P < 0.05$) are indicated

crude extracts. At the biochemical and molecular level, some studies also reported changes in photosynthetic activity and pigment contents (Sedmak & Eleršek, 2006; Mohamed, 2008), detoxification and antioxidant responses (Pietsch et al., 2001; Ou et al., 2005; Mohamed, 2008), and morphology and ultrastructure of microalgae (Ou et al., 2005; Sedmak & Eleršek, 2006). In the case of CYN, to our knowledge there are no data regarding its potential effects on microalgae apart from the work of Bar-Yosef et al. (2010) that showed some effects of CYN on microalgae.

In this work, log-phase growing microalgae were exposed to cyanobacterial crude extracts containing MC-LR and CYN and respective purified toxins for 7 days, aiming to study the potential toxic effects on their growth that could result from a cyanobacterial bloom scenario. Three- to four-day exposure times are generally recommended for algal growth inhibition tests by some standard guidelines (EPS, 1992, 1996; OECD, 2006). However, according to the literature, the effects of toxins became more apparent and pronounced after long-term exposures (Babica et al., 2006, 2007). For this reason, 4- and 7-day exposures were chosen to evaluate the toxic effects of cyanobacterial crude extracts and purified toxins on the growth rates of microalgae. Exposure times longer than 7 days were not considered in this study in order to maintain unrestricted exponential growth under nutrient-sufficient conditions. Nonetheless, we noticed a slight difference between the growth rates recorded after 4 and 7 days of exposure, which generally became more evident under higher toxin concentrations. This difference may express a more pronounced decrease of the growth rate between the fourth and seventh days as the toxin concentrations increase. These decreases were particularly observed in the cases of *M. aeruginosa* crude extract for *C. vulgaris*, purified MC-LR for *C. reinhardtii*, and both pure toxins for *Nannochloropsis* sp. (data not shown). Since no toxin degradation was verified and microalga species were in log phase at the end of 7 days, the observed growth rate decreases may possibly be a result of nutrient depletion.

Our experiments demonstrated that cyanobacterial crude extracts containing MC-LR and CYN at high concentrations could affect the growth of the microalga species, although with different trends. These cyanobacterial crude extracts clearly induced a stimulatory effect on the microalgal growth rates over the 7-day exposure period, with the exception to *A. ovalisporum* crude extract containing 2.5 mg l^{-1} of CYN. This growth rate stimulation may be related to the presence of other organic products or nutrients in the medium provided by the cyanobacterial crude extracts. Similarly to our results, a few studies have reported stimulatory effects of crude extracts with low MC content (<1.06 mg l^{-1}) (B-Béres et al., 2012) or low purified MC concentrations (0.1–4 mg l^{-1}) (Sedmak & Kosi, 1998; Ou et al., 2005). Our results are not in agreement with other studies which also investigated the effect of cyanobacterial crude extracts containing MCs on microalga species. In a laboratory experiment with 14-day exposure, Mohamed (2008) observed that *M. aeruginosa* crude extract containing MCs caused growth inhibition of *C. vulgaris* and *Scenedesmus quadricauda* at a concentration range lower than those used in our work (0.001–0.1 mg l^{-1}) after 3 days of exposure. Kearns and Hunter (2000) also observed growth inhibition of *C. reinhardtii* by a cell extract from *Anabaena flos-aquae* containing a lower concentration (0.01 mg l^{-1}) of semipurified MCs after 12 days of exposure. Similarly, Mahmoud (2005) observed growth inhibition of *C. vulgaris* and *Chlorococcum humicola* by cyanobacterial crude extracts from two different species: *M. aeruginosa* (strain PCC 7806) and *Nodularia harvenya* (strain PCC 7804). Nevertheless, what is noteworthy in the present study is that the early growth rate stimulation of *C. vulgaris* by *M. aeruginosa* crude extract containing 2.5 mg l^{-1} of MC-LR was observed during the first 4 days of exposure only (with reproducibility within bioassays), but not during the remaining period of the experiment, contrary to what happened in the case of *C. reinhardtii* and *Nannochloropsis* sp., where the increases of growth rates continued and persisted until the seventh day of exposure. In fact, our results showed only minor, nonsignificant inhibition of growth rate of *C. vulgaris* after 7 days of exposure, suggesting that longer exposure periods may lead to a significant decrease in the growth rate of microalga species.

