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DOI
10.1111/1462-2920.15429

Publication date
2021

Document Version
Final published version

Published in
Environmental Microbiology

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Interspecific protection against oxidative stress: green algae protect harmful cyanobacteria against hydrogen peroxide

Erik F. J. Weenink, Hans C. P. Matthijs,† J. Merijn Schuurmans, Tim Piel, Maria J. van Herk, Corrien A. M. Sigon, Petra M. Visser and Jef Huisman ©

Department of Freshwater and Marine Ecology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, P.O. Box 94240, Amsterdam, GE, 1090, The Netherlands.

Summary

Oceanographic studies have shown that heterotrophic bacteria can protect marine cyanobacteria against oxidative stress caused by hydrogen peroxide (H$_2$O$_2$). Could a similar interspecific protection play a role in freshwater ecosystems? In a series of laboratory experiments and two lake treatments, we demonstrate that freshwater cyanobacteria are sensitive to H$_2$O$_2$ but can be protected by less-sensitive species such as green algae. Our laboratory results show that green algae degrade H$_2$O$_2$ much faster than cyanobacteria. Consequently, the cyanobacterium Microcystis was able to survive at higher H$_2$O$_2$ concentrations in mixtures with the green alga Chlorella than in monoculture. Interestingly, even the lysate of destructed Chlorella was capable to protect Microcystis, indicating a two-component H$_2$O$_2$ degradation system in which Chlorella provided antioxidant enzymes and Microcystis the reductants. The level of interspecific protection provided to Microcystis depended on the density of Chlorella. These findings have implications for the mitigation of toxic cyanobacterial blooms, which threaten the water quality of many eutrophic lakes and reservoirs worldwide. In several lakes, H$_2$O$_2$ has been successfully applied to suppress cyanobacterial blooms. Our results demonstrate that high densities of green algae can interfere with these lake treatments, as they may rapidly degrade the added H$_2$O$_2$ and thereby protect the bloom-forming cyanobacteria.

Introduction

Some species play a key role in ecological communities by protecting other species against harmful conditions. For example, the marine heterotrophic bacterium Alteromonas sp. protects the cyanobacterium Prochlorococcus sp. against cell damage caused by oxidative stress (Morris et al., 2008, 2011). This example has led to the Black Queen Hypothesis, an evolutionary theory that refers to the card game ‘Hearts’ and assumes that some species have lost a costly function (the Black Queen) (Morris et al., 2012; Mas et al., 2016). This function is essential for survival, however, and is still carried out by other species known as helpers. In this case, Prochlorococcus lacks a large number of antioxidant genes in comparison to other microorganisms (Bernroitner et al., 2009; Morris et al., 2012). However, Alteromonas acts as a helper by degrading H$_2$O$_2$ and other reactive oxygen species (ROS) and thereby provides protection to nearby Prochlorococcus cells. Similar interspecific protection against oxidative stress might be more widespread than previously recognized (Jakubovics et al., 2008; Bobadilla Fazzini et al., 2010; Zipser, 2011).

High sensitivity of cyanobacteria to oxidative stress has not only been reported for Prochlorococcus in the oligotrophic ocean but also for freshwater cyanobacteria in eutrophic lakes (Drábková et al., 2007a; Matthijs et al., 2012; Barrington et al., 2013; Lürling et al., 2014; Weenink et al., 2015; Daniel et al., 2019). Some of these cyanobacteria can form dense and often toxic blooms during warm periods in summer, deteriorating water quality with severe negative impacts on drinking water reservoirs, irrigation and recreation (Chorus and Bartram, 1999; Carmichael, 2001; Codd et al., 2005; O’Neil et al., 2012; Huisman et al., 2018). Different methods have been developed to suppress cyanobacterial dominance (Ibelings et al., 2016; Paerl et al., 2018). One promising approach is based on the observation that bloom-
forming cyanobacteria tend to be more sensitive to H$_2$O$_2$ than many eukaryotic phytoplankton taxa including green algae, diatoms and dinoflagellates (Barroin and Feuillade, 1986; Drábková et al., 2007a; Barrington and Ghadouani, 2008; Matthijs et al., 2012; Weenink et al., 2015; Yang et al., 2018; Wang et al., 2019; Lusty and Gobler, 2020), most likely because cyanobacteria display a lower antioxidant activity than eukaryotic algae (Matthijs et al., 2016). Hence, low concentrations of H$_2$O$_2$ have been successfully applied to suppress harmful cyanobacterial blooms in lakes (Matthijs et al., 2012, 2016) and wastewater ponds (Barrington et al., 2013). The applied H$_2$O$_2$ concentrations (1–5 mg L$^{-1}$; Matthijs et al., 2012, 2016) are two to three orders of magnitude higher than natural H$_2$O$_2$ concentration in lakes (1–50 μg L$^{-1}$; Cooper and Lean, 1989; Håkkinen et al., 2004; Cory et al., 2017). Yet, the applied H$_2$O$_2$ concentrations are still far below the H$_2$O$_2$ sensitivity thresholds of aquatic macroinvertebrates and fish (Rach et al., 1997; Gaikowski et al., 1999; Matthijs et al., 2012; Burson et al., 2014), although some zooplankton taxa are sensitive to H$_2$O$_2$ concentrations in the applied range (Meinertz et al., 2008; Matthijs et al., 2012; Reichwaldt et al., 2012; Yang et al., 2018). Hence, this mitigation method acts selectively against cyanobacteria, while environmental impacts on most of the eukaryotic organisms in the ecosystem are kept to a minimum. Furthermore, in comparison to other algicides, application of H$_2$O$_2$ has the advantage that it breaks down to water and oxygen within a few days, and therefore leaves no long-term chemical traces in the environment (Matthijs et al., 2012).

