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Effects on growth, antioxidant enzyme activity and levels of extracellular proteins in the green alga *Chlorella vulgaris* exposed to crude cyanobacterial extracts and pure microcystin and cylindrospermopsin

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ABSTRACT

Toxic cyanobacteria and cyanotoxins have been pointed as important players in the control of phytoplankton diversity and species abundance, causing ecological unbalances and contamination of the environment. *In vitro* experiments have been undertaken to address the impact of toxic cyanobacteria in green algae. In this regard the aim of this work was to compare the toxicity of two cyanobacteria species, *Aphanizomenon ovalisporum* and *Microcystis aeruginosa,* to the green alga *Chlorella vulgaris* by assessing culture growth when exposed for three and seven days to (I) cyanobacterial cell extracts and (II) pure toxins microcystin-LR (MC-LR) and cylindrospermopsin (CYN). The biochemical response of the green alga to pure toxins was also characterized, through the activity of the antioxidant markers glutathione S-transferase (GST) and glutathione peroxidase (GPx) and the expressed extracellular proteins in seven-day exposed cultures. *A. ovalisporum* crude extracts were toxic to *C. vulgaris*. Pure toxins up to 179.0 μg/L, on the other hand, stimulated the green alga growth. Growth results suggest that the toxicity of *A. ovalisporum* extracts is likely due to a synergistic action of CYN and other metabolites produced by the cyanobacterium. Regarding the green alga antioxidant defense mechanism, CYN at 18.4 and 179.0 μg/L increased the activity of GPx and GST while MC-LR inhibited the enzymes' activity at a concentration of 179.0 μg/L demonstrating a contrasting mode of action. Moreover the identification of F-ATPase subunit, adenylate cyclase, sulfate ABC transporter, putative porin, aspartate aminotransferase, methylene-tetrahydrofolate dehydrogenase and chlorophyll a binding proteins in the culture medium of *C. vulgaris* indicates that biochemical processes involved in the transport of metabolites, photosynthesis and amino acid metabolism are affected by cyanobacterial toxins and may contribute to the regulation of green alga growth.

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1. Introduction

Cyanobacterial blooms are regarded as environmental hazards driven by the production and release of secondary metabolites capable to induce toxic reactions in other organisms and humans. Among the toxins produced by cyanobacteria are the cyclic heptapeptides microcystins (MCs), produced by species of the genera *Anabaenopsis, Aphanocapsa, Microcystis*, *Oscillatoria*, *Nostoc* and *Anabaena*, and the tricyclic alkaloid cylindrospermopsin (CYN), produced by filamentous cyanobacteria species such as *Cylindrospermopsis raciborskii, Umezaka natans* and *Aphanizomenon ovalisporum* (Van Apeldoorn et al., 2007). Microcystins comprise a large group of chemical variants. Most frequent variations are amino acid substitutions in two fixed positions and demethylation of D-MeAsp and/or Mdha (Van Apeldoorn et al., 2007). The most representative variant is MC-LR presenting in the structure

Abbreviations: MC-LR, microcystin-LR; CYN, cylindrospermopsin; GST, glutathione s-transferase; GPx, glutathione peroxidase.

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respectively the variable amino acids leucine and arginine. MCs act through the specific inhibition of serine/threonine protein phosphatases PPP1 and PPP2A (Pereira et al., 2011). Moreover, in animal cells, this toxic effect is mediated by the organic anion transporter polypeptides (OATP) that allow the toxin to enter the cells (Campos and Vasconcelos, 2010). On the other hand the specific molecular targets of CYN have not been characterized. One hypothesis advanced regards the inhibition of glutathione and protein synthesis in the target cells likely through the interaction of CYN with ribosomal proteins (Froscio et al., 2008). Moreover the CYN toxicity is considered to be mediated by P-450 generated metabolites (Humpage et al., 2005).

Regarding microalgae allelopathic effects have been reported in species exposed to the toxins or crude cyanobacterial extracts containing MCs or CYN, reinforcing the hypothesis that cyanotoxins have a role in phytoplankton species abundance and composition (Sedmak and Kosi, 1998). Furthermore evidences have been provided underlining that phytoplankton species respond heterogeneously to the toxin (Sedmak and Kosi, 1998; Babica et al., 2007) and possibly display differential susceptibility. Sedmak and Kosi (1998) reported that, in *in vitro* conditions and under low light, MC-RR at 10^{-7} M stimulated the growth of *Chroococcus minutus* nevertheless exposure to high light led to growth inhibition thus evidencing that microalgae response may be influenced by environmental factors such as light intensity. Babica et al. (2007) reported that environmentally relevant concentrations of MC-LR and MC-RR (up to 10 μg/L) had no influence in the growth of several microalgae species. Nevertheless while *Pseudokirchneriella subcapitata* growth was inhibited after four days of exposure to MC-LR or MC-RR in concentrations equal or higher than 1000 μg/L, *S. quadricauda* was affected only at 25,000 μg/L MC-LR. The authors also reported that *C. reinhardtii* and *Chlorella kessleri* showed higher sensitivity to the variant MC-RR contrasting the responses of other microalgae.

In vitro experiments have also demonstrated that MCs affect the physiology and metabolism of microalgae. Cell aggregation, increase in cell volume, and overproduction of photosynthetic pigments were recorded in *Microcystis aeruginosa* and *S. quadricauda* exposed to environmentally relevant concentrations (5×10^{-7} M) MC-LR, -RR, and -YR (Sedmak and Elersek, 2006). Moreover Perron et al. (2012) reported that MCs may affect photosynthetic efficiency and the flow of energy through photosystem II in green algae, when present at concentrations superior to 10 mg/L. Such variations can be detected within only 15 min of exposure (Perron et al., 2012). Pietsch et al. (2001) reported variations in the activity of detoxification enzymes peroxidase (POD) and glutathione **S**-transferase (GST) and photosynthesis in *Scenedesmus armatus* emphasizing the biochemical differences in the response to crude cyanobacterial extracts and purified MCs. Furthermore variability in the biochemical responses to the oxidative stress was recorded in *P. Subcapitata* (Bártová et al., 2011). Pure MCs at 300 μg/L had no influence in green alga growth, nevertheless glutathione reductase (GR) activity increased in the first hours of exposure (3 and 24 h) suggesting the activation of an antioxidant defense mechanism. Other oxidative stress biomarkers (levels of glutathione, GSH, and activities of GST and glutathione peroxidase, GPx) were not affected by cyanobacterial samples (Bártová et al., 2011).

