Combined treatment of toxic cyanobacteria *Microcystis aeruginosa* with hydrogen peroxide and microcystin biodegradation agents results in quick toxin elimination

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Under some conditions the growth of toxic cyanobacteria must be controlled by treatment with algicidal compounds. Hydrogen peroxide has been proposed as an efficient and relatively safe chemical which can remove cyanobacteria from the environment selectively, without affecting other microorganisms. However, the uncontrolled release of secondary metabolites, including toxins may occur after such a treatment. Our proposal presented in this paper concerns fast biodegradation of microcystin released after cell lysis induced by hydrogen peroxide. The effectiveness of both, *Sphingomonas* sp. and heterologously expressed MlrA enzyme, in the removal of the toxin from *Microcystis aeruginosa* culture was investigated. The results indicate that neither *Sphingomonas* cells nor MlrA are affected by hydrogen peroxide at the concentrations which stop the growth of cyanobacteria. A several-fold reduction in microcystin levels was documented in the presence of these agents with biodegradation ability. Our results provide evidence that such a combined treatment of water reservoirs dominated by microcystin-producing cyanobacteria may be a promising alternative which allows fast elimination of both, the bloom forming species and toxins, from the environment.

**Key words:** cyanobacteria, microcystins, hydrogen peroxide, microcystinase, *Sphingomonas*

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**Abbreviations:** ACN, acetonitrile; CAT, catalase; H$_2$O$_2$, hydrogen peroxide; MC, microcystins; MC-LR/LR, microcystin LR, MlrA, microcystinase; ROS, reactive oxygen species; TFA, trifluoroacetic acid

**INTRODUCTION**

Different treatment techniques for cyanobacterial bloom control and cyanobacterial cell/metabolite removal from water have been suggested which may also have negative consequences (e.g. release of intracellular metabolites into the water). Several laboratory studies indicated that cyanobacteria are more sensitive to hydrogen peroxide (H$_2$O$_2$) than eukaryotic phytoplankton. This is due to the differences in the metabolism of O$_2$. The eukaryotic phototrophs utilize O$_2$ formed during water photolysis *via* the Mehler reaction and the production of H$_2$O$_2$ as an intermediate. Thus, they developed an efficient system of H$_2$O$_2$ utilization. Cyanobacterial flavodiiron proteins involved in the regulation of photosynthetic electron transport (Allahverdiyeva *et al.*, 2015) may fully reduce O$_2$ to water, without producing internal reactive oxygen species (ROS). However, this mechanism does not require expression of enzymes involved in the degradation of H$_2$O$_2$. As a consequence, the weakness of cyanobacteria is their relatively low resistance to H$_2$O$_2$. Therefore, the H$_2$O$_2$ treatment has been proposed as an effective option of controlling cyanobacterial bloom formation. For example, Matthijs and coworkers (2012) documented in a field experiment that homogenous introduction of 2 mg l$^{-1}$ (60 mM) H$_2$O$_2$ into the whole volume of Lake Koetshuis (the Netherlands) resulted in selective elimination of cyanobacteria and did not impact eukaryotic phytoplankton, zooplankton, or macrofauna. Monitoring of the cyanobacterial population for 7 weeks following the treatment indicated it remained at a low abundance. Recently, the impact of H$_2$O$_2$ and other chemicals (CuSO$_4$, chlorine, KMnO$_4$, ozone) on cyanobacteria and the production/release of toxins has also been studied (Fan *et al.*, 2014).

Contradictory results reported by several authors suggest that the problem of MCs (microcystins) release after treatment with different chemicals is not fully understood. The advantages and disadvantages of treatment with H$_2$O$_2$ were indicated both in laboratory and field studies. In the paper of Matthijs and coworkers (2012), besides indicated rapid collapse of the cyanobacterial population, a 99% reduction in MC concentration within a few days of treatment was documented. Similarly, a laboratory experiment indicated that treatment of *M. aeruginosa* culture with H$_2$O$_2$ (doses ranging from 0 to 102 mg l$^{-1}$) did not cause an associated increase in the amount of dissolved toxins (Fan *et al.*, 2014). The authors suggested that toxin oxidation rates were faster than release rates. The application of such a treatment to remove cyanobacteria and MCs from waste stabilization ponds and hypereutrophic systems resulted in a decreased total MC content (to 8% of the initial concentration) after 5 days (Barrington *et al.*, 2013). However, the concentration of intracellular MCs increased to above the initial level after 25 days of treatment.