On the other hand, *A. ovalisporum* crude extract containing 2.5 mg l^{-1} of CYN strongly inhibited the growth rate of all microalga species during the exposure time (>60% inhibition), whilst for intermediate CYN concentrations, enhancement of their growth rates occurred, being statistically higher than those of the control from 0.025 to 0.5 mg l^{-1} of CYN.

This pattern may be related to a hormetic response, which has already been documented as a common dose–response relationship for exposure to several stressors (Calabrese, 2002). The prevalence of the stimulating effect at lower CYN concentrations could again be attributed to the extra nutrients provided in the cyanobacterial crude extract. In contrast, the algal growth rate inhibition under *A. ovalisporum* crude extract containing 2.5 mg l^{-1} of CYN should be mainly due to the toxicity of other bioactive compounds potentially present in the cyanobacterial extract or even a synergistic effect resulting from the action of CYN and other bioactive compounds. Higher toxicity has been demonstrated, for example, in *C. raciborskii* crude extract in comparison with pure CYN (Berry et al., 2009). Nevertheless, to our knowledge, no other bioactive compounds have been reported for this species or for *A. ovalisporum*. As a consequence, the effects observed in this work regarding *A. ovalisporum* should be interpreted taking into consideration these features of the cyanobacterial crude extracts. However, further studies are needed to investigate the possible relationship with such a hormesis effect of cyanobacterial crude extracts or whether such a hormesis effect could be a biological response of microalga species to a cyanobacterial bloom.

The differences observed between the results of our experiments and previous works concerning cyanobacterial crude extracts may be attributed to differences in the composition of the crude extracts and/or the presence of other bioactive compounds. The composition of the crude extracts will depend on the extraction procedures as well as the cyanobacterial species or strain; For example, Mahmoud (2005) extracted the toxins from *M. aeruginosa* PCC 7806 and *N. harvenya* PCC 7804 using Bold's basal medium, while in our study we used 50% MeOH solution to extract MC-LR from *M. aeruginosa*. Therefore, it is possible that growth inhibition of microalgae observed in previous works could be mediated by the action of other bioactive metabolites in combination with MCs.

The results of our experiments also demonstrate that purified MC-LR and CYN at higher concentrations ($\geq$8 mg l^{-1}) could stimulate growth rates of microalga species. In general, the growth rate increases did not exceed 26%, even though growth rate increases higher than 36% relative to control were registered for *C. reinhardtii* and *Nannochloropsis* sp. on exposure to 0.8 and 1.6 mg l^{-1} of purified MC-LR. Similarly to our results, a few studies have reported significant increases of growth of microalgae on exposure to MC concentrations lower than or similar to 0.5 mg l^{-1}. In a laboratory experiment with purified MC-RR at concentrations of 0.104 and 0.519 mg l^{-1} (with 14 days of exposure), Sedmak and Kosi (1998) demonstrated that the green alga *Coelastrum microporum* exhibited early growth stimulation after 10 days of exposure followed by subsequent growth inhibition in the remaining days. Using a similar experimental design with a different exposure time (16 days), the authors also observed growth stimulation of the green alga *Monoraphidium contortum* at MC-RR concentration of 0.104 mg l^{-1}. Ou et al. (2005) showed that the growth of the grazing chrysophyte *Posterioochromonas* sp. increased remarkably in the presence of MC-LR and MC-RR at concentrations between 0.1 and 4 mg l^{-1} within 17 days of exposure.

Pure CYN slightly inhibited the growth rate of *C. vulgaris* (<10% inhibition) after 4 days of exposure to concentrations of 8.5 and 16.7 mg l^{-1}. After 7 days of exposure, no growth rate inhibition was observed, suggesting the loss of CYN activity. One plausible explanation for this loss of activity is the activation of detoxification mechanisms and biotransformation of CYN by the microalgae via the activity of glutathione transferase (GST), as demonstrated for MC (Kondo et al., 1992). Nevertheless, the glutathione–CYN conjugate has not been identified so far. The rest of the results show that CYN has no significant effects on the growth of the three species. To our knowledge, this study provides the first evidence that CYN at environmentally occurring concentrations ($\leq$0.8 mg l^{-1}) does not cause significant negative effects on the growth of three phytoplankton species (Griffiths & Saker, 2003; Bogialli et al., 2006; Rücker et al., 2007; Gallo et al., 2009).