Further advances in this area of investigation regard the comparison of the toxic potential of different cyanobacteria species in green algae, and the factors that influence their toxicity, such as the chemical diversity of the bioactive compounds produced by each species and the synergistic effects in target organisms and phytoplankton. In this context this work aimed to compare the toxicity of two cyanobacterial species, *A. ovalisporum* and *M. aeruginosa* to the green alga *Chlorella vulgaris*, by assessing (I) growth in cultures exposed to cyanobacterial cell extracts and (II) growth, GST and GPx antioxidant enzyme activities and the expressed extracellular proteins in cultures exposed to the pure toxins MC-LR and CYN. The repertory of extracellular proteins present in *C. vulgaris* culture medium provides additional information on the metabolic processes that characterize *C. vulgaris* response to CYN and MC-LR.

2. Materials and methods

2.1. Biological material

C. vulgaris (LEGE Z-001) is from Algoteca, University of Coimbra – ACOI – 879 and was maintained as axenic culture in the laboratory. *M. aeruginosa* (LEGE 91094) was isolated from Lagoa de Mira, Portugal in the laboratory and *A. ovalisporum* (LEGE X-001) from Lake Kinnerett, Israel. Species identification was confirmed in the laboratory by morphology. Microalgae were grown in Z8 medium (Kotai, 1972), at 25 °C, 22 $\mu Em^{-2} s^{-1}$ light intensity with a light/dark period of 14/10 h, in 500 ml flasks (*C. vulgaris*) or 5 L or 15 L containers (*A. ovalisporum* and *M. aeruginosa* strains) (Saker et al., 2003) under sterile conditions. After 45 days of culturing, cyanobacterial biomasses were collected by filtration (*A. ovalisporum*) or centrifugation (*M. aeruginosa*) and thereafter frozen at −80° C and lyophilized. *C. vulgaris* cultures were renewed after eleven to fourteen days of growth (stationary phase) with 5×10^5 cells/mL cell density. Absence of bacterial contamination was confirmed by optical microscopy (Pinheiro et al., 2013).

2.2. Cyanobacterial cell extracts

Cyanobacterial biomasses were collected by centrifugation for 10 min at 40,000*g* and resuspended in distilled water (0.2 g biomass/mL). Cells were subsequently lysed by sonication in bath for 15 min followed by a second treatment with vibracell at 60 HZ, with 5 cycles of 1 min. The crude cell extracts were stored at −20 °C until subsequent use in *C. vulgaris* exposure experiments.

2.3. Purification and quantification of CYN and MC-LR

Cylindrospermopsin (CYN) was extracted from *A. ovalisporum* following a modified version of the method described by Welker et al. (2002). Freeze dried cells (0.7 g) were lysed with ultrasound in bath for 15 min in 5 mL trifluoracetic acid (TFA, 0.1%, v/v), followed by a second treatment with vibracell at 60 HZ, with 5 cycles of 1 min. The homogenate was stirred for 1 h at room temperature, centrifuged (20,000*g*, 4 °C, 20 min) and the supernatant collected and stored at −20 °C. CYN was thereafter purified by a Waters Alliance e2695 HPLC system (Milford, Massachusetts, USA) coupled with a PDA 2998 on a semi-preparative Gemini C18 column (250 mm × 10 mm i.d., 5 μm) from Phenomenex (Torrance, California, USA) kept at 40 °C. The isocratic elution utilized a 5% (v/v) methanol (MeOH) solution containing 2 mM of sodium 1-heptanesulfonate monohydrate with a flow rate of 3 mL/min and 500 μL injection volume. Standard CYN (0.08–5.0 μg/mL) (Alexis, San Diego, CA, USA) was prepared in water. Chromatographic purity of CYN at 262 nm was of 98 percent.

The CYN purified fractions were then quantified in the same HPLC system on a Atlantis® HILLIC phase column (250 mm × 4.6 mm i.d., 5 μm) from Waters kept at 40 °C, with same isocratic elution and flow of 0.9 mL/min and 10 μL injection volume. 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 (25, 20, 10, 5, 2, 1 and 0.5 μg/mL) in ultrapure water.

MC-LR was extracted and purified as described by Pinheiro et al. (2013). Briefly, the lyophilized *M. aeruginosa* biomass was extracted with MeOH 75% (v/v) and lysed with ultrasound in ice at 60 Hz for 5 × 1 min (VibraCell 50-sonics & Material Inc. Danbury, CT, USA). The homogenate was centrifuged at 10,000*g* for 15 min and the supernatant collected and applied to a solid-phase extraction (SPE). The toxin MC-LR was eluted using MeOH 80% (v/v) and concentrated by rotary evaporation at 35 °C (Pinheiro et al., 2013). The MC-LR was thereafter purified and quantified by HPLC-PDA. The MC-LR semi-preparative 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 MeOH and water with 0.1% (v/v) TFA and flow rate 2.5 mL/min. The injected volume was 500 μL. Peak purity and percentage of purified MC-LR were calculated at 214 nm and 238 nm. The MC-LR fraction was then evaporated with air nitrogen and the residue resuspended in distilled water to the desired concentration. Chromatographic purity of MC-LR was of 97 percent.

The MC-LR purified fractions were then quantified in the same HPLC system on a Merck Lichrospher RP-18 endcapped column (250 mm × 4.6 mm i.d., 5 μm) (Whitehouse Station, New Jersey, USA) equipped with a guard column (4 × 4 mm, 5 μm) both kept at 45 °C. The PDA range was 210–400 nm with a fixed wavelength of 238 nm. The linear gradient elution consisted of (A) MeOH+0.1% TFA and (B) H_2O

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+0.1% TFA (55% A and 45% B 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) with a flow rate of 0.9 mL/min. The MC-LR was identified by comparison of spectra and retention time with a standard of MC-LR (Cyano Biotech GmbH, Berlin, Germany). The system was calibrated using a set of seven dilutions of MC-LR standard (0.5–20 μg/mL) in MeOH 50%. The minimum amount of MC-LR that can be detected in water is 0.2 μg/mL, based on a signal-to-noise ratio of 3. The retention time of the MC-LR peak was 10.44 min. Chromatograms and spectra of the purified CYN and MC-LR are presented in Supplemental data (Appendix A).

