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Microcystin interferes with defense against high oxidative stress in harmful cyanobacteria

J. Merijn Schuurmans[a,b], Bregje W. Brinkmann[a], A. Katharina Makower[c,1], Elke Dittmann[c], Jef Huisman[a,*], Hans C.P. Matthijs[a,2]

[a] Department of Freshwater and Marine Ecology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, P.O. Box 94248, 1090 GE, The Netherlands
[b] Department of Aquatic Ecology, Netherlands Institute of Ecology, Droevendaalsesteeg 6, Wageningen, The Netherlands
[c] Department of Microbiology, Institute for Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Str. 24/25, Potsdam, Germany

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ABSTRACT

Harmful cyanobacteria producing toxic microcystins are a major concern in water quality management. In recent years, hydrogen peroxide (H_2O_2) has been successfully applied to suppress cyanobacterial blooms in lakes. Physiological studies, however, indicate that microcystin protects cyanobacteria against oxidative stress, suggesting that H_2O_2 addition might provide a selective advantage for microcystin-producing (toxic) strains. This study compares the response of a toxic *Microcystis* strain, its non-toxic mutant, and a naturally non-toxic *Microcystis* strain to H_2O_2 addition representative of lake treatments. All three strains initially ceased growth upon H_2O_2 addition. Contrary to expectation, the non-toxic strain and non-toxic mutant rapidly degraded the added H_2O_2 and subsequently recovered, whereas the toxic strain did not degrade H_2O_2 and did not recover. Experimental catalase addition enabled recovery of the toxic strain, demonstrating that rapid H_2O_2 degradation is indeed essential for cyanobacterial survival. Interestingly, prior to H_2O_2 addition, gene expression of a thioredoxin and peroxiredoxin was much lower in the toxic strain than in its non-toxic mutant. Thioredoxin and peroxiredoxin are both involved in H_2O_2 degradation, and microcystin may potentially suppress their activity. These results show that microcystin-producing strains are less prepared for high levels of oxidative stress, and are therefore hit harder by H_2O_2 addition than non-toxic strains.

1. Introduction

Harmful cyanobacterial blooms cause major water quality problems in many lakes, reservoirs and estuaries (Chorus and Bartram, 1999; Guo, 2007; Bullerjahn et al., 2016; Huisman et al., 2018), and their global occurrence is likely to be stimulated by continued eutrophication, rising CO_2 levels and global warming (Paerl and Huisman, 2008; O'Neil et al., 2012; Sandrini et al., 2016; Visser et al., 2016). Cyanobacterial blooms increase the turbidity of the water column, suppressing growth of other phytoplankton and submerged macrophytes (Scheffer, 1998). Moreover, cyanobacteria can produce a variety of toxins that pose a serious threat to the safety of recreational waters and drinking water reservoirs (Chorus and Bartram, 1999; Carmichael, 2001; Codd et al., 2005; Huisman et al., 2018). In particular, the cyclic peptide microcystin is widespread, acts as inhibitor of protein phosphatases, and is a potent liver toxin for birds and mammals including humans (MacKintosh et al., 1990; Nishiwaki-Matsushima et al., 1992; Carmichael, 2001; Meriluoto et al., 2017).

Why cyanobacteria produce microcystins is still under debate. One plausible explanation is that microcystins act as defense against grazing, as indicated by co-evolutionary adaptations between microcystin-producing cyanobacteria and zooplankton (Hairston et al., 2001; Lemaire et al., 2012; Jiang et al., 2016). Yet, molecular genetic evidence indicates that microcystins originated prior to the origins of their zooplankton predators (Rantala et al., 2004), and therefore microcystins may have (had) another function as well. Several recent studies indicate that microcystins protect cyanobacterial cells against oxidative stress, by binding covalently with the cysteines of RuBisCO and several

* Corresponding author: Department of Freshwater and Marine Ecology, IBED/University of Amsterdam, P.O. Box 94248, 1090 GE Amsterdam, The Netherlands.
E-mail address: j.huisman@uva.nl (J. Huisman).
[1] Present address: Department of Experimental Limnology, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Alte Fischerhuette 2, 16775 Stechlin, Germany.
[2] Deceased, April 17, 2016.