Contrary to our results, previous works have shown that pure MCs may have adverse effects on the growth of microalgae. Babica et al. (2007) demonstrated that the growth of five planktonic photoautotrophic representatives of Chlorophyta (*C. reinhardtii*, *Chlorella kesslerii*, *Pediastrum duplex*, *Pseudokirchneriella subcapitata*, and *S. quadricauda*) was strongly inhibited by pure MC-LR and MC-RR at concentration of 25 mg l^{-1} during an exposure period of 11 days. *P. subcapitata* was shown to be the most sensitive species, being strongly inhibited by both MC variants

at concentrations above 1 mg l^{-1}. In another study (14-day exposure experiment), lower concentrations of pure MC-LR (0.001–0.1 mg l^{-1}) were also reported to inhibit the growth of *C. vulgaris* and *S. quadricauda* after 3 days of exposure, with subsequent recovery during the remaining period of the experiment (Mohamed, 2008).

In general, cyanobacterial crude extracts caused more pronounced effects than the pure toxins on the growth rate of microalga species in the present study. This outcome was also consistently demonstrated in previous studies reporting the adverse effects of cyanobacterial crude extracts compared with pure MCs on aquatic organisms, including microalgae (Pietsch et al., 2001; Mohamed, 2008). These results confirm that a mixture of different metabolites with different chemical properties in the cyanobacterial crude extract may interact in a synergistic way and induce higher toxicity compared with that observed for pure toxins. Several compounds of the cell crude extracts, including the known cyanotoxins, are soluble in water. After senescence and/or cell lysis of a cyanobacterial bloom, these soluble compounds as well as the cyanotoxins are released into the water, becoming immediately available for uptake. Once in natural waters, these compounds may interact with each other and cause adverse toxic effects on aquatic organisms. This hydrophilic property is therefore crucial for the potential toxic action on aquatic organisms, including microalgae. The impact on the environment and animal health may be increased considering the relative stability of CYN and MCs (Tsuji et al., 1994; Chiswell et al., 1999) and, in turn, their persistence in the water after cell lysis (Eaglesham et al., 1999).

Our experiments also showed that the growth responses of the microalgae were species specific and dependent on the type and concentration of toxin applied. On the one hand, *Nannochloropsis* sp. was the species most sensitive to *A. ovalisporum* crude extract, with the lowest IC_{50} values for 4 and 7 days of exposure (2.33 and 1.43 mg l^{-1}, respectively). This sensitivity may be explained based on the aquatic environment where this microalga can be found. Although it is well known primarily from marine environments, *Nannochloropsis* sp. has recently been reported in fresh and brackish waters (Fawley & Fawley, 2007), including estuaries. In European brackish waters, *Nannochloropsis* sp. can predominantly co-occur with cyanobacteria belonging to the planktonic genera *Nodularia*, *Aphanizomemon*, *Microcystis*, and *Anabaena*, but also the benthic genera *Anabaena*. Most of these genera are reported to be hepatotoxin (MCs and nodularins) producers (Lopes & Vasconcelos, 2011). Recent studies have reported *M. aeruginosa* cyanobacterial blooms in estuarine waters, where MC-LR and demethyl MC-LR were the most dominant congeners detected (Sobrino et al., 2004; Lehman et al., 2005, 2008). In marine waters, *Nannochloropsis* sp. may be in contact with toxins that reach the ocean. Recently, Miller et al. (2010) demonstrated that MCs from a toxic bloom of *Microcystis* of a Californian estuary had the potential to enter marine coastal ecosystems and cause death of marine mammals from MC intoxication. Despite confirmation of outflows of MC-contaminated fresh water to the ocean, the occurrence of toxic cyanobacteria producing MC-LR and CYN in marine environments is effectively low. As a consequence, *Nannochloropsis* sp. will not frequently be exposed to these toxins in its natural habitat (marine environment) and, in turn, does not develop effective defense mechanisms against the adverse action of either toxin. As MC-LR and CYN are typical cyanotoxins from freshwater environments and blooms of cyanobacteria responsible for production of these toxins have increased worldwide in fresh waters, the microalgae *C. reinhardtii* and *C. vulgaris* are putatively more exposed to both toxins and consequently may have developed protective mechanisms against the toxic effects of these compounds. On the other hand, *C. vulgaris* was shown to be the species most sensitive to exposure to pure CYN, even though its growth rate was slightly inhibited at 8.5 and 16.7 mg l^{-1} of CYN.