2.4. Exposure experiments

C. vulgaris (5×10^5 cells/mL) growing in 150 ml glass flasks with 50 ml Z8 medium, with shaking, were exposed during 7 days to (I) *M. aeruginosa* crude cell extracts with MC-LR and *A. ovalisporum* crude cell extracts with CYN and (II) pure MC-LR or CYN at concentrations of 5.0, 18.4 and 179.0 μg/L. Crude cell extracts were prepared as described above using cyanobacterial biomasses equivalent to cellular densities of 10^6 and 10^7 cells/mL in order to mimic a real situation of exposure to the cellular contents of a toxic cyanobacteria outbreak. Final toxin concentrations in *M. aeruginosa* crude extracts were 3.0 and 41.5 μg/L and in *A. ovalisporum* crude extracts 32.0 and 333.0 μg/L CYN. The experiments with the pure toxins were done in order to discard the matrix effects of the crude extracts. Three replicate cultures were performed for each experimental condition ($n=3$). Controls were represented by growing *C. vulgaris* in Z8 medium in the absence of toxin. *C. vulgaris* cell density was estimated with Neubauer chamber and optical microscopy after three and seven days of exposure. Specific growth rate (μ) and percentage reduction of growth rate (%I) were then calculated to assess green alga growth as described in Pinheiro et al. (2013). At the end of the experiments *C. vulgaris* cells were separated from the culture medium by centrifugation for 10 min at 4000*g* and both fractions (cells and culture medium) stored at −80 °C.

2.5. Enzyme activity

Proteins from *C. vulgaris* cells were obtained by sonication (18 cycles of 5 s, at 60 HZ) in phosphate buffer (20 mM, pH 7.0) in a ratio of 0.1 g of cells per 2 ml of buffer. The homogenates were centrifuged at 4600*g* for 20 min at 4 °C, the supernatants were collected and protein concentration was determined by the Bradford (1976) method. Protein samples were thereafter stored at −80 °C until further analysis. Cytosolic GST and GPx activities were determined in microplates according to the methods of Habig et al. (1974) and Lawrence and Burk (1976), respectively. Both reactions were performed with 0.1 mg prot/mL, and the enzymatic activities were expressed in nkat/mg prot.

2.6. Analysis of proteins from C. vulgaris culture medium

Aliquots (50 ml) of *C. vulgaris* culture medium from all group replicates in the experiments with pure MC-LR and CYN were collected and stored at −80 °C. The culture medium of *C. vulgaris* exposed to pure MC-LR was freeze dried and the proteins thereafter solubilized in phosphate buffer (20 mM, pH 7.0). In alternative culture medium from *C. vulgaris* exposed to pure MC-LR was filtered using Amicon Ultra centrifugal devices with 15 mL capacity and membrane molecular weight (MW) cut-off of 10 kDa (Millipore, Billerica, MA, USA). Proteins with MW superior to 10 kDa were therefore concentrated to a final volume between 0.5 and 1.0 mL. Total protein content was determined by the Bradford method (Bradford, 1976). Protein samples were mixed with loading buffer with Tris–HCl (0.25 M) pH 6.8, SDS (6%, w/v), glycerol (30%, v/v), β-mercaptoethanol (6.25%, v/v) and proteins separated by SDS-PAGE according to Laemmli (1970). Gels were stained with colloidal coomassie blue (Neuhoff et al., 1988), gel images acquired using GS-800 calibrated scanner (Bio-Rad, Hercules, CA, USA) and the protein profiles analyzed using the Quantity One software (Bio-Rad, Hercules, CA, USA). Protein abundance in SDS-PAGE gel was calculated as relative intensity (percentage of the sum of the optical densities of the same protein band in all experimental conditions). Optical densities were retrieved by the software Quantity One (Bio-Rad, Hercules, CA, USA).

2.7. Two-dimensional gel electrophoresis (2DE)

Concentrated protein samples (0.7 μg prot.) were diluted to 125 μL in urea (7 M), thiourea (2 M), 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) (4%, w/v), dithiothreitol (65 mM) and ampholytes, pH 4–7 (0.8%, v/v) (Bio-Rad, Hercules, CA, USA) and proteins separated by 2DE essentially as described by Puerto et al. (2011). Protein samples were loaded on to 7 cm, pH 4–7 immobiline dry-strips (Bio-Rad, Hercules, CA, USA) and proteins separated by isoelectric focusing (IEF) in a Protean IEF Cell (Bio-Rad, Hercules, CA, USA) with the following program: 16 h at 50 V (strip rehydration); step 1, 15 min at 250 V; step 2, 2 h voltage gradient to 4000 V (linear ramp); step 3, 4000 V until achieving 20,000 V/h (linear ramp). After the first dimension IEF gel strips were equilibrated using 10 mg/mL dithiothreitol and 25 mg/mL iodoacetamide in urea (6 M), glycerol (30%, v/v), SDS (2%, w/v) (Puerto et al., 2011). Subsequently IEF gel strips were placed on top of 12% (w/v) acrylamide SDS-PAGE slab gels and proteins separated in a miniProtean II cell (Bio-Rad, Hercules, CA, USA) at 150 V. Gel staining for protein visualization was performed as described by Neuhoff et al. (1988).