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other redox-sensitive proteins (Zilliges et al., 2011; Meissner et al., 2013). The binding of microcystin to cyanobacterial proteins is strongly enhanced during oxidative stress (Zilliges et al., 2011; Meissner et al., 2013), and appears to prevent proteolytic degradation of the bound protein (Zilliges et al., 2011). In line with these findings, it was shown that a microcystin-producing strain of the cyanobacterium *Microcystis* was less affected by naturally occurring concentrations of H_2O_2 (34 µg/L) than its microcystin-deficient mutant (Zilliges et al., 2011). It has therefore been hypothesized that increasing levels of oxidative stress in surface water will favor the growth of microcystin-producing (toxic) over non-toxic cyanobacterial strains (Dziallas and Grossart, 2011; Paerl and Otten, 2013).

In recent years, hydrogen peroxide (H_2O_2) has been used to suppress harmful cyanobacterial blooms (Matthijs et al., 2012, 2016; Barrington et al., 2013), especially when immediate action is urgent and other means of cyanobacterial control are not feasible. H_2O_2 is a reactive oxygen species (ROS) found in low concentrations of 1–50 µg/L in the surface waters of lakes (Cooper and Zika, 1983; Häkkinen et al., 2004; Cory et al., 2017). H_2O_2 and other ROS are produced by photolysis of organic matter (Cooper and Zika, 1983) and as by-product of cellular metabolism such as the Mehler reaction of phytoplankton photosynthesis (Mehler, 1951; Asada, 1999). Interestingly, cyanobacteria lack the Mehler reaction of eukaryotic phototrophs. Instead, they deploy a Mehler-like reaction involving flavodiiron proteins that produces water instead of H_2O_2 (Helman et al., 2003; Allahverdiyeva et al., 2013). This subtle difference in photosynthetic physiology likely explains laboratory observations that cyanobacteria are more sensitive to H_2O_2 than eukaryotic phytoplankton species (Barroin and Feuillade, 1986; Drábková et al., 2007a,b; Barrington and Ghadouani, 2008; Weenink et al., 2015). Inspired by these observations, Matthijs et al. (2012) applied H_2O_2 to a recreational lake at a final concentration of 2 mg/L in 2009, which resulted in a rapid collapse of the cyanobacterial bloom whereas eukaryotic phytoplankton, zooplankton and fish were hardly affected by the treatment. Since then, H_2O_2 has been successfully applied to suppress more than 20 cyanobacterial blooms in lakes in The Netherlands and elsewhere. H_2O_2 degrades to water and oxygen within hours to days, depending on organic matter concentrations, redox-sensitive metals like iron and the biological activity of H_2O_2-degrading organisms (Cooper and Zepp, 1990; Häkkinen et al., 2004). Hence, H_2O_2 addition leaves no long-term chemical traces in the environment.

If microcystin offers protection against oxidative stress, however, and if oxidative stress will favor the growth of microcystin-producing (toxic) over non-toxic cyanobacterial strains, this would call for prudent use of H_2O_2 treatments against cyanobacterial blooms. Because H_2O_2 added to lakes degrades within a few days, repeated H_2O_2 treatments may be necessary if cyanobacterial blooms recur. If the presumptions about the protective role of microcystins in oxidative stress are correct, there is a risk that repeated H_2O_2 addition to lakes might select for toxic cyanobacterial strains in a similar way as repeated antibiotics treatments may select for resistant bacteria in hospitals. Yet, it is unclear whether the protective role of microcystin identified by Zilliges et al. (2011) also applies to H_2O_2 treatments of lakes. In particular, the H_2O_2 concentration used by Zilliges et al. (2011) was much lower than the lethal H_2O_2 concentrations that have been reported for bloom-forming cyanobacteria (Drábková et al., 2007a, b; Ding et al., 2012; Mikula et al., 2012) and that have been applied in lake treatments (Matthijs et al., 2012).