It is conceivable, however, that the less H$_2$O$_2$ sensitive eukaryotic phytoplankton act as helpers that degrade the added H$_2$O$_2$ and thereby protect the targeted cyanobacteria. If so, this would provide a freshwater example of interspecific protection akin to the Black Queen Hypothesis, with practical implications for the mitigation of cyanobacterial blooms. To our knowledge, examples of interspecific protection against oxidative stress have not yet been described in lakes.

To investigate our hypothesis that eukaryotic phytoplankton can protect cyanobacteria against oxidative stress, we performed a series of laboratory experiments with freshwater cyanobacteria and green algae. First, we studied interspecific differences in H$_2$O$_2$ sensitivity and H$_2$O$_2$ degradation capacity between three common bloom-forming cyanobacteria (Microcystis, Anabaena and Planktothrix) and six common green algae (Chlorella, Desmodesmus, Kirchneriella, Ankistrodesmus, Monoraphidium and Chlamydomonas) in monoculture experiments exposed to different H$_2$O$_2$ concentrations. Subsequently, we applied different H$_2$O$_2$ concentrations to mixtures of Microcystis and Chlorella to investigate whether H$_2$O$_2$ degradation by green algae enhanced the survival of the cyanobacteria. We also examined whether H$_2$O$_2$ degradation by green algae is mediated by extracellular or intracellular antioxidant activity. Finally, to assess the relevance of these laboratory findings for the mitigation of cyanobacterial blooms in lakes, we compared the effectiveness of H$_2$O$_2$ treatments in two lakes with different relative abundances of cyanobacteria and green algae.

**Results**

Experiment 1: Cyanobacteria are more sensitive to H$_2$O$_2$ than green algae

The cyanobacteria Microcystis, Anabaena and Planktothrix differed in their sensitivity to H$_2$O$_2$ (Fig. 1A–C). The photosynthetic yield of Microcystis and Anabaena was not affected at H$_2$O$_2$ concentrations of 1 and 2 mg L$^{-1}$ but strongly declined after the addition of ≥3.5 mg L$^{-1}$ of H$_2$O$_2$ (Fig. 1A and B). The cyanobacterium Planktothrix showed the highest sensitivity. Its photosynthetic yield was not affected at 1 mg L$^{-1}$ of H$_2$O$_2$ but strongly declined after the addition of ≥2 mg L$^{-1}$ of H$_2$O$_2$ (Fig. 1C).

The six species of green algae were all much more resistant to H$_2$O$_2$ than the investigated cyanobacteria. The photosynthetic yield of the green algae was not affected by H$_2$O$_2$ concentrations up to 12 mg L$^{-1}$ and was mildly suppressed in some taxa after the addition of 20 mg L$^{-1}$ (Fig. 1D–I). The photosynthetic yield of Chlorella, Desmodesmus, Kirchneriella, Ankistrodesmus and Monoraphidium strongly declined after the addition of ≥35 mg L$^{-1}$ H$_2$O$_2$ (Fig. 1D–H), whereas the photosynthetic yield of Chlamydomonas resisted even higher H$_2$O$_2$ concentrations and was strongly suppressed only after the addition of ≥75 mg L$^{-1}$ (Fig. 1I). The underlying data of the minimum fluorescence ($F_{m}$) and maximum fluorescence ($F_{m}$) of the species are reported in Figs S1 and S2.

The H$_2$O$_2$ degradation capacity was much higher in axenic monocultures of the green algae than in those of the cyanobacteria (Fig. 2). All three cyanobacteria completely degraded 1 mg L$^{-1}$ of H$_2$O$_2$ within a few hours, Microcystis and Anabaena also completely degraded 2 mg L$^{-1}$ of H$_2$O$_2$, and Anabaena completely degraded 3.5 mg L$^{-1}$ (Fig. 2A–C). However, H$_2$O$_2$ added at higher concentrations was only partially degraded and still remained in the cyanobacterial cultures after 24 h. In contrast, all six species of green algae completely degraded even the highest H$_2$O$_2$ concentration of 100 mg L$^{-1}$ within 24 h (Fig. 2D–I).