2.8. Protein identification

Protein identification was performed based in the method described by Santos et al. (2009). Protein spots were excised from gels and proteins subjected to in-gel digestion using the protease trypsin (Pandey and Mann, 2000). The tryptic digests were desalted and concentrated using reversed phase microcolumns (Gobom et al., 1999). The peptides were eluted directly onto the MALDI plate using the matrix α-cyano-4-hydroxycinnamic acid (5 mg/mL) prepared in acetonitrile (70%, v/v) and TFA (0.1%, v/v). Samples were analyzed using a 4700 Proteomics Analyzer MALDI-TOF/TOF (AB SCIEX, Foster City, CA, USA). Peptide mass fingerprint (PMF) data was collected in positive MS reflector mode in the range of 700–4000 (m/z) and was calibrated internally using trypsin autolysis peaks. Several of the highest intensity and/or relevant non tryptic peaks were selected for MS/MS analysis. Both MS and MS/MS spectra were analyzed using the software GPS Explorer (Version 3.6; AB SCIEX), and were searched together against a locally stored copy of the two sections, Swiss-Prot and TrEMBL, of the UniProtKB protein sequence knowledgebase (release 2011_12) using the Mascot search engine (Version 2.1.04) (Perkins et al., 1999). The search included peaks with a signal-to-noise ratio greater than 10 and allowed for up to two missed trypsin cleavage sites. To be considered a match, a confidence interval (CI) of at least of 99 percent, calculated by AB SCIEX GPS Explorer software, was required.

2.9. Statistical analysis

All experiments were performed in triplicate. Data are presented with average value and respective standard deviation (SD). Statistical analysis was performed with the T-student test, considering a confidence level of 95 percent ($P<0.05$).

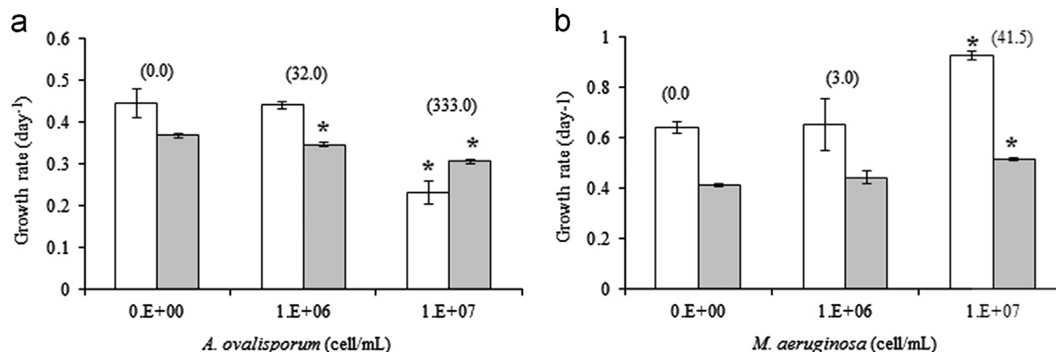

Fig. 1. Growth rate of *C. vulgaris* when exposed to *A. ovalisporum* (a) or *M. aeruginosa* (b) cell extracts varying regarding the cell density (10^6 and 10^7 cell/mL) and toxin concentration. In parenthesis are the concentrations of the toxins CYN and MC-LR (μg/mL) present in *A. ovalisporum* and *M. aeruginosa* extracts respectively. White columns refer to 3-day old and gray columns to 7-day old cultures. Significant differences in the specific growth rate in respect to control (C), at $P<0.05$ (*).

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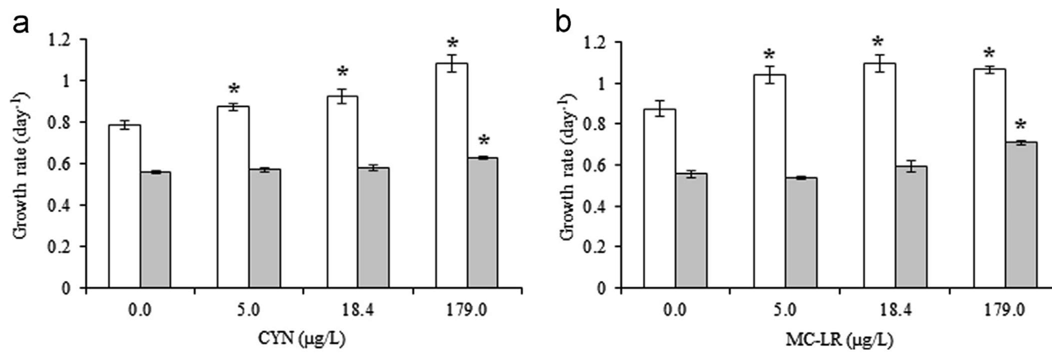

Fig. 2. Growth rate of *C. vulgaris* when exposed to different concentrations of the purified cyanotoxins CYN (a) or MC-LR (b). White columns refer to 3-day and gray columns to 7-day old cultures. Significant differences in the specific growth rate in respect to control (C), at $P < 0.05$ (*).

3. Results

3.1. Growth of *C. vulgaris* in the presence of *A. ovalisporum* and *M. aeruginosa* cell extracts

A significant reduction in *C. vulgaris* growth was registered after seven days of exposure to *A. ovalisporum* cell extract with 10^6 cells/mL (32.0 μg/L CYN) or, after three and seven days to cell extract with 10^7 cells/mL (333.0 μg/L CYN) (Fig. 1a). Highest growth rate inhibition (−48 percent) was observed after three days in cultures exposed to 10^7 cells/mL *A. ovalisporum* cell extract (Fig. 1a). In contrast a significant increase in growth of *C. vulgaris* was registered after exposure for three and seven days to *M. aeruginosa* cell extract with 10^7 cells/mL (41.5 μg/L MC-LR) (Fig. 1b). Highest growth stimulation (45 percent) was observed in the third day of exposure. The results suggest that toxicity of *A. ovalisporum* cell extracts increases with the concentration of the toxin CYN. However this effect may also be potentiated by the presence of other putative bioactive metabolites present in the cell extracts.

3.2. Growth of *C. vulgaris* in the presence of purified MC-LR and CYN

C. vulgaris growth increased after three days of exposure to purified CYN at concentrations of 5.0, 18.4 and 179.0 μg/L. Nevertheless after seven days of exposure only cultures with highest CYN concentration displayed increased growth relative to control (Fig. 2a). Highest stimulation (37 percent) was verified after three days of exposure to 179.0 μg/L CYN. A similar pattern of response was observed in *C. vulgaris* cultures exposed to pure MC-LR. This cyanotoxin stimulated *C. vulgaris* growth during the first three days, in all concentrations tested. After seven days of exposure only the cultures with highest MC-LR concentration displayed increased growth in regard to control (Fig. 2b). Highest stimulation (27 percent) was verified after seven days of exposure to 179.0 μg/L MC-LR.

