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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_2O_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_2O_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_2O_2 as an intermediate. Thus, they developed an efficient system of H_2O_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_2O_2. As a consequence, the weakness of cyanobacteria is their relatively low resistance to H_2O_2. Therefore, the H_2O_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_2O_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_2O_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_2O_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_2O_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_2O_2 (in the range of 0−60 mg l^{-1}) caused very limited MC degradation. It was concluded that H_2O_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_2O_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_2O_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 *mlrA* 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; Dziga *et al*., 2016b). 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_2O_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_2O_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_2O_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_2O_2, (ii) the impact of H_2O_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_2O_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_{600}=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_2O_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_2O_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_2O_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_2O_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_2O_2 or/and Sphingomonas sp. Ten-day-old culture of *M. aeruginosa*, OD_{750}=0.85 (approx. 10^7 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_2O_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_2O_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_2O_2; culture C: *M. aeruginosa* plus *Sphingomonas* sp.; culture D: *M. aeruginosa* with 10 mg l^{-1} H_2O_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 (Hielscher 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*); H_2O_2 treated *M. aeruginosa* and three cultures of *M. aeruginosa* treated with H_2O_2 and MlrA. The tested dilutions of the enzyme were MlrA1, MlrA2 and MlrA3 which corresponds to 80, 8 and 0.8 mU ml^{-1} of *M. aeruginosa* culture, respectively. The H_2O_2 concentration was 10 mg l^{-1}. A portion of the enzyme was added to the *M. aeruginosa* culture, OD_{750}=0.7, directly after the addition of H_2O_2. The samples were analysed by HPLC after 0.5, 1.0, 2.0, 3.0, and 24 h of treatment with H_2O_2 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 H_2O_2 concentrations. Twenty four hours of incubation with 5 and 50 mg l^{-1} of H_2O_2 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 H_2O_2 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} H_2O_2, 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 H_2O_2 (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 H_2O_2 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 H_2O_2. 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 H_2O_2. 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 H_2O_2 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 H_2O_2 (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

Table 1. The influence of different doses of H_2O_2 on the viability *Sphingomonas* sp. cells, its activity against MC-LR and MlrA activity; ± indicates standard deviation.

	Parameters after exposure to H_2O_2		
H_2O_2 concentration (mg l^{-1})	Viability of *Sphingomonas* (number of CFU x 10^8 ml^{-1})	*Sphingomonas* activity against MC-LR (mU ml^{-1})	MlrA activity (U ml^{-1})
control	3.9 ± 2.2	0.34 ± 0.09	1.01 ± 0.34
5	4.1 ± 0.8	0.50 ± 0.06	1.08 ± 0.39
50	3.7 ± 1.1	0.55 ± 0.12	1.06 ± 0.35
150	1.3 ± 0.5*	0.59 ± 0.12	0.45 ± 0.24
500	0.6 ± 0.6*	0.56 ± 0.13	1.25 ± 0.13

*Indicates statistically significant difference of CFU number between control, 5, and 50 mg l^{-1}; $p<0.025$

Table 2. The impact of H_2O_2 on the release of MC variants and the effect of treatment with *Sphingomonas* sp.
Time (h) indicates the length of incubation with H_2O_2 or/and the bacterial cells. The % of MC increase was calculated in relation to group A, whereas % of MC degradation in groups C and D was calculated in relation to groups A and B, respectively.

Type of culture	Time (h)	Concentration of dissolved MC variants (μg ml^{-1}) and % of MC increase (+) / degradation (–)							
		-LR		-LW		-LF		-LY	
		μg ml^{-1}	%	μg ml^{-1}	%	μg ml^{-1}	%	μg mL^{-1}	%
M. aeruginosa, control (A)	1	0.365		0.000		0.000		0.088	
	24	0.413		0.000		0.000		0.106	
M. aeruginosa + H_2O_2 (B)	1	0.519	+42.1	0.087		0.000		0.129	+46.2
	24	0.763	+68.0	0.202		0.125		0.107	+1.0
M. aeruginosa + *Sphingomonas* sp. (C)	1	0.301	–17.5	0.115		0.058		0.075	–14.8
	24	0.239	–42.1	0.092		0.000		0.060	–43.2
M. aeruginosa + H_2O_2 *Sphingomonas* sp. (D)	1	0.312	–39.8	0.089	0.0	0.067		0.083	–35.4
	24	0.413	–45.6	0.161	–20.1	0.123	0.0	0.066	–36.5