An opposite observation was documented by Huo and coworkers (2015), whose study also included the results of cell disintegration kinetics. The production of hydroxyl radicals during *M. aeruginosa* exposure to H$_2$O$_2$ (in the range of 0–60 mg l$^{-1}$) caused very limited MC degradation. It was concluded that H$_2$O$_2$ alone does not degrade MCs effectively. A similar observation was reported by Lürling and coworkers (2014). The efficiency of peroxide and ultrasound in reducing cyanobacterial biomass and potential release of MCs was tested in laboratory assays. Doses of 4 and 8 mg l$^{-1}$ of H$_2$O$_2$ reduced the total MC concentrations by 23%, however, the dissolved MC con-
centrations were 9- and 12-fold higher than in the control, respectively. These reports indicate that the decrease of MC concentration in the cyanobacterial bloom after H$_2$O$_2$ application is not obvious.

There is a growing number of reports documenting the microbial degradation of cyanotoxins (Dziga et al., 2013; Dziga et al., 2016a). Such a biodegradation is thought to be an important process regulating the concentration of these secondary metabolites in the natural environment (Dziga et al., 2017). A well-known pathway utilized by strains capable of MC degradation, such as *Sphingomonas*, is based on a cluster containing the *mlr* genes; the *mlr*A gene encodes microcystinase (*MlrA*), a crucial protein in MC-degradation (Bourne et al., 2001). *Mlr* proteins have been recently heterologously expressed which has allowed to conduct experiments with recombinant enzymes (Dziga et al., 2012a; Dziga et al., 2012b). Despite the lack of an efficient *MlrA* purification method, the production of cell lysates with a high *MlrA* activity may offer a tool for practical application of this protein.

It has been suggested that under natural conditions the amount of MCs released after cell lysis induced by H$_2$O$_2$ may be reduced by environmental factors, such as microbiological activity, UV radiation, photosensitized transformation in the presence of humic substances and pigments, or adsorption to particles (Barrington et al., 2013). However, MCs are relatively resistant to sun irradiation which results from the fact that they absorb at wavelengths of 238–240 nm (Lawton et al., 1999). Furthermore, the actual impact of biodegradation on the MC concentration in natural environments is difficult to estimate. The calculated efficiency of this process varies greatly between the isolated strains and seems to be relatively slow (Dziga et al., 2013).

In this paper, we are proposing a novel approach which combines the biodegradation of toxic MCs released from cyanobacterial cells after H$_2$O$_2$ exposure. Our hypothesis is that such a cooperative activity of MC-degrading bacteria or *MlrA* should result in a rapid elimination of toxins from the water column. The conducted experiments assessed: (i) the impact of H$_2$O$_2$ on the viability of *Sphingomonas* cells and both *MlrA* and *Sphingomonas* sp. cell activity against MC-LR after exposure to different doses of H$_2$O$_2$; (ii) the impact of H$_2$O$_2$ on the release of MC variants from *M. aeruginosa* cells; (iii) *MlrA* and *Sphingomonas* sp. capability for fast biodegradation of MCs released from *M. aeruginosa* cells after H$_2$O$_2$ treatment.

**MATERIALS AND METHODS**

**Chemicals and strains.** Trifluoroacetic acid (TFA) was purchased from Sigma (St Louis, MO, USA), hydrogen peroxide was obtained from Krakchemia (Krakow, Poland), C18 Purospher column and acetonitrile (ACN) were obtained from Merck (Darmstadt, Germany). MC-LR, -LF and -LW used as standards were extracted and purified from a culture of *Microcystis aeruginosa* PCC 7813 strain (the Pasteur Institute, Paris) while MC-RR was isolated from the *Microcystis* NIES 107 strain (Gajdek et al., 2001).

*Escherichia coli* BL21(DE3) (Novagen, an Affiliate of Merck KGaA, Darmstadt, Germany) with pET21a-mlrA used for the expression of recombinant protein was grown at 37°C in LB broth supplemented with ampicillin (100 µg ml$^{-1}$). *MlrA* expressed in *E. coli* BL21pET21a was produced as described by Dziga and coworkers (Dziga et al., 2012b). The average recombinant *MlrA* activity was 1550 U ml$^{-1}$ of the culture OD$_{600nm}$=2 *Sphingomonas* sp. ACM 3962, obtained from the Australian Collection of Microorganisms, was cultured in a recommended peptone yeast extract medium (299) at 28°C overnight. After one day, the cells were centrifuged and washed with 50 mM PBS buffer, pH 7. The strain of *M. aeruginosa* PCC 7813 was cultivated in a Z-medium at 20°C in 40 µmol m$^{-2}$ s$^{-1}$ of photosynthetically active radiation (provided in a light/dark cycle; 12/12 h).