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Experiment 2: Chlorella protects Microcystis against H$_2$O$_2$

As a next step, we performed monoculture and mixture experiments with Microcystis and Chlorella at six H$_2$O$_2$ concentrations to investigate the potential for interspecific protection (Fig. 3). Monoculture experiments confirmed that Chlorella degraded H$_2$O$_2$ faster than Microcystis (Fig. 3A and C), in agreement with the previous results (Fig. 2). Microcystis monocultures were strongly suppressed after H$_2$O$_2$ addition of 10 mg L$^{-1}$ and collapsed at 15 and 20 mg L$^{-1}$ (Fig. 3B). Flow cytometer counts illustrate that Microcystis cells exposed to these high H$_2$O$_2$ concentrations were destroyed to cellular debris (Fig. 4A and B). Conversely, Chlorella monocultures sustained growth at all applied H$_2$O$_2$ additions (Figs 3D and Fig. 4C,D).

In mixtures of Microcystis and Chlorella, H$_2$O$_2$ degradation was even slightly faster than in the monoculture experiments of Chlorella and both species sustained growth even at the high H$_2$O$_2$ additions of 15 and 20 mg L$^{-1}$ (Figs 3E,F and 4E,F). Note that, at all applied H$_2$O$_2$ concentrations, Chlorella reached a consistently lower population density in mixtures with Microcystis (Fig. 3F) than in the Chlorella monoculture (Fig. 3D),
indicating that *Microcystis* was an effective competitor restraining the population density of *Chlorella*.

When *Microcystis* was grown in a mineral medium to which we added spent medium of the *Chlorella* cultures, the H$_2$O$_2$ degradation rate was comparable to that in the *Microcystis* monoculture (compare Fig. 3G and A) and the *Microcystis* population collapsed at H$_2$O$_2$ additions of 15 and 20 mg L$^{-1}$ (Figs 3H and 4G,H). However, when *Microcystis* was grown in mineral medium to which we added lysate of the *Chlorella* cultures, containing the cellular debris of *Chlorella*, the H$_2$O$_2$ degradation rate was comparable to that in the mixture of *Microcystis* and *Chlorella* cells (compare Fig. 3I and E) and the *Microcystis* population sustained growth even at the high H$_2$O$_2$ additions of 15 and 20 mg L$^{-1}$ (Figs 3J and 4I,J).

**Experiment 3: Protection by Chlorella is density-dependent**

H$_2$O$_2$ degradation was faster in high-density *Chlorella* cultures than in low-density *Chlorella* cultures (Fig. 5A–C). To investigate whether this density-dependent H$_2$O$_2$ degradation affects interspecific protection, we performed experiments with *Microcystis* and three different population densities of *Chlorella* (Fig. 5D–F). In mixed cultures...
Fig. 3. Legend on next page.
with low *Chlorella* densities, *Microcystis* sustained population densities at 45% and 21% of the control after addition of 10 and 20 mg L\(^{-1}\) of H\(_2\)O\(_2\) respectively but was suppressed to <3% of the control at 40 mg L\(^{-1}\) of H\(_2\)O\(_2\) (Fig. 5D). At intermediate *Chlorella* densities, *Microcystis* sustained higher population densities (67% and 40% of the control respectively) at 10 and 20 mg L\(^{-1}\) of H\(_2\)O\(_2\) but was still suppressed to <5% of the control at 40 mg L\(^{-1}\) of H\(_2\)O\(_2\) (Fig. 5E). At high *Chlorella* densities, *Microcystis* sustained population densities that were 90%, 53% and 25% of the control at 10, 20 and 40 mg L\(^{-1}\) of H\(_2\)O\(_2\) respectively (Fig. 5F). Hence, *Microcystis* survival improved with increasing population densities of *Chlorella*.

Experiment 4: Rapid H\(_2\)O\(_2\) degradation is mediated by living cells

In the absence of living cells, the H\(_2\)O\(_2\) degradation rate by the lysate and spent medium of both species was very low (Fig. S1). In particular, the lysate of *Chlorella* by itself hardly degraded any H\(_2\)O\(_2\) (Fig. S1a), whereas the lysate of *Chlorella* did show a fast H\(_2\)O\(_2\) degradation rate in the presence of *Microcystis* cells (Fig. S1).

Lake treatments

Lake Oosterduinse Meer was dominated by a dense *Aphanizomenon* bloom in June 2016 (Fig. 6A and B). On the day of the H\(_2\)O\(_2\) treatment, prior to H\(_2\)O\(_2\) addition, the lake contained a cyanobacterial biovolume of 99.0 \(\times\) 10\(^6\) \(\mu\)m\(^3\) ml\(^{-1}\) (99.6% of total phytoplankton), consisting almost entirely of *Aphanizomenon flos-aquae* (99.6%) with minor contributions by *Microcystis* spp. (0.02%) and *Dolichospermum* spp. (0.01%). Eukaryotic phytoplankton contributed only 0.35 \(\times\) 10\(^5\) \(\mu\)m\(^3\) ml\(^{-1}\) (0.35%), consisting mainly of green algae (0.17%) and diatoms (0.11%) (Table S1).