3.3. Activity of GST and GPx in *C. vulgaris* cells exposed to purified MC-LR and CYN

After seven days of exposure to the cyanotoxins MC-LR and CYN at 5.0, 18.4 or 179.0 μg/L *C. vulgaris* cells were analyzed regarding the activity of enzymes GST and GPx. An increase in the activity of GST was verified in the cells of the green alga exposed to 18.4 and 179.0 μg/L CYN as shown in Fig. 3a. Nevertheless activity of this enzyme was reduced in *C. vulgaris* when exposed to the highest MC-LR concentration (Fig. 3a). A similar pattern of activity was registered for GPx, increasing in *C. vulgaris* cells exposed to 18.4 μg/L CYN but decreasing after seven days of exposure to 179.0 μg/L MC-LR (Fig. 3b).

3.4. *C. vulgaris* extracellular proteins

Regarding the exposure experiments with pure MC-LR we had difficulty to achieve a good solubilization of the proteins from the freeze dried culture medium material. Consequently interpretable (reproducible) patterns of extracellular proteins were achieved only for the replicate cultures exposed to 5.0 and 179.0 μg/L MC-LR. The protein patterns are presented in Fig. 4a. A 65 kDa extracellular protein was resolved in SDS-PAGE gels from *C. vulgaris* cultures exposed to 5 μg/L MC-LR and six proteins with different molecular mass (MM) from the cultures exposed to the highest MC-LR concentration (Fig. 4a). An alternative filtration protocol was thereafter applied to concentrate protein samples from *C. vulgaris* cultures exposed to CYN, therefore minimizing any irreversible precipitation of proteins that can occur during the process of freeze drying. With this approach reproducible protein patterns in all samples from the CYN experiment were achieved (Fig. 4b). All samples were characterized by the presence of the same 65 kDa protein (Fig. 4b). Nevertheless the relative intensity of the protein band in SDS-PAGE gels decreased in culture medium samples with 179.0 μg/L CYN (Fig. 4c) whilst other proteins with lower MM were detected. A 43 kDa protein was detected in the medium of *C. vulgaris* cultures exposed to 18.4 and 179.0 μg/L CYN. Moreover six other proteins, with MM between 48 kDa and 29 kDa were detected in the medium of *C. vulgaris* cultures exposed to the highest concentration of CYN (Fig. 4b). The separation of extracellular proteins from the 179.0 μg/L CYN by two-dimensional gel electrophoresis was also performed (Fig. 5). With this technique the previously reported 65 kDa protein was resolved in two major protein spots with 59 and 62.5 kDa. Another group of proteins could be detected with MM between 29 and 31 kDa. This technique showed to be important to further complement the repertory of proteins identified by mass spectrometry.

3.5. Protein identification

Twenty protein bands/spots were excised from the SDS-PAGE and 2DE gels, digested with the protease trypsin and the peptides analyzed by MALDI-TOF/TOF mass spectrometry. The combination of peptide mass fingerprint data and MS/MS peptide sequencing allowed identifying twelve proteins with functions displayed in Table 1. Six proteins are putatively involved in ion transport and are located in the plasma membrane, vacuole or thylakoid membranes (F-ATPase, putative ion transporter - porin, sulfate ABC transporter). Another putative plasma membrane protein, involved in signal transduction, was identified (adenylate cyclase) and two other proteins putatively involved in the metabolism of purines and amino acids (aspartate aminotransferase and methylenetetrahydrofolate dehydrogenase). Three isoforms of chlorophyll a-b binding proteins (light harvesting complex proteins,

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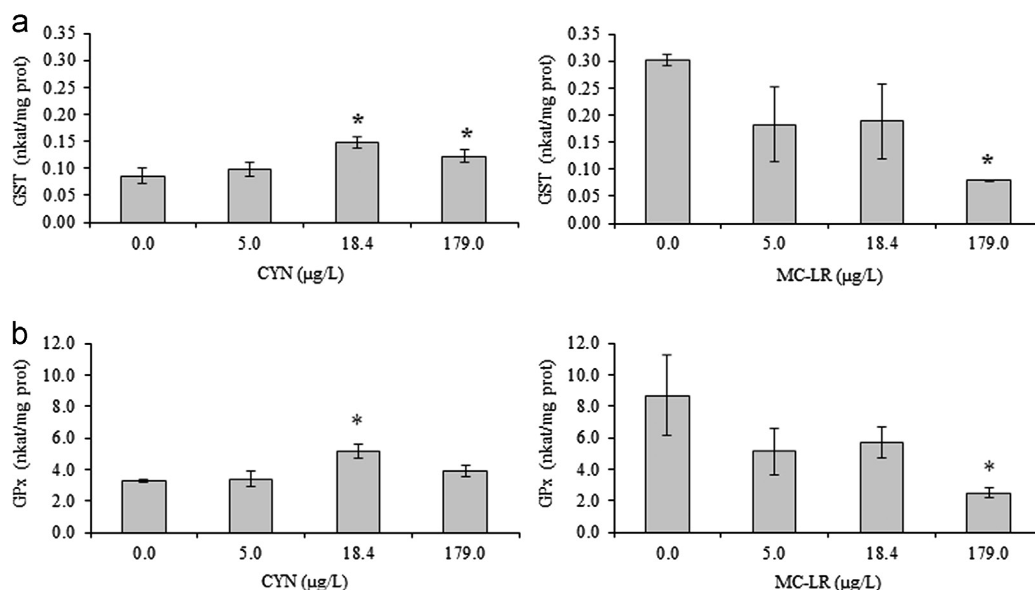

Fig. 3. Activities of the enzymes GST (a) and GPx (b) in *C. vulgaris* cells growing for 7 days at different concentrations of the cyanotoxins CYN and MC-LR. Significant differences in the activity of the enzymes in respect to control $P < 0.05$ (*).