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 OD_{750}=0.6–1.0. In the samples which were treated with H_2O_2 at different phases of growth, the MC concentration was significantly higher than in

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.
Group (A) – untreated control samples; group (B) – samples treated with 10 mg l^{-1} H_2O_2, (C) – samples treated with *Sphingomonas* sp. cells, (D) – samples treated with 10 mg l^{-1} H_2O_2 and *Sphingomonas* sp. cells. The time of treatment was 2, 5 and 24 h. All analyses were made in triplicate.

		Type of experimental group			
Day of sampling	Time (h)	*M. aeruginosa*, control (A)	*M. aeruginosa* + H_2O_2 (B)	*M. aeruginosa* + *Sphingomonas* (C)	*M. aeruginosa* + H_2O_2 + *Sphingomonas* (D)
		MC concentration (μg ml^{-1})			
1	2	0.071±0.01	0.078±0.02	0.068±0.07	0.030±0.00***
	5	0.092±0.02	0.084±0.02	0.019±0.01	0.008±0.01
	24	0,099±0.05	0.360±0.02*	0.005±0.01	0.003±0.00
2	2	0.117±0.08	0.079±0.02	0.028±0.00**	0.042±0.02***
	5	0.115±0.05	0.132±0.01	0.003±0.00	0.010±0.01
	24	0.140±0.05	0.587±0.06*	0.013±0.02	0.008±0.01
3	2	0.190±0.03	0.241±0.09	0.075±0.01**	0.074±0.02***
	5	0.239±0.06	0.332±0.03	0.044±0.02	0.073±0.02
	24	0.210±0.07	0.701±0.13*	0.017±0.01	0.012±0.02
4	2	0.347±0.07	0.344±0.09	0.128±0.03**	0.097±0.02***
	5	0.290±0.07	0.375±0.03	0.107±0.01	0.078±0.03
	24	0.360±0.06	0.613±0.22*	0.071±0.05	0.044±0.02
5	2	0.333±0.02	0.356±0.03	0.089±0.05**	0.109±0.01***
	5	0.330±0.05	0.333±0.05	0.078±0.02	0.088±0.01
	24	0.301±0.02	0.697±0.10*	0.044±0.02	0.022±0.02
11	2	0.382±0.04	0.311±0.05	0.086±0.01**	0.092±0.02***
	5	0.467±0.09	0.348±0.01	0.088±0.01	0.089±0.03
	24	0.315±0.06	0.383±0.12	0.052±0.02	0.051±0.03

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.

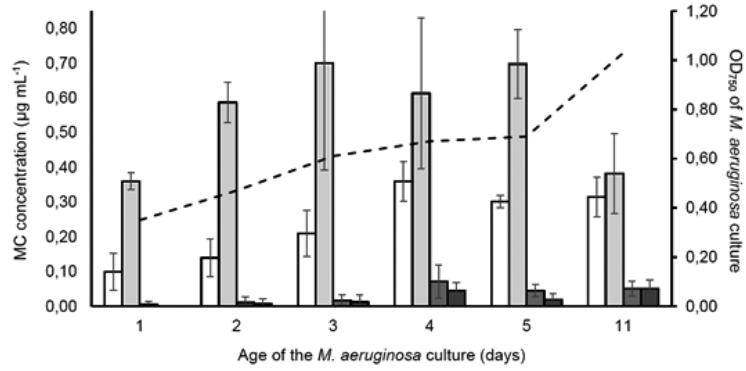

Figure 1. MC concentration at different stages of growth of the *M. aeruginosa* culture after treatment with H_2O_2 and *Sphingomonas* sp.
White bars indicate control culture, light-grey bars – H_2O_2 treated *M. aeruginosa,* grey bars – culture of *M. aeruginosa* treated with *Sphingomonas* sp., dark-grey bars – culture of *M. aeruginosa* treated with H_2O_2 and *Sphingomonas* sp. Dashed line indicates the OD_{750} of the *M. aeruginosa* culture. Line bars indicate S.D. values.