During the treatment of Lake Oosterduinse Meer, the target H\(_2\)O\(_2\) concentration of 5.0 mg L\(^{-1}\) was reached \(\sim\) 3 h after the H\(_2\)O\(_2\) addition started (Fig. 6A). High H\(_2\)O\(_2\) concentrations \(\geq\) 2.5 mg L\(^{-1}\) were maintained for several hours, although the added H\(_2\)O\(_2\) had disappeared after 24 h. The cyanobacterial biovolume decreased by 64.0% (relative to the initial cyanobacterial biovolume) at 5 h after the start of the H\(_2\)O\(_2\) addition and almost completely disappeared 2 days later (>99.8% decline) (Fig. 6B). The cyanobacterial biovolume remained very low (<0.02%) during the first week after the treatment, while eukaryotic phytoplankton (including the green algae) increased almost 20-fold.

Lake Krabbeplas consisted of a mixture of cyanobacteria and eukaryotic phytoplankton (Fig. 6C and D). On the treatment day prior to H\(_2\)O\(_2\) addition, Lake Krabbeplas contained a cyanobacterial biovolume of 33.2 \(\times\) 10\(^6\) \(\mu\)m\(^3\) ml\(^{-1}\) (57.5% of total phytoplankton), including *Limnococcus limneticus* (29.1%), *Pseudanabaena limnetica* (28.1%), *Planktothrix agardhii* (0.8%) and *Coelomoror pusillum* (0.6%). Eukaryotic phytoplankton was slightly less abundant than the cyanobacteria, with a eukaryotic biovolume of 24.5 \(\times\) 10\(^5\) \(\mu\)m\(^3\) ml\(^{-1}\) (42.5%) consisting of a rich diversity of green algae (17.1%) including *Monoraphidium contortum*, *Desmodesmus* spp. and *Chlamydomonas* spp., and other taxa such as diatoms, dinoflagellates and cryptomonads (Table S2).

In Lake Krabbeplas, H\(_2\)O\(_2\) degradation was much faster than in Lake Oosterduinse Meer (Fig. 6C). Although the total amount of H\(_2\)O\(_2\) administered to the lake was equivalent to 4.6 mg L\(^{-1}\), the maximum H\(_2\)O\(_2\) concentration measured in the lake was only 3.5 mg L\(^{-1}\). Within 2 h after the start of the treatment, the H\(_2\)O\(_2\) concentration in Lake Krabbeplas had decreased to <2 mg L\(^{-1}\), and within 5 h all added H\(_2\)O\(_2\) had disappeared. One day after the treatment, the cyanobacterial biovolume had declined by 82.1% (relative to the initial cyanobacterial biovolume). Contrary to Lake Oosterduinse Meer, however, cyanobacteria in Lake Krabbeplas did not decline further but remained present at a stable biovolume of \(\sim\) 6 \(\times\) 10\(^7\) \(\mu\)m\(^3\) ml\(^{-1}\) during the first week after the treatment and subsequently increased (Fig. 6D).

Discussion

Differences in H\(_2\)O\(_2\) sensitivity between cyanobacteria and green algae

Oxygenic phototrophic organisms have developed antioxidant defences to degrade ROS and, thereby, to protect themselves against oxidative damage (Bernrother *et al.*, 2009; Latifi *et al.*, 2009; Dietz, 2011; Foyer and Shigeoka, 2011). Our results show considerable

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Fig. 3. The green alga *Chlorella* protects the cyanobacterium *Microcystis* against high H\(_2\)O\(_2\) concentrations. H\(_2\)O\(_2\) was added at different initial concentrations to (A, B) *Microcystis* monocultures, (C, D) *Chlorella* monocultures, (E, F) mixtures of *Microcystis* and *Chlorella*, (G, H) mixtures of *Microcystis* with spent medium of *Chlorella* and (I, J) mixtures of *Microcystis* with the lysate of destructed *Chlorella* cells. Left panels show H\(_2\)O\(_2\) degradation during the first 2 h after the addition of different H\(_2\)O\(_2\) concentrations (2.5, 5, 10, 15 and 20 mg L\(^{-1}\)). Right panels show cell counts of *Microcystis* (blue bars) and *Chlorella* (green bars) after 4 days. nd = not determined. Horizontal dashed lines indicate the initial population densities of *Microcystis* (panels B, H and J) and *Chlorella* (panel D) when the H\(_2\)O\(_2\) was added (at \(t = 0\)). Initial population densities of *Microcystis* and *Chlorella* in panel F were the same as in panels B and D respectively.

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interspecific variation in H$_2$O$_2$ sensitivity among cyanobacteria and green algae representative of eutrophic lakes (Figs 1 and 2). Among the cyanobacteria, *Planktothrix* was more sensitive than *Microcystis* and *Anabaena*. This result aligns with other recent studies in which *Planktothrix* was more sensitive to H$_2$O$_2$ than *Microcystis* (Yang et al., 2018; Lusty and Gobler, 2020). Among the green algae, *Chlamydomonas reinhardtii* was more resistant than the five other taxa that we investigated. Similarly, Drábková et al. (2007a) reported that a *C. reinhardtii* strain was more H$_2$O$_2$ resistant than the green algae *Scenedesmus quadricauda* and *Pseudokirchneriella subcapitata*.