**Preliminary experiments with hydrogen peroxide.** The viability of *Sphingomonas* sp. cells after the exposure to hydrogen peroxide. The resistance of *Sphingomonas* sp. to H$_2$O$_2$ (3% stock solution, stabilized) was monitored based on the bacterial cell traditional plate counting. Two ml of 20 h-old culture (OD$_{600nm}$=0.8) were washed twice with the PBS buffer and incubated in three replicates with 0 (control), 5, 50, 150, 500 mg l$^{-1}$ of H$_2$O$_2$ for 5 h. After incubation, the cells were serially diluted and plated on agar plates ($10^{5}–10^{7}$ dilutions).

**MlrA activity in the presence of hydrogen peroxide.** The *MlrA* extract was incubated with 0 (control) 5, 50, 150, 500 mg l$^{-1}$ of H$_2$O$_2$ for 1 h (three replicates). After the incubation period, a typical activity assay was performed using varying enzyme dilutions (as described below).

**Activity of *Sphingomonas* sp. against MC-LR.** Two ml of 20 h-old culture (OD$_{600nm}$=0.8) were washed twice with the PBS buffer and incubated with 0 (control), 5, 50, 150, 500 mg l$^{-1}$ of H$_2$O$_2$ for 5 h (3 replicates for each concentration). After the incubation period, the cells were washed with the PBS buffer and concentrated in 200 µl of the PBS buffer; 20 µl of such a suspension were incubated with 180 µl of 1 µg ml$^{-1}$ MC-LR. After 1 and 2 h, 100 µl of the suspension were centrifuged to remove cells and the supernatant was analysed by HPLC.

**Simultaneous treatment of *M. aeruginosa* culture with hydrogen peroxide and *Sphingomonas* sp. cells.** The fate of different MC variants after treatment with H$_2$O$_2$ or/and *Sphingomonas* sp. Ten-day-old culture of *M. aeruginosa*, OD$_{600nm}$=0.85 (approx. 10$^{9}$ of cells ml$^{-1}$) was cultivated under standard conditions (see section 2.1), under visible light intensity of 40 µmol of photons m$^{-2}$ s$^{-1}$ for 1 day. Culture A (control): *M. aeruginosa* PCC 7813 strain cells cultivated alone; culture B: the cells exposed to 10 mg l$^{-1}$ of H$_2$O$_2$; culture C: *M. aeruginosa* cells treated with *Sphingomonas* sp. (final cell concentration 5 × 10$^{6}$ cells ml$^{-1}$) culture D: *M. aeruginosa* cells treated with H$_2$O$_2$ (10 mg l$^{-1}$) and *Sphingomonas* sp. (5 × 10$^{6}$ cells ml$^{-1}$). All of the groups were analysed in 3 independent replicates. The MC variants were analysed after 1 and 24 h of treatment.

**The activity of *Sphingomonas* sp. at different stages of cyanobacterial growth.** The experiment started (day 0) when a fresh *M. aeruginosa* culture reached OD$_{750}$ = 0.2 (approx. 2.5 × 10$^{6}$ of cells ml$^{-1}$). On the 1, 2, 3, 4, 5 and 11 day, four samples were taken and exposed to different conditions: culture A (control): without treatment; culture B: *M. aeruginosa* with 10 mg l$^{-1}$ H$_2$O$_2$; culture C: *M. aeruginosa* plus *Sphingomonas* sp.; culture D: *M. aeruginosa* with 10 mg l$^{-1}$ H$_2$O$_2$ plus *Sphingomonas* sp. (5 × 10$^{6}$ cells ml$^{-1}$). The samples (made in triplicate) were cultivated under standard conditions (see Materials and Methods) and after 2, 5 and 24 h, 100 µl of the culture were centrifuged and collected for further HPLC analysis.