This study therefore investigates the hypothesis advanced by earlier studies (e.g., Zilliges et al., 2011; Dziallas and Grossart, 2011; Paerl and Otten, 2013) that microcystin-producing (toxic) cyanobacteria are more resistant to high oxidative stress than non-toxic cyanobacteria. For this purpose, the photosynthetic efficiency, growth kinetics, H_2O_2 degradation and transcriptome response of a toxic *Microcystis* strain, its non-toxic mutant, and a non-toxic *Microcystis* strain are compared. The applied H_2O_2 concentrations are representative for lake treatments of cyanobacterial blooms, which are two to three orders of magnitude higher than naturally occurring H_2O_2 concentrations in lakes.

2. Materials and methods

2.1. Strains and growth conditions

All experiments were carried out with axenic cultures of the toxic strain *Microcystis aeruginosa* PCC 7806, its non-toxic microcystin-deletion mutant Δ*mcyB* (Dittmann et al., 1997), and the non-toxic strain *Microcystis aeruginosa* PCC 7005. Batch cultures containing 400 mL of BG-11 mineral medium (Rippka et al., 1979) with 10 mM $NaNO_3$ were grown in 2 L Erlenmeyers placed in an incubator (Innova 43, New Brunswick Scientific, Enfield, USA) at 25 °C and shaken at 100 rpm. Light intensity in the incubator was set to a low continuous level of 13 ± 3 µmol photons $m^{-2} s^{-1}$ of fluorescent white light to avoid light-induced oxidative stress.

The batch cultures were initiated at an optical density (OD_{750}) of 0.03, obtained from precultures grown under the same light and medium conditions. The experiment started 5 days after inoculation of the batch cultures, when 10 mg/L of H_2O_2 was added to the treatments, whereas no H_2O_2 was added to the controls. This high H_2O_2 concentration was used because of the high cell density of the cultures, to achieve a similar dose per cell as in lake treatments with H_2O_2. At the start of the experiments, cell densities for *Microcystis* PCC 7806 and its Δ*mcyB* mutant were $\sim 1 \times 10^7$ cells/mL, whereas cell densities for *Microcystis* PCC 7005 were slightly lower at 0.6×10^7 cells/mL because it had a slightly lower growth rate.

Samples were taken just prior to H_2O_2 addition (t = 0), immediately after H_2O_2 addition and after 1, 2, 4, 24, 48 and 120 h. In the catalase addition experiment the last samples were taken after 72 instead of 120 h. All experiments were carried out in triplicate.

2.2. Microcystin analysis

Microcystis PCC 7806 produces two microcystin variants, MC-LR and [D-Asp^3]MC-LR (Tonk et al., 2009), whereas its Δ*mcyB* mutant and *Microcystis* PCC 7005 produce no microcystins. Methanol extractable intracellular and extracellular microcystin concentrations were determined by filtering 5–10 mL of culture over a 1.2 µm pore size 25 mm GF/C filter (Whatman GmbH, Dassel, Germany). The filters and filtrates were stored at −20 °C before further analysis. Filters were freeze-dried and subsequently intracellular microcystins were extracted with 75% MeOH and quantified by HPLC according to Van de Waal et al. (2011), with a Shimadzu LC-20AD HPLC system with an SPD-M20 A photodiode array detector (Shimadzu, Kyoto, Japan). Peaks of [D-Asp^3] MC-LR and MC-LR could not be completely separated and were summed as the total free intracellular microcystin fraction. Extracellular microcystins were quantified by directly injecting the filtrate into the HPLC system.

2.3. Photosynthetic yield

Photosynthetic yield was measured by filtering 1–10 mL (depending on culture density) of culture over a 1.2 µm pore size 25 mm diameter GF/C filter (Whatman GmbH, Dassel, Germany). Filters were kept in the dark for 3 min and subsequently fluorescence was measured using a mini-PAM (Walz, Effeltrich, Germany). Photosynthetic yield was measured as $\Phi_{PSII} = (F_m - F_0)/F_m$, where F_0 and F_m are the minimum and maximum fluorescence, respectively (Genty et al., 1989; Matthijs et al., 2012).

2.4. Hydrogen peroxide quantification and catalase addition

H_2O_2 was quantified using a colorimetric assay with 5 mM *p*-nitrophenylboronic acid (*p*-NPBA) in a carbonate buffer at pH 8.9. In alkaline solution p-NPBA reacts with H_2O_2 and forms stable yellow