However, the most striking difference in sensitivity was observed between cyanobacteria and green algae. Our results show that, in monoculture, the freshwater cyanobacteria were all more sensitive to H$_2$O$_2$ than the green algae. The photosynthetic yield of the bloom-forming cyanobacterium *Planktothrix* collapsed at $\geq 2$ mg L$^{-1}$ H$_2$O$_2$ and *Microcystis* and *Anabaena* at $\geq 3.5$ mg L$^{-1}$ H$_2$O$_2$, whereas the investigated green algae were hardly affected up to at least 20 mg L$^{-1}$ H$_2$O$_2$ (Fig. 1). These results are in agreement with previous studies, which have also shown that cyanobacteria are more sensitive to H$_2$O$_2$ than eukaryotic phytoplankton including green algae (Barroin and Feuillade, 1986; Drábková et al., 2007a; Drábková et al., 2007b; Matthijs et al., 2012; Weenink et al., 2015; Yang et al., 2018; Wang et al., 2019; Lusty and Gobler, 2020).

The difference in H$_2$O$_2$ sensitivity between cyanobacteria and green algae appears to be related to differences in H$_2$O$_2$ degradation rates, since all six species of green algae degraded the added H$_2$O$_2$ much faster than the cyanobacterial species (Fig. 2). This might be explained by a key difference in photophysiology between cyanobacteria and eukaryotic algae (Helman et al., 2003; Allahverdiyeva et al., 2015; Matthijs et al., 2016). When eukaryotic phototrophs are exposed to high light or low inorganic carbon concentrations, excess electrons generated by photosynthesis are transferred to oxygen (O$_2$) producing the superoxide anion (O$_2^-$) via the Mehler reaction (Mehler, 1951; Asada, 1999). The highly reactive superoxide is rapidly converted to H$_2$O$_2$ by superoxide dismutase, which is subsequently converted to water by peroxidases and catalases to protect the cells against oxidative damage (Asada, 1999; Shigeoka et al., 2002; Apel and Hirt, 2004). In contrast, cyanobacteria deploy a ‘Mehler-like reaction’, in which excess electrons are transferred to oxygen by the flavodiiron proteins Flv1 and Flv3, which produce water directly without intermediary production of O$_2^-$ and H$_2$O$_2$ (Helman et al., 2003; Allahverdiyeva...
et al., 2015). This implies that cyanobacteria will produce much less H$_2$O$_2$ during photosynthesis than eukaryotic phytoplankton. Accordingly, in evolutionary terms, cyanobacteria may not have had the need for a similarly high H$_2$O$_2$ degradation capacity as eukaryotic phytoplankton.

**Green algae protect cyanobacteria against high oxidative stress**

Our results show that, in combination with the green alga *Chlorella*, the cyanobacterium *Microcystis* can survive at much higher H$_2$O$_2$ concentrations than in monoculture, at least up to 20 mg L$^{-1}$ of H$_2$O$_2$ (Figs 3 and 4). Apparently, the high H$_2$O$_2$ degradation rate by *Chlorella* protects *Microcystis* against oxidative stress.

In several ways, these results resemble the previously described example of protection against oxidative stress of *Prochlorococcus* by *Alteromonas* in the oligotrophic ocean (Morris et al., 2011, 2012). For example, our results show that degradation of H$_2$O$_2$ is essential for survival, and that *Microcystis* relies on the H$_2$O$_2$ degradation activity of *Chlorella* when H$_2$O$_2$ concentrations become high. Using the terminology of the Black Queen Hypothesis, the green alga serves as helper and the cyanobacterium as the beneficiary.
There are also some interesting differences, however. In particular, the observed interspecific protection of freshwater bloom-forming cyanobacteria by green algae occurs at much higher H$_2$O$_2$ concentrations than the protection of the marine cyanobacterium *Prochlorococcus* by *Alteromonas*. Marine *Prochlorococcus* spp. lack most antioxidant enzymes, and therefore they are very sensitive to H$_2$O$_2$ (Morris *et al*., 2012). Experiments have shown that *Alteromonas* may already provide protection to *Prochlorococcus* at H$_2$O$_2$ concentrations as low as 0.007 mg L$^{-1}$ (Morris *et al*., 2011), which is of similar magnitude as the natural H$_2$O$_2$ concentration in surface waters of the oligotrophic open ocean (Yuan and Shiller, 2001; Gerringa *et al*., 2004; Yuan and Shiller, 2005). In contrast, most freshwater cyanobacteria, including *Microcystis*, contain genes encoding anti-ROS enzymes such as peroxiredoxins (Franguel *et al*., 2008; Bernroitner *et al*., 2009; Schuurmans *et al*., 2018). Our results show that these bloom-forming cyanobacteria are much less H$_2$O$_2$ sensitive than *Prochlorococcus*. Without interspecific protection, they can survive up to 1–2 mg L$^{-1}$ of H$_2$O$_2$ (Fig. 1; see also Drábková *et al*., 2007a; Barrington *et al*., 2013; Lürling *et al*., 2014; Piel *et al*., 2020). For this reason, the H$_2$O$_2$ concentrations applied in lake treatments greatly exceed the H$_2$O$_2$ concentrations that they would naturally