**Microcystin degradation by *MlrA* following *M. aeruginosa* exposure to hydrogen peroxide.** The extracts of a heterologous *Escherichia coli* strain were obtained by sonication with an ultrasonic processor UP100H (Hielserch Ultrasonics). The centrifuged lysates were used as a source of the *MlrA* enzyme. The *MlrA* activity assays were performed as follow: 5 µl of the en-
zyme in different dilutions was added to 45 µl of the MC solution. The enzyme and MCs were suspended in the PBS buffer, pH 7.0. The final MC concentration was 1 µg ml⁻¹. The incubation temperature was 20°C and the reaction was stopped after 1 h by the addition of 5 µl of 1% TFA. Samples were cooled to 5°C and analysed by HPLC.

In the experiment on biodegradation of MC-LR released by *M. aeruginosa*, five experimental groups were analysed in three replicates: control (untreated *M. aeruginosa*); H₂O₂ treated *M. aeruginosa* and three cultures of *M. aeruginosa* treated with H₂O₂ and MlrA. The tested dilutions of the enzyme were MlrA1, MlrA2 and MlrA3 which corresponds to 80, 0.8 and 0.34 mU ml⁻¹ of *M. aeruginosa* culture, respectively. The H₂O₂ concentration was 10 mg l⁻¹. A portion of the enzyme was added to the *M. aeruginosa* culture, OD₅₅₀=0.7, directly after the addition of H₂O₂. The samples were analysed by HPLC after 0.5, 1.0, 2.0, 3.0, and 24 h of treatment with H₂O₂ or/and MlrA.

**HPLC analyses.** HPLC analyses were performed as described by Meriluoto and Spoof (2005) using an Agilent 1220 Infinity Gradient DAD LC System with a gradient pump and an integrated degassing unit, an autosampler, a column oven and a diode array detector. MC-LR and its degradation product (acyclic MC-LR) were separated and quantified using a Purospher STAR RP-18 endcapped column (55 mm × 4 mm, 3 µm particles). The mobile phase consisted of a gradient of 0.05% aqueous TFA (solvent A) and 0.05% TFA in acetonitrile (solvent B). The assays were performed with the following linear gradient programme: 0 min 25% B, 5 min 70% B, 6 min 70% B, and 6.1 min 25% B. The retention times of MC-LR (substrate) and acMC-LR (product of MlrA and Sphingomonas activity) were 3.7 and 3.2 min, respectively. The retention times of other MC variants were: 5.2 min (-LW), 5.4 min (-LF) and 4.1 min (-LY). The variants were verified by LC-MS.

**Statistical analysis.** Statistically significant differences were determined by the Anova test. Anova and Tukey test were used to analyse the diversity between experimental groups.

**RESULTS**

**The impact of hydrogen peroxide on the viability of Sphingomonas sp. cells and the activity of Sphingomonas sp. and MlrA against MC-LR**

The viability of Sphingomonas sp. was determined after its exposure to different H₂O₂ concentrations. Twenty four hours of incubation with 5 and 50 mg l⁻¹ of H₂O₂ did not change the cell viability compared to the control. The only statistically significant decrease in viable cells was observed at the 150 and 500 mg l⁻¹ concentrations (Table 1).

The impact of hydrogen peroxide on the activity of Sphingomonas sp. and MlrA alone towards MC-LR was determined by measuring the acyclic MC-LR concentration, the product of MlrA activity. In the whole tested range of H₂O₂ concentrations, both Sphingomonas sp. and the MlrA expressed the same activity as in the control. It should be pointed out that even after exposure to 500 mg l⁻¹ H₂O₂, the activity was not affected (Table 1).

**The impact of hydrogen peroxide on the release of MC variants by *M. aeruginosa* PCC 7813 strain and the role of Sphingomonas sp. in the regulation of MC concentration**

The *M. aeruginosa* strain cells cultivated alone (culture A) released two MC variants, -LR and -LY. The exposure of the culture (B) to H₂O₂ (1 and 24 h of treatment) caused an increased release of these variants. Furthermore, two other MC variants (-LW and -LF) were observed after 1 and/or 24 h of H₂O₂ treatment (Table 2). The application of Sphingomonas sp. decreased the concentration of -LR and -LY variants in the *M. aeruginosa* cultures that were both, untreated (C) and treated (D) with H₂O₂. The range of their reduction after 1 h varied from 14.8 to 39.8%, whereas after 24 h from 36.5–45.6%. The presence of the -LW and -LF variants was also recorded in the *M. aeruginosa* cultures C and D, treated with Sphingomonas sp. or/and H₂O₂. The decrease in concentration of these variants was either not observed or was about 2 times slower (-LW vs -LR and -LY).