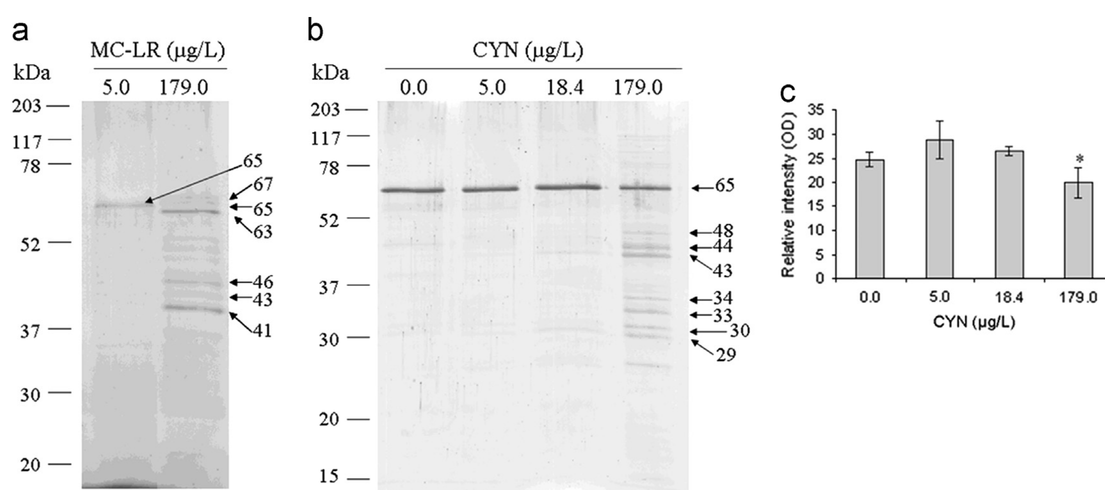

Fig. 4. SDS-PAGE profile of the proteins present in the medium of *C. vulgaris* cultures grown for 7 days and exposed to different concentrations of the toxins MC-LR (a) and CYN (b) and the variation in the relative intensity of the 65 kDa protein band resolved in SDS-PAGE gel b (c). Arrows indicate the reproducible protein bands detected in the culture media and respective molecular mass. The total amount of protein loaded in each lane was 0.7 µg. Significant differences in the relative intensity of the protein in regard to control for $P < 0.05$ (*).

LHCP) were also identified from the 2DE gels. The most abundant protein in the culture medium was F-ATPase which, in 2DE gels, was separated in two isoforms. Another protein present in the MC-LR and CYN cultures (estimated MM 41 and 43 kDa respectively) is a putative ion transporter. Six proteins were identified that are expressed by different bacteria species. A blast search with those proteins was performed, allowing to reveal homologous sequences with a maximum identity between 29% and 75%, from plants and green algae (Table 1), suggesting that homologous proteins are putatively expressed in species of these taxonomic groups.

4. Discussion

4.1. Effects of cyanobacterial crude extracts and pure toxins in C. vulgaris Growth

Results have shown that *A. ovalisporum* cell extracts, replicating a senescent bloom with 10^6 and 10^7 cells/mL, reduce *C. vulgaris* growth. The CYN concentrations estimated by HPLC in these extracts were 32.0 and 333.0 µg/L respectively. On the other hand *M. aeruginosa* extracts simulating also a senescent bloom with equivalent cell densities and with 3.0 and 41.5 µg/L MC-LR, stimulated *C. vulgaris* growth. Purified CYN and MC-LR, between 5.0 and 179.0 µg/L, also led to the increase of *C. vulgaris* growth. These results underline (1) the toxic potential of *A. ovalisporum* in regard to *M. aeruginosa* extracts and (2) the putative presence of other bioactive metabolites in *A. ovalisporum* that may act synergistically with the toxin CYN. Among the putative metabolites are the saxitoxin (STX), anatoxin-a (ATX) and lipopolysaccharides (LPS) reported in *Aphanizomenon* genera (Ballot et al., 2010; Bernardová et al., 2008; Wiese et al., 2010). In a recent study our group reported growth stimulation of *C. reinhardtii* and *Nannochloropsis* sp. to *M. aeruginosa* extracts (25–2500 µg/L MC-LR) (Pinheiro et al., 2013). *C. vulgaris* cultures, on the other hand, showed no clear response to *M. aeruginosa* extracts. Moreover *A. ovalisporum* extracts inhibited green algae growth only when CYN was present at 2500 µg/L. In the same study pure CYN and MC-LR

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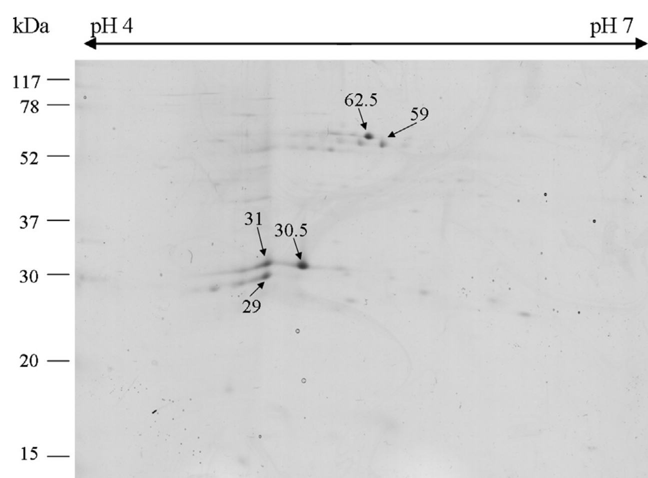

Fig. 5. Two-dimensional gel electrophoresis of extracellular proteins from *C. vulgaris* cultures exposed to 179.0 μg/L CYN. Total amount of protein loaded in the gel was 0.7 μg. Estimated molecular masses are displayed for the most abundant proteins.

at concentrations between 400 and 37,300 μg/L stimulated of *C. reinhardtii* and *Nannochloropsis* sp. growth however *C. vulgaris* growth was inhibited after three days of exposure to CYN (8500–16700 μg/L). The present study thus corroborates the previously reported tolerance of green algae to pure CYN and MC-LR. However *C. vulgaris* cultures used in the present study showed increased sensitivity, with significant stimulation being registered, for instance, at 5.0 μg/L pure toxins and growth inhibition in *A. ovalisporum* cell extracts with 32.0 μg/L CYN. This sensitivity was more evident in the first three days of exposure, with some of the differences in growth being reported for this period and not at the end of the experiment (seven days of exposure). This might suggest the green alga adaptation to the cyanobacterial compounds, possibly modulated by the activation of defense mechanisms, or the toxin concentration decay during the exposure period. In only one situation (exposure to *A. ovalisporum* 10^7 cells/ml extract) growth was inhibited after seven days of exposure but no effects were registered in the first three days.