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**Fig. 6.** H$_2$O$_2$ treatments of two lakes with different relative abundances of cyanobacteria and eukaryotic phytoplankton. (A, B) Lake Oosterduinse Meer was dominated by cyanobacteria (99.6%) with only a minor contribution by eukaryotic phytoplankton (0.35%) prior to the treatment. In this lake, (A) the added H$_2$O$_2$ remained in the lake for several hours, and (B) the cyanobacterial bloom was effectively suppressed. (C, D) Lake Krabbeplas consisted of a mixture of cyanobacteria (57.5%) and eukaryotic phytoplankton (42.5%). In this lake, (C) the added H$_2$O$_2$ concentration declined much faster, and (D) the cyanobacteria were much less suppressed than in Lake Oosterduinse Meer. Data in (A) and (C) show mean ± standard error (n = 2–10 per data point); the horizontal dashed line indicates the target H$_2$O$_2$ concentration (i.e. the expected concentration based on the total amount of H$_2$O$_2$ added to the lake). Red arrows in (B) and (D) indicate the start of H$_2$O$_2$ addition. See Tables S1 and S2 for detailed changes in phytoplankton community composition.
Green algae protect cyanobacteria against H$_2$O$_2$

encounter in lakes (0.001–0.050 mg L$^{-1}$; Cooper and Lean, 1989; Häkkinen et al., 2004; Cory et al., 2017). Although the protective mechanism shows many similarities with the Prochlorococcus–Alteromonas example of the Black Queen Hypothesis, it is thus unlikely that protection of bloom-forming cyanobacteria by green algae has developed from a co-evolutionary interaction between the species.

Furthermore, the ecological relationship between helper and beneficiary is different. Although reciprocity is not required for the Black Queen Hypothesis, the interaction between Alteromonas and Prochlorococcus might be beneficial for both species. The heterotrophic bacterium Alteromonas provides protection against extracellular H$_2$O$_2$ to Prochlorococcus cells, while the phototrophic Prochlorococcus in turn fixes atmospheric carbon that partly becomes available as a public good (Morris et al., 2012). A major novelty of our study is that one phototrophic species protects another phototropic species against oxidative stress. In our experiments, Chlorella protects Microcystis but Microcystis does not seem to return a favour to Chlorella. Instead, cyanobacteria and green algae compete with each other for limiting resources, and our data suggest that the survival of Microcystis may even suppress the Chlorella population to some extent (as indicated by the difference in population densities of Chlorella between Fig. 3D and F). In a sense, Chlorella facilitates its competitor.

Which card is being played?

The antioxidant activity of Chlorella is essential for H$_2$O$_2$ degradation and hence for the interspecific protection of Microcystis. Interestingly, the lysate of Chlorella by itself caused a very slow H$_2$O$_2$ degradation (Fig. S1), whereas the lysate of Chlorella in combination with Microcystis cells enabled a much faster H$_2$O$_2$ degradation (Fig. 3I). Apparently, Microcystis can activate the H$_2$O$_2$ degradation activity in lysate of Chlorella. Moreover, in the presence of Microcystis cells, the lysate of Chlorella had a much higher H$_2$O$_2$ degradation rate than spent medium of Chlorella (Fig. 3G and I). The latter result indicates that the H$_2$O$_2$-degrading enzymes are contained in the Chlorella cells rather than being exported extracellularly. So, what is the expensive Black Queen card that Chlorella holds by which Microcystis is protected, and what essential component for H$_2$O$_2$ degradation may be missing in the lysate of destructed Chlorella cells that is provided by Microcystis?

Our results suggest that H$_2$O$_2$ degradation in this system requires two components. The cells of Chlorella likely contain H$_2$O$_2$-degrading enzymes. These enzymes function well in living Chlorella cells (Fig. 3C and D). The enzymes can also function in the lysate (Figs 3I,J and 4I,J), but there they cannot degrade H$_2$O$_2$ by themselves (Fig. S1). Apparently, a second compound is required that fuels H$_2$O$_2$ degradation, most likely a flow of reductants needed by the enzymes for H$_2$O$_2$ reduction (e.g. Shigeoka et al., 2002). These reductants can be provided by living Chlorella cells, but our results indicate that in the absence of living Chlorella cells the provision of reductants can also be taken over by Microcystis (Figs 3I,J and 4I,J).

Microcystis PCC 7806 does not produce catalase (Franguel et al., 2008; Schurmans et al., 2018). Whether Chlorella sorokiniana 211-8k produces catalase is not known. However, catalase can scavenge large amounts of H$_2$O$_2$ without the requirement for an electron-donating substrate (Apel and Hirt, 2004), which does not fit the two-component system observed in our data. Moreover, in our experiments the cell-free lysate of Chlorella had a very low H$_2$O$_2$ degradation rate (Fig. S1), suggesting the absence or only a low potency of catalase mediated anti-ROS activity in Chlorella sorokiniana 211-8k.