**The efficiency of MC degradation by Sphingomonas sp. cells following the treatment of *M. aeruginosa* PCC 7813 culture with H₂O₂ at different phases of growth**

The results of this experiment are presented in Table 3 which indicates the summarized concentration of all variants detected in the *M. aeruginosa* cultures (the most abundant MC-LR variant varied from 50–90% of the total MCs). It should be emphasized that an increased release of MCs from cyanobacterial cells was observed only 24 h after the application of H₂O₂ (group a vs group b). A significant decrease of MC-LR level related to the biodegradation process carried out by bacterial cells (group c and d) occurred already after 2 h of microbial activity. However, continued incubation with

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**Table 1. The influence of different doses of H₂O₂ on the viability Sphingomonas sp. cells, its activity against MC-LR and MlrA activity:** ± indicates standard deviation.

<table>
<thead>
<tr>
<th>H₂O₂ concentration (mg l⁻¹)</th>
<th>Viability of Sphingomonas sp. (number of CFU x 10⁶ ml⁻¹)</th>
<th>Sphingomonas activity against MC-LR (mU ml⁻¹)</th>
<th>MlrA activity (U ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>3.9 ± 2.2</td>
<td>0.34 ± 0.09</td>
<td>1.01 ± 0.34</td>
</tr>
<tr>
<td>5</td>
<td>4.1 ± 0.8</td>
<td>0.50 ± 0.06</td>
<td>1.08 ± 0.39</td>
</tr>
<tr>
<td>50</td>
<td>3.7 ± 1.1</td>
<td>0.55 ± 0.12</td>
<td>1.06 ± 0.35</td>
</tr>
<tr>
<td>150</td>
<td>1.3 ± 0.5*</td>
<td>0.59 ± 0.12</td>
<td>0.45 ± 0.24</td>
</tr>
<tr>
<td>500</td>
<td>0.6 ± 0.6*</td>
<td>0.56 ± 0.13</td>
<td>1.25 ± 0.13</td>
</tr>
</tbody>
</table>

*Indicates statistically significant difference of CFU number between control, 5, and 50 mg l⁻¹; p<0.025
**Table 2.** The impact of H₂O₂ on the release of MC variants and the effect of treatment with *Sphingomonas* sp.

<table>
<thead>
<tr>
<th>Type of culture</th>
<th>Time (h)</th>
<th>Concentration of dissolved MC variants (µg ml⁻¹) and % of MC increase (+) / degradation (−)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-LR</td>
</tr>
<tr>
<td><em>M. aeruginosa</em>, control (A)</td>
<td>1</td>
<td>0.365</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.413</td>
</tr>
<tr>
<td><em>M. aeruginosa</em> + H₂O₂ (B)</td>
<td>1</td>
<td>0.519</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.763</td>
</tr>
<tr>
<td><em>M. aeruginosa</em> + <em>Sphingomonas</em> sp (C)</td>
<td>1</td>
<td>0.301</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.239</td>
</tr>
<tr>
<td><em>M. aeruginosa</em> + H₂O₂ + <em>Sphingomonas</em> sp (D)</td>
<td>1</td>
<td>0.312</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.413</td>
</tr>
</tbody>
</table>

*Sphingomonas* sp. (up to 24 h) enabled further reduction in toxin concentration. To indicate more clearly significant differences between the experimental groups, the results of the 24 h treatment are shown in Fig. 1. In the control culture, the concentration of MCs released from the cells varied from 0.09 to 0.24 µg ml⁻¹ during the experiment and was similar for OD₇₅₀ = 0.6–1.0. In the samples which were treated with H₂O₂ at different phases of growth, the MC concentration was significantly higher than in the control culture.

**Table 3.** The extracellular MC concentration in the samples taken from the culture of *M. aeruginosa* PCC7813 cultures at various stages of growth and various types of treatment.