Environmental concentrations of dissolved MCs are commonly below 0.01 mg l^{-1}, and values ranging from 5×10^{-5} to 5×10^{-3} mg l^{-1} are considered to be typical for aquatic ecosystems with massive cyanobacterial development (Babica et al., 2006, 2007). However, the occurrence of dissolved MCs at concentrations above 0.01 mg l^{-1} might exceptionally occur in water after the collapse of cyanobacterial blooms or the application of algicides. Jones and Orr (1994) detected concentrations of MCs between 1.3 and 1.8 mg l^{-1} in algicide-treated recreational lake water. Kemp and John (2006) measured total MC concentrations as high as 8.43 mg l^{-1} in scum samples. In other cases, even higher MC concentrations (up to 25 mg l^{-1}) have been observed in natural bloom samples (Nagata et al., 1997; Fastner et al., 1999;

Máthé et al., 2007). For CYN, environmentally occurring concentrations with a cyanobacterial bloom may vary considerably from 4×10^{-3} to 0.8 mg l^{-1} in Australian waters (Shaw et al., 1999; Griffiths & Saker, 2003), but Falconer and Humpage (2006) suggested a more frequent concentration range of 1×10^{-3} to 0.01 mg l^{-1} in aquatic ecosystems. In European waters, some monitoring studies reported variations in total CYN content (intra- and extracellular) between 4.1×10^{-4} and 18.4×10^{-3} mg l^{-1} depending on the month and the depth of sampling (Bogialli et al., 2006) and dissolved CYN at concentrations between 8×10^{-5} and 11.75×10^{-3} mg l^{-1} (Rücker et al., 2007; Gallo et al., 2009). In our experiments, toxin concentrations were high compared with the concentrations commonly detected in the environment. However, concentrations corresponding to environmental conditions were found to be completely ineffective in all microalga species, suggesting that the discussed allelopathic effects against microalgae or aquatic plants are unlikely to occur, at least concerning growth of microalgae (Pflugmacher, 2002; Hierro & Callaway, 2003; LeBlanc et al., 2005; Babica et al., 2006, 2007; Sedmak & Eleršek, 2006; B-Béres et al., 2012).

Actually, the allelopathic effects in conditions of low cyanobacterial cell densities, as commonly found in freshwater communities, were recently considered an infrequent process (Leao et al., 2009). On the contrary, some studies reported significant effects of MC-LR on microalgae at concentrations as low as 0.01 mg l^{-1} (Kearns & Hunter, 2000, 2001).

At the level of natural ecosystems, Sedmak and Kosi (1998) reported no alterations in phytoplanktonic species diversity during outbreaks of cyanobacteria with low water MC contents (<0.01 mg l^{-1}). Nevertheless, authors verified a strong negative impact on species diversity and community structure in situations of high cyanobacterial cell densities and high MC values (>0.01 mg l^{-1}). High concentrations of MCs (>0.01 mg l^{-1}) were also reported to cause morphological and physiological changes in microalgae such as cell aggregation, increase of cell volume, vacuolization, and swelling of chloroplasts, as well as to influence metabolism (Ou et al., 2005; Sedmak & Eleršek, 2006). These cell changes and alterations of species diversity and microalgal communities at high MC concentrations may be related to other environmental and biological factors, including the presence of different bioactive compounds other than toxins alone.

Conclusions

This work approached, for the first time, the effects of cyanobacterial crude extracts containing CYN and pure CYN on growth of microalgae. The work evidences that short-term exposure (4–7 days) to cyanobacterial crude extracts and pure toxins can generally lead to an increase of growth rate. A severe decrease of the growth rates of microalgae was obtained only with the highest CYN concentration of *A. ovalisporum* crude extracts, while a slight decrease of the growth rate was observed only for *C. vulgaris* at the highest CYN concentrations. This clearly shows that CYN at concentrations commonly detected in the environment does not affect negatively the growth of *Nannochloropsis* sp., *C. reinhardtii*, and *C. vulgaris*. Concerning MC-LR and MC-LR-containing extracts, the effects obtained showed growth stimulation in some species for concentrations higher than 0.025 mg l^{-1}. *Nannochloropsis* sp. was more sensitive than *C. reinhardtii* or *C. vulgaris*, but only in the case of CYN-containing *A. ovalisporum* crude extracts and only at the highest concentration. The effects on growth rates were species specific and occurred only at the highest concentrations. The results of our study indicate that MC-LR and CYN at environmentally occurring concentrations were unable to affect negatively growth of microalgae, and therefore these molecules may play roles other than allelopathy in natural ecosystems.

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