A more plausible explanation is that Chlorella contains glutathione peroxidase (GPX) and/or ascorbate peroxidase (APX) to scavenge H$_2$O$_2$. In case of GPX, H$_2$O$_2$ is reduced to H$_2$O by oxidizing glutathione (GSH) to glutathione disulfide (GSSG). Subsequently, GSSG is regenerated to GSH by the glutathione-reductase cycle, which requires an influx of reductants from, e.g. NADPH. APX works in a similar way using ascorbate as reductant, which is coupled to glutathione via the glutathione-ascorbate cycle. Many Chlorella strains including Chlorella sorokiniana 211-8k contain GPX and/or APX, usually in combination with glutathione reductase (Pelah and Cohen, 2005; Wang and Xu, 2012; Sablitiati et al., 2015). By contrast, sequencing of the genome of Microcystis PCC 7806 did not reveal GPX or APX, but it does contain glutathione synthetase and reductase to produce and regenerate glutathione (Franguel et al., 2008; Straub et al., 2011; Zilliges et al., 2011). Glutathione can be actively exported to and imported from the extracellular environment in both its reduced (GSH) and oxidized (GSSG) forms (Couto et al., 2016). Hence, a plausible hypothesis for our results is that the high H$_2$O$_2$-degrading activity of peroxidases produced by Chlorella protects both Chlorella and Microcystis against oxidative stress (Fig. 7A). When Chlorella cells were destructed, their peroxidase enzymes remained intact in the lysate but now their activity required the delivery of reductants by Microcystis (Fig. 7B). Further research may help to elucidate this mechanism in full detail, for instance by investigating whether peroxidases such as GPX or APX are indeed active in Chlorella, and whether addition of these peroxidases in pure form to monocultures of Microcystis would provide similar protection against oxidative stress as the addition of lysate of Chlorella.
Implications for lake treatments

In several lakes and ponds, H₂O₂ addition has been applied as an emergency method to suppress toxic cyanobacterial blooms (Matthijs et al., 2012, 2016; Barrington et al., 2013). What constitutes a successful treatment? The suppression of toxic cyanobacteria should be strong enough to avoid health risks associated with recreation or drinking water supply. Furthermore, the cyanobacterial bloom should be sufficiently suppressed to prevent rapid regrowth from the remaining cyanobacterial population. In practice, this implies that successful treatments should aim at an almost complete elimination of the cyanobacterial bloom.

As shown by our laboratory results, green algae and presumably also other eukaryotic phytoplankton can counteract the treatment success by protecting cyanobacteria against oxidative stress. Although whole-lake treatments are expensive and cannot be controlled to a similar degree as laboratory experiments, the results of the H₂O₂ treatments in Lake Oosterduinse Meer and Lake Krabbeplas are consistent with these laboratory findings (Fig. 6). In Lake Oosterduinse Meer, the abundance of eukaryotic phytoplankton including green algae was very low, the added H₂O₂ concentration remained above 2.5 mg L⁻¹ for at least 5.5 h, and the dense cyanobacterial population was completely suppressed by the H₂O₂ treatment (Fig. 6A and B). Lake Krabbeplas initially contained less cyanobacteria than Lake Oosterduinse Meer but a much higher abundance of eukaryotic phytoplankton. In this case, the added H₂O₂ was degraded rapidly, and a substantial fraction (~15%) of the cyanobacterial population survived the H₂O₂ treatment (Fig. 6C and D). Consequently, the H₂O₂ treatment was more successful in Lake Oosterduinse Meer than in Lake Krabbeplas.

Similar results were obtained by a recent study by Lusty and Gobler (2020). They applied H₂O₂ to incubation experiments with natural phytoplankton assemblages sampled from lakes. Mill Pond was dominated by a high cyanobacterial biomass (expressed as Chl a L⁻¹), whereas the biomass of green algae was below detectable levels at the start of the experiment. Addition of H₂O₂ to incubation experiments containing the phytoplankton community of this lake caused a major collapse of 96% of the cyanobacterial population (their Fig. 4A). In contrast, in Roth Pond the initial green algal biomass was more than twice the cyanobacterial biomass. In this case, H₂O₂ addition to the incubation experiments of Roth Pond reduced the cyanobacteria by only 34% (their Fig. 6A). Hence, the results for Mill Pond and Roth Pond obtained by Lusty and Gobler (2020) support our observations that a high abundance of green algae in the phytoplankton community may reduce the efficiency of H₂O₂ to suppress cyanobacterial blooms.

The density-dependent nature of this interspecific protection implies that, at a higher concentration of green algae, a higher H₂O₂ dosage will be required to suppress cyanobacterial blooms. One could argue to raise the H₂O₂ dosage until the desired collapse of the cyanobacterial population is achieved. From a water management perspective, however, there are limits on the amount of H₂O₂ that can be applied to lakes, because high H₂O₂ concentrations can be lethal to sensitive non-target organisms such as zooplankton (Matthijs et al., 2012; Reichwaldt et al., 2012; Burson et al., 2014; Yang et al., 2018). For this reason, Matthijs et al. (2016)
generally recommended H$_2$O$_2$ concentrations $\leq$5 mg L$^{-1}$ as a precautionary limit for lake applications. As a consequence, extensive H$_2$O$_2$ scavenging by high abundances of green algae and other eukaryotic phytoplankton can protect bloom-forming cyanobacteria against oxidative stress, and thereby prevent successful suppression of toxic cyanobacterial blooms by H$_2$O$_2$ treatments.