<table>
<thead>
<tr>
<th>Type of experimental group</th>
<th>Day of sampling</th>
<th>Time (h)</th>
<th><em>M. aeruginosa</em>, control (A)</th>
<th><em>M. aeruginosa</em> + H₂O₂ (B)</th>
<th><em>M. aeruginosa</em> + <em>Sphingomonas</em> sp (C)</th>
<th><em>M. aeruginosa</em> + H₂O₂ + <em>Sphingomonas</em> sp (D)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MC concentration (µg ml⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>0.071±0.01</td>
<td>0.078±0.02</td>
<td>0.068±0.07</td>
<td>0.030±0.00***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.092±0.02</td>
<td>0.084±0.02</td>
<td>0.019±0.01</td>
<td>0.019±0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>0.099±0.05</td>
<td>0.360±0.02**</td>
<td>0.005±0.01</td>
<td>0.003±0.00</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>0.117±0.08</td>
<td>0.079±0.02</td>
<td>0.028±0.00**</td>
<td>0.042±0.02***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.115±0.05</td>
<td>0.132±0.01</td>
<td>0.003±0.00</td>
<td>0.010±0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>0.140±0.05</td>
<td>0.587±0.06*</td>
<td>0.013±0.02</td>
<td>0.008±0.01</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
<td>0.239±0.06</td>
<td>0.332±0.03</td>
<td>0.044±0.02</td>
<td>0.073±0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.210±0.07</td>
<td>0.701±0.13*</td>
<td>0.017±0.01</td>
<td>0.012±0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>0.347±0.07</td>
<td>0.344±0.09</td>
<td>0.128±0.03**</td>
<td>0.097±0.02***</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2</td>
<td>0.290±0.07</td>
<td>0.375±0.03</td>
<td>0.107±0.01</td>
<td>0.078±0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.290±0.07</td>
<td>0.375±0.03</td>
<td>0.107±0.01</td>
<td>0.078±0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>0.360±0.06</td>
<td>0.701±0.13*</td>
<td>0.017±0.01</td>
<td>0.012±0.02</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td>0.333±0.02</td>
<td>0.356±0.03</td>
<td>0.089±0.05**</td>
<td>0.109±0.01***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.330±0.05</td>
<td>0.333±0.05</td>
<td>0.078±0.02</td>
<td>0.088±0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>0.301±0.02</td>
<td>0.697±0.10**</td>
<td>0.044±0.02</td>
<td>0.022±0.02</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>2</td>
<td>0.382±0.04</td>
<td>0.311±0.05</td>
<td>0.086±0.01**</td>
<td>0.092±0.02***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.467±0.09</td>
<td>0.348±0.01</td>
<td>0.088±0.01</td>
<td>0.089±0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>0.315±0.06</td>
<td>0.383±0.12</td>
<td>0.052±0.02</td>
<td>0.051±0.03</td>
</tr>
</tbody>
</table>

Asterisks indicate statistically significant differences of the MCs concentration: *after 24 h between the relevant groups (a) and (b), p<0.01; **after 2 h between the relevant groups (a) and (c), p<0.001; ***after 2 h between the relevant groups (b) and (d), p<0.001.
the control culture. This is more evident during the first phase of growth (1–3 days, 3.2 times higher average MC concentration in comparison to the control) than in days 4–11 (1.7 times higher MC concentration, on average). A 24 h incubation of H$_2$O$_2$ treated and untreated *M. aeruginosa* culture with *Sphingomonas* sp. resulted in a drastic decrease in the MC-LR level. Independently of growth phase, the bacterial cells were efficient and enabled an almost complete elimination of the toxin from the environment. The average reduction in MC concentration during the whole experiment was 86% (i.e. 7-fold) in group C in comparison with group A, and 94% (i.e. 25-fold) in group D vs the group B.

The potential of recombinant MlrA in MC degradation following the treatment of *M. aeruginosa* PCC 7813 culture with H$_2$O$_2$

In this experiment the *M. aeruginosa* culture treated with H$_2$O$_2$ was additionally supplemented with recombina

Figure 2. The MC-LR concentration in *M. aeruginosa* culture after subsequent hours of treatment with H$_2$O$_2$ or/and MlrA. White bars indicate control culture, light-grey bars – H$_2$O$_2$ treated *M. aeruginosa*, grey bars – culture of *M. aeruginosa* treated with H$_2$O$_2$ and 8 mU ml$^{-1}$ MlrA, dark-grey bars – *M. aeruginosa* treated with H$_2$O$_2$ and 0.8 mU ml$^{-1}$ MlrA. Line bars indicate S.D. values.