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_2O_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_2O_2

In this experiment the *M. aeruginosa* culture treated with H_2O_2 was additionally supplemented with recombinant MlrA produced in an *E. coli* strain (see Materials and Methods). After preliminary experiments (not shown) two dilutions of MlrA2 and MlrA3 enzymes (8 and 0.8 mU of MlrA per ml of *M. aeruginosa* culture) were tested (Fig. 2). The concentration of MC-LR in the culture of *M. aeruginosa* treated with H_2O_2 decreased 6- and 20-fold after 3 h and 24 h of incubation with MlrA2, respectively. An enzyme dilution that was ten times higher (MlrA3) was less efficient in fast reduction of MC-LR concentration, however, after 24 h of incubation the amount of toxin was reduced 4-fold.

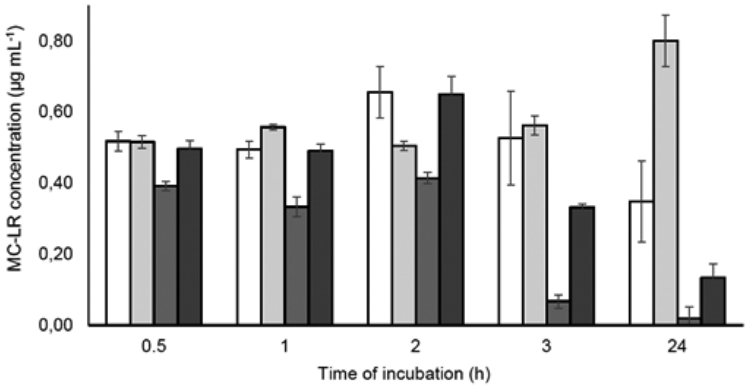

Figure 2. The MC-LR concentration in *M. aeruginosa* culture after subsequent hours of treatment with H_2O_2 or/and MlrA.
White bars indicate control culture, light-grey bars – H_2O_2 treated *M. aeruginosa*, grey barslocs – culture of *M. aeruginosa* treated with H_2O_2 and 8 mU ml^{-1} MlrA, dark-grey bars – *M. aeruginosa* treated with H_2O_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_2O_2 is that it does not significantly impact the eukaryotic phytoplankton, zooplankton and macrofauna at concentrations which affect cyanobacteria. Additionally, H_2O_2 is degraded spontaneously within hours or a few days and thus does not contaminate the environment. Diluted H_2O_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_2O_2 provided an expected observation that *Sphingomonas* sp. was not affected by 50 mg l^{-1} of H_2O_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_2O_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_2O_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_2O_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_2O_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_2O_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_2O_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 oxidant 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_2O_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_2O_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_2O_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 also confirmed increased proportion of these more hydrophobic variants when cells were lysed by H_2O_2. As was noted by the authors, more hydrophobic variants are better associated with the lipid layer. Fan and coworkers (2014) reported that H_2O_2 and other chemicals ($CuSO_4$, chlorine, ozone) induce a loss of cyanobacterial membrane integrity which leads to an enhanced MCs release by *M. aeruginosa*. We can assume that cell and membrane disintegration caused by hydrogen peroxide is responsible for the occurrence of -LW and -LF variants (as a dissolved fraction) documented in the present work. Interestingly, the concentration of -LW was much less affected by *Sphingomonas* cells than the concentration of -LR and -LY (about 20 and 40% degradation within 24 h, respectively), whereas the concentration of -LF did not change in the presence of bacteria. Several reports indicated that bacteria with a *mlr* cluster may degrade different MC variants (Edwards *et al.*, 2008; Zhang *et al.*, 2010; Imanishi *et al.*, 2005). Furthermore, it was documented that recombinant MlrA is active against both, more and less 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_2O_2 impacts cyanobacteria. Fan and coworkers (2013) documented that residual H_2O_2 was present in cyanobacteria cultures even 5 days after treatment, which suggests slow action of H_2O_2 on cyanobacteria under laboratory condition (with no UV radiation). Faster and more effective H_2O_2 action is likely to take place in reservoirs due to natural UV radiation from sunlight. The concentration of H_2O_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_2O_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_2O_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 reser-

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 degradation 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 m^3 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 m^3 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 supress 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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