Regarding the effects of MCs in green algae, Mohamed (2008) reported initial inhibitory response of *C. vulgaris* and *S. quadricu-lata* cultures after three days of exposure to crude MCs or purified

Table 1
List of proteins identified in *C. vulgaris* culture media, using MALDI-TOF/TOF mass spectrometry. Details regarding the identification are presented in the subsequent Appendix B.

Protein # (kDa)	Protein name	Accession number (UniProtKB)	Organism	Molecular mass (kDa)[a]	pI[a]	Biological function	Subcellular location	Homology[b]
MC-LR cultures								
65	F-ATPase family transporter: protons (vacuolar)	A4S429	*Ostreococcus lucimarinus*	68.4	5.0	Hydrogen ion transporting ATP synthase activity	Vacuolar membrane	Nd[c]
63	Adenylate cyclase	E0SDN8	*Dickeya dadantii*	50.2	5.8	Signal transduction; conversion of ATP to 3′,5'-cyclic AMP (cAMP) and pyrophosphate	Plasma membrane	putative F-box protein (29%) (Q9ZPR5)
46	Putative uncharacterized protein	F5RDN7	*Methyloversatilis universalis*	40.3	5.01	Ion transporter, porin	Membrane	predicted protein (35%) (B9NJM1)
41	Sulfate ABC transporter periplasmic	F3LSJ9	*Rubrivivax benzoatilyticus*	36.5	9.15	Sulfate transmembrane-transporting ATPase activity	Outer membrane	sulfate-binding protein precursor, putative (74%) (B9TB09)
CYN cultures								
62.5	F-ATPase subunit alpha	D3WD49	*Berberidopsis corallina*	55.5	5.2	Hydrogen ion transporting ATP synthase activity	Thylakoid membrane	Nd
59	F-ATPase subunit alpha	Q332Y4	*Lactuca sativa*	55.5	5.3	Hydrogen ion transporting ATP synthase activity	Thylakoid membrane	Nd
44	Aspartate aminotransferase	F5R976	*Methyloversatilis universalis*	43.6	7.0	Amino acid degradation and biosynthesis, transaminase activity	Cytosol, mitochondria	Aspartate aminotransferase (47%) (A8I263)
43	Putative uncharacterized protein	F5RDN7	*Methyloversatilis universalis*	40.3	5.01	Ion transporter, porin	Membrane	Predicted protein (35%) (B9NJM1)
33	NADP-dependent methylenetetrahydromethanopterin dehydrogenase	F5R8M3	*Methyloversatilis universalis*	29.8	7.01	Folate and derivative biosynthesis	mitochondria	Methylenetetrahydrofolate dehydrogenase, putative (31%) (B9T6E2)
31; 30.5	Chlorophyll a-b binding protein	Q9M600	*Euphorbia esula*	28.5	5.6	Photosynthesis, metal binding	Thylakoid membrane	Nd
29	Chlorophyll a-b binding protein	Q10HD0	*Oryza sativa*	28.5	5.6	Photosynthesis, metal binding	Thylakoid membrane	Nd

[a] Protein theoretical molecular mass and isoelectric point.
[b] Protein with highest similarity using blast search restricted to green plant gene sequences.
[c] Not determined.

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MC-LR. Green algae cultures were able to recover to cellular densities comparable to control in the subsequent four days of culture in part due to the activation of protective mechanisms such as the production of intra and extra cellular polysaccharides and oxidative stress defense enzymes. Bártová et al. (2011) reported that purified MC-LR, the congener MC-RR and *M. aeruginosa* crude extracts containing 300.0 μg/L MCs have no influence on *P. subcapitata* growth. Nevertheless growth was stimulated by *M. aeruginosa* exudates with 300 μg/L MCs. Moreover Babica et al. (2007) reported that MC-LR or MC-RR in the range of 100–5000 μg/L were ineffective to the species *C. reinhardtii, C. kessleri, Pediastrum duplex* and *S. quadricauda*. Only *P. subcapitata* was strongly inhibited by MC-LR or -RR in concentrations equal or superior to 1000 μg/L. Together these studies underline the differential susceptibility of green algae species to MCs and *M. aeruginosa* crude extracts. Moreover provide a conjunct of evidences that leads to the assumption that ecologically relevant toxin concentrations, between 1 and 100 μg/L, may not impair green algae growth. On the other hand Sedmak and Kosi (1998) reported a negative correlation between the total MC content and the number of phytoplankton species in a study undertaken to assess the relationship between the species diversity, the toxic cyanobacterial blooms and MC content in natural water bodies. Moreover 10 μg/L MCs were referred as the boundary level above which species diversity decreased to a minimal level (Sedmak and Kosi, 1998). In this regard one can consider the hypothesis that environmental variables, e.g. light, temperature, pH, nutrient availability and water contaminants, may play a role in the phytoplankton susceptibility to cyanobacterial toxins. These variables are important for investigation and should be considered in the assessment of the toxic potential of cyanobacterial toxins. Variations in temperature, for instance, have shown to modulate the toxicity of silver nanoparticles and copper on aquatic alga (Oukarroum et al., 2012a,b). Moreover pH and dissolved organic carbon (DOC) were considered as important metal toxicity modifying factors (Heijerick et al., 2005).

4.2. Oxidative stress

The enzymes GST and GPx showed a similar pattern of activity for each toxin. Nevertheless contrasting enzyme activities were reported when comparing CYN and MC-LR exposed cultures. The results therefore suggest that green alga cells exposed to CYN induce antioxidant enzymes as a protective mechanism to cope with oxidative stress. Moreover GST may play a dual function, serving as a key detoxification enzyme by catalyzing the binding of glutathione to CYN. GST and GPx have been used to monitor the toxic effects of CYN in a variety of organisms and cell types (Gutiérrez-Praena et al., 2011; Prieto et al., 2011; Puerto et al., 2011). However these biochemical markers were not sensitive to MC-LR indicating that *C. vulgaris* has a distinct biochemical response to the two cyanotoxins. The decrease of GST activity in green alga exposed to MC-LR was unexpected since the involvement of the enzyme in MC detoxification is currently assumed (Campos and Vasconcelos, 2010; Pflugmacher et al., 2007).