DISCUSSION

To prevent cyanobacterial blooms, a reduction of the nutrient load in surface waters should be the primary goal, but this is not always feasible. A pre-treatment of water reservoirs with chemicals possessing algicidal activity may be necessary in some circumstances. It has been suggested that to prevent an overgrowth of toxic strains and to reduce the release of metabolites, the algicides should be used when cyanobacteria are in their early growth phase. However, it requires continuous monitoring of the susceptible water reservoirs. Hydrogen peroxide is one of the most commonly proposed chemicals efficient in the elimination of toxic cyanobacteria. According to Matthijs and coworkers (2012), the main advantage of H$_2$O$_2$ is that it does not significantly impact the eukaryotic phytoplankton, zooplankton and macrofauna at concentrations which affect cyanobacteria. Additionally, H$_2$O$_2$ is degraded spontaneously within hours or a few days and thus does not contaminate the environment. Diluted H$_2$O$_2$ was proposed to be used for “the selective elimination of harmful cyanobacteria from recreational lakes and drinking water reservoirs, especially when immediate action is urgent and/or cyanobacterial control by reduction of eutrophication is currently not feasible” (Matthijs et al., 2016).

Our investigation of MC-degrading strain’s sensitivity to H$_2$O$_2$ provided an expected observation that *Sphingomonas* sp. was not affected by 50 mg l$^{-1}$ of H$_2$O$_2$ (Table 1) which is about 10 times higher than the recommended concentration to eliminate cyanobacteria. What is particularly important, the activity of bacterial cells and MlrA against MC-LR was not affected even after exposure to 500 mg l$^{-1}$ of H$_2$O$_2$. This means that in the presence of hydrogen peroxide, MC biodegradation based on MlrA activity may occur and is not affected by the reagent which causes lysis of the cyanobacterial cells.

Our results stand in opposition to the findings presented in a recent paper of Kansole and Lin (2017). In laboratory batch experiments, the impact of both, H$_2$O$_2$ and copper sulfate on MC degrading bacteria Bacillus sp., was evaluated. Both chemicals (at the concentration of 5 and 1 mg l$^{-1}$, respectively) were lethal to *Bacillus* sp. population. It suggests that
different bacterial strains (including those with MC-degradation capability) may have a different level of tolerance to the oxidative stress caused by H$_2$O$_2$. Unfortunately, the impact of hydrogen peroxide on the capability of MC degradation of the investigated strain was not analysed.

As was mentioned in the introduction, the impact of H$_2$O$_2$ on toxin release by the treated cyanobacterial cells may be different and several contradictory results have been reported. Generally, it is hard to predict how the toxic cyanobacterial strains respond to oxidative stress caused by H$_2$O$_2$. Some papers present unexpected effects related to the production of MCs under the stress conditions. Zilliges and coworkers (2011) compared a Microcystis strain capable of MC production with its mutant unable to produce this toxin. The results clearly indicated that the mutant defective in MC production had increased sensitivity under high light conditions after H$_2$O$_2$ treatment. The authors suggested a new role of MCs in the modulation of protein metabolism and in protection against oxidative stress. Furthermore, M. aeruginosa may rapidly initiate antioxidant defence and change the MC content (Giannuzzi et al., 2016). This could lead to dominance in the blooms of the M. aeruginosa population which contains cells with higher MC production. Interestingly, the response of toxic strains may also involve other physiological changes. The authors documented a high potential of M. aeruginosa to respond to ROS. The M. aeruginosa strain isolated from a temperate environment was able to activate an enzymatic antioxidant catalase (CAT) after an exposure to an increased level of antioxidant species caused by higher temperature (Giannuzzi et al., 2016). Finally, the authors suggested that the formation of OH$^-$ may be significantly inhibited by CAT. This finding indicates that M. aeruginosa may have a greater competitive advantage over other species at higher mean water temperatures.

Thus, the rapid reduction of MC concentration in blooming water bodies after H$_2$O$_2$ application may be questioned and several physiological alterations may lead to an increased MC production. These examples indicated the need to combine the H$_2$O$_2$ treatment with other agents which allow to reduce the MC concentration even if cyanobacterial response to oxidative stress causes an enhanced production of toxins.

The treatment of M. aeruginosa culture with H$_2$O$_2$ resulted in a 2-fold increase in the extracellular MC concentration after 24 h (Table 2). Additionally to the most abundant -LR, as well as -LY variants, observed in all experimental cultures, hydrogen peroxide caused the release of other MCs: -LW and -LF. This complies with the data shown by Lürling and coworkers (2014) which confirmed increased proportion of these more hydrophobic MCs (-LR, -RR, -YR, -LY, -LF and -LW, Dziga et al., 2012b). However, the specificity of the enzyme towards these substrates is different (lower degradation rate of -LW and -LF, not published). Different experimental conditions may cause slight conformational changes of the enzyme, resulting in lower activity against the more hydrophobic variants. Further research should document, whether in the natural environment the strains possessing MlrA are able to hydrolyse all of the major MC variants with similar efficiency.

The indication of a significant increase in extracellular MC concentration (including all detected variants) after 24 h of treatment with hydrogen peroxide (Table 3) suggests that the toxins are released from cyanobacterial cells gradually. It indicates that within at least one day H$_2$O$_2$ impacts cyanobacteria. Fan and coworkers (2013) documented that residual H$_2$O$_2$ was present in cyanobacteria cultures even 5 days after treatment, which suggests slow action of H$_2$O$_2$ on cyanobacteria under laboratory condition (with no UV radiation). Faster and more effective H$_2$O$_2$ action is likely to take place in reservoirs due to natural UV radiation from sunlight. The concentration of H$_2$O$_2$ required to eliminate cyanobacteria could be reduced even by an order of magnitude when UV radiation is applied (Barrington et al., 2013). On the other hand, UV radiation is attenuated with depth and its penetration through a blooming surface would be very limited. Nevertheless, a prolonged presence of H$_2$O$_2$ requires both, longer monitoring and the employment of agents which may remove the toxins efficiently within at least one day. Both Sphingomonas sp. and MlrA alone meet this condition. They act the most efficiently within few hours of addition, but further MC-LR reduction may be observed within 24 h. Moreover, independently of the stage of cyanobacterial growth, biodegradation related to bacterial cell or enzyme activity is efficient (Table 3, Fig. 1).

Iwinski and coworkers (2017) investigated the impact of copper on the rate of bacteria mediated degradation of MC-LR, including relative abundance and diversity of bacteria identified in the samples of M. aeruginosa culture isolated from a natural pond. A decrease in bacterial diversity was observed following copper-exposures greater than 0.1 mg l$^{-1}$. However, some groups of MC-degrading bacteria were less sensitive to copper exposure and their relative abundance increased. It was concluded that the copper formulation at the concentration registered for use did not significantly alter degradation rates or bacterial composition. Future experiments should also confirm the impact of H$_2$O$_2$ on the composition and growth rate of bacterial population, with particular emphasis on the strains with MC-degradation abilities.

The proposed combined treatment offers an alternative approach and a possible application of such a strategy may include for instance MC decontamination of fish ponds. The domination of MC-producers in fish ponds is common in several European countries and is well recognized in Serbia (Drobac et al., 2016). It may cause histopathological damage of fish tissues and create a health problem for humans. Rapid and efficient MC degradation may be helpful in solving this problem. Below is an estimation which clarifies the range of volumes of cell culture or MlrA lysate required to quickly decontaminate small water rese-
voirs from MCs. MlrA produced heterologously seems to be much more efficient in MC decontamination than the cells of natural strains with a relatively low rate of degradation (Dziga et al., 2012b). The amount of enzyme necessary for efficient MC decontamination depends on the initial concentration of these toxins. For example, if we assume that under natural conditions MlrA activity is similar to that indicated under laboratory conditions, efficient purification of a 5000 m³ fish pond contaminated with 5 µg l⁻¹ of MCs would require approximately 2.7 l of the enzyme produced as described in Materials and Methods, which means that about 90 l of E. coli culture must be prepared. In a recent proposal – an open column bioreactor filled with BL21(DE3)-mlrA cells immobilized in alginate beads – the documented degradation rate calculated for 1 l of the carrier was 30 µg of MCs per 1 h and was much higher than the degradation rates documented in other reports (Dziga et al., 2014) where microbes are employed to remove unwanted chemicals. However, it means that using an alginate bead carrier produced from 900 l of E. coli-mlrA culture, about 110 mg of MCs could be degraded within 24 h, i.e. 22 m³ of water contaminated with 5 µg l⁻¹ of MCs. If we compare these results, it is obvious that direct application of MlrA to a water column is much more efficient than water treatment using a column with alginate entrapped E. coli-mlrA.

CONCLUSION

Our results provide a proposal of combined treatment of water reservoirs contaminated with cyanobacteria capable of MC production. In our opinion, the treatment of cyanobacteria with hydrogen peroxide or other chemicals should be supplemented with agents which allow removal of MCs released from the lysed cells. Independently of the chemicals used to suppress and/or to kill cyanobacteria, monitoring of cyanotoxin concentration after such a treatment should be followed by a rapid degradation of toxins released from the cells disrupted by chemical activity of algicides. Such an approach may be desired especially when a fast and direct action is necessary.

Conflicts of interest

The authors declare no conflict of interest.

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