4.3. Extracellular proteins

The analysis of the proteins present in *C. vulgaris* culture medium aimed to gather additional biochemical information about the green alga response to cyanotoxins. The extracellular proteins were, recently, object of a proteomics study in *C. reinhardtii* cell wall-less strain, enabling to disclose main metabolic changes associated to green alga adaptation to high CO_2 levels (Baba et al., 2011). We wanted also to test the hypothesis that *C. vulgaris* expresses and delivers to the culture medium enzymes that confer protection to cyanotoxins. Enzymes with putative protective functions, e.g. hydrolases, transferases, oxidoredutases, have been reported in the culture medium of *C. reinhardtii* cell wall-less strain (Baba et al., 2011) and in plant extracellular matrix (Lee et al., 2004; Liu et al., 2007; Tian et al., 2009). Two distinct F-ATPase isoforms were identified by 2DE. The plasma membrane homologous protein (H^+-ATPase) is a proton pump that energizes the uptake of nutrients and regulates intracellular pH. This protein is essential for the active transport across membranes (Palmgren, 2001). Taking in consideration the levels of this protein reported in the culture medium we may expect an alteration of active transport mechanisms and likely the uptake of nutrients in CYN exposed *C. vulgaris* cells. A putative sulfate ABC transporter was identified in *C. vulgaris* exposed to 179.0 μg/L MC-LR culture medium. ABC proteins are importers or exporters of a variety of substrates across cellular membranes including nutrients, rare elements such as molibdenum and toxic compounds and drugs (Hollenstein et al., 2007). In plants several sulfate transport systems have been characterized (Lindberg and Melis, 2008) and in green alga *C. reinhardtii* four putative sulfate transporters of the $H^+/SO_4{}^{2-}$ co-transporter type and three of the $Na^+/SO_4{}^{2-}$ type have been identified with functions assigned only to the chloroplasts (Lindberg and Melis, 2008). The increase in the level of this protein in the culture medium may suggest an alteration of its expression and activity and, in turn, a change in the flux of sulfate in the cells of the green alga exposed to 179.0 μg/L MC-LR. A putative porin was identified in C. vulgaris cultures exposed to the highest CYN and MC-LR concentrations. Porins are voltage-gated diffusion pores being involved in the transport of small hydrophillic molecules across the outer membrane of bacteria, mitochondria and chloroplasts (De Pinto et al., 2010; Bölter and Soll, 2001), with putative functions also in the plasma membrane (De Pinto et al., 2010). The homolog protein identified in C. vulgaris cultures highlights the putative modulation of cell membrane transport systems. Adenylate cyclase is a component of the G protein signaling cascade, located in the plasma membrane and converts ATP in cAMP. In plant cells is thought to have a role in biotic or abiotic stress sensing, defense responses and apoptosis (Gehring, 2010). Activity of adenylyl cyclase has been also reported for example in the green alga Volvox carteri (Nass et al., 1994). The presence of this protein in C. vulgaris cultures exposed to MC-LR suggests that the protein may have a role in the toxin chemical sensing. Aspartate aminotransferase (AST) catalyzes the reversible transfer of an α-amino group between aspartate and glutamate playing a key role in the metabolic regulation of carbon and nitrogen metabolism in all organisms (Wu et al., 2011). Isoforms can be present in the cytoplasm as well as in the mitochondria and chloroplasts (Wilkie et al., 1996; Wu et al., 2011). Moreover extracellular isoforms were identified in *C. reinhardtii* (Baba et al., 2011). Methylenetetrahydrofolate dehydrogenase participates in one carbon metabolism supporting, for instance, cytosolic purine synthesis during embryonic development and in cells undergoing rapid growth (Christensen and Mackenzie, 2008). On the other hand chlorophyll a-b binding proteins are components of the Photosystems I and II and participate in light dependent reactions of photosynthesis (Campos et al., 2010). The levels of AST, Methylenetetrahydrofolate dehydrogenase and LHC proteins in *C. vulgaris* cultures exposed to CYN can suggest alterations in the carbon and nitrogen metabolism and photosynthesis in the green alga driven by the toxin.

Extracellular phosphatases were shown to be modulated by the toxin CYN (Bar-Yosef et al., 2010). This represents a putative mechanism by which CYN producers like *A. ovalisporum* enhance the access to inorganic phosphate (Pi) (Bar-Yosef et al., 2010). Surprisingly we were not able to identify this enzyme in *C. vulgaris* culture media. One possible reason is that the enzyme is preferentially localized in *C. vulgaris* periplasm, as has been suggested for *C. reinhardtii* (Quisel et al., 1996). Moreover it is well known that the expression and activity of this enzyme is regulated by the internal phosphorus pool as well as the external Pi concentrations (Quisel et al., 1996; Gage and

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Gorham, 1985; Chróst and Overbeck, 1987) thereby *C. vulgaris* cell could have maintained a low expression of the enzyme due to the high availability of Pi in the culture medium.

We were not able to assign the hypothesized toxin defense functions for the extracellular proteins. This result however does not exclude our hypothesis and new extracellular protein functions may be explored in the future by refining the proteomic methods and increasing their sensitivity.

5. Conclusions

In this work a conjunct of data is presented that reinforces the hypothesis of tolerance of the green alga *C. vulgaris* to ecologically relevant CYN and MC-LR concentrations (between 5.0 and 179.0 μg/L) and reveals the susceptibility to *A. ovalisporum* crude extracts. The biochemical response of *C. vulgaris* to CYN and MC-LR was differentiated by the activities of cytosolic GPx and GST, with CYN causing a significant increase in the activity of both enzymes while and MC-LR a decrease. The analysis of extracellular proteins was useful to reveal putative biomarkers of green alga response to cyanobacterial toxins. Functions carried out by these proteins regard the transport of solutes across plasma membrane, the metabolism of essential organic molecules, proteins and photosynthesis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2013.04.019.

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