**Experimental procedures**

Species and pre-culture conditions

Axenic cultures of *Microcystis aeruginosa* strain PCC 7806 (hereafter Microcystis), *Planktothrix agardhii* PCC 7811 (Planktothrix) and *Anabaena* PCC 7938 (Anaabaena) were kindly provided by the Institute Pasteur Collection (Paris, France). *Chlorella sorokiniana* SAG 211-8k, *Kirchneriella contorta* SAG 11.81, *Monoraphidium griffithii* SAG 202-13, *Chlamydomonas reinhardtii* SAG 77.81, *Desmodesmus armatus* SAG 276-4e and *Ankistrodesmus falcatus* SAG 202-9 were all kindly provided by the SAG Culture Collection of Algae (Göttingen, Germany).

Cells were pre-cultured under axenic conditions in 500 ml Erlenmeyer flasks with 100 ml culture volume on a rotary shaker at 100 rpm, in nutrient-rich BG-11 medium (Rippka et al., 1979) at 20°C under continuous illumination of 30 μmol photons m$^{-2}$ s$^{-1}$ provided by white fluorescent tubes (TL 34 lamps, Philips, Eindhoven). Inocula for the experiments were taken from the exponential growth phase. Axenic conditions were confirmed by microscopic examination and flow cytometry analysis of the cultures.

**Experiment 1: Sensitivity of cyanobacteria and green algae to H$_2$O$_2$**

In Experiment 1, we tested whether cyanobacteria and green algae would differ in their sensitivity to H$_2$O$_2$ and H$_2$O$_2$ degradation capacity. First, we exposed monocultures of the cyanobacteria *Microcystis*, *Anabaena* and *Planktothrix* to different H$_2$O$_2$ concentrations. For each species, 12-well plates (Corning, Kennebunk, USA) were inoculated with 4.3 ml aliquots from pre-cultures with similar biovolumes, to which 0.2 ml of H$_2$O$_2$ was added at different concentrations. Population biovolumes at the start of the experiment were 0.60 ± 0.05 mm$^3$ mL$^{-1}$ (mean ± standard error, n = 12). H$_2$O$_2$ was added to the well plates with cyanobacteria to obtain final H$_2$O$_2$ concentrations of 0 (control), 1, 2, 3.5, 5, 8 and 12 mg L$^{-1}$.

Subsequently, well plates with monocultures of the green algae *Chlorella*, *Kirchneriella*, *Monoraphidium*, *Chlamydomonas*, *Desmodesmus* and *Ankistrodesmus* with initial population biovolumes of 0.58 ± 0.03 mm$^3$ mL$^{-1}$ (mean ± standard error, n = 23) were exposed to different H$_2$O$_2$ concentrations. Because previous studies indicated that green algae are less sensitive to H$_2$O$_2$ than cyanobacteria (Barroin and Feuillade, 1986; Drábková et al., 2007a; Matthijs et al., 2012; Weenink et al., 2015), we applied 10-fold higher final H$_2$O$_2$ concentrations of 0, 12, 20, 35, 50, 75 and 100 mg L$^{-1}$ to the wells with green algae. All treatments were performed with six replicates.

Well plates were put on top of a laboratory bench at room temperature (~20°C), away from direct sunlight and evenly illuminated by fluorescent tubes (TL5-49 W, Philips) at ~10 μmol photons m$^{-2}$ s$^{-1}$. The wells were carefully mixed by rotary movement with a clean spatula once every hour, except during night-time. Samples (300 μl) from three replicates were taken at t = 0, 1, 2, 3, 4, 5 and 24 h after H$_2$O$_2$ addition for analysis of the H$_2$O$_2$ concentration. Simultaneously, photosynthetic yield in the other three replicates was measured by pulse-amplitude modulation (PAM) fluorometry.

**Experiment 2: Interspecific protection against H$_2$O$_2$ addition**

In Experiment 2, we investigated the response of mixtures of *Microcystis* and *Chlorella* to different H$_2$O$_2$ concentrations. The experiment was performed in 50 ml incubation flasks and consisted of five different treatments:

1. *Microcystis* monocultures (5 ml preculture of *Microcystis* and 20 ml BG-11 medium),
2. *Chlorella* monocultures (5 ml preculture of *Chlorella* and 20 ml BG-11 medium),
3. mixtures of *Microcystis* and *Chlorella* (5 ml preculture of *Microcystis*, 5 ml preculture of *Chlorella* and 15 ml BG-11 medium),
4. mixtures of *Microcystis* with spent medium of *Chlorella* (5 ml preculture of *Microcystis*, 5 ml spent medium of *Chlorella* and 15 ml BG-11 medium),
5. mixtures of *Microcystis* with the lysate of *Chlorella* cells (5 ml preculture of *Microcystis*, 5 ml lysate of *Chlorella* and 15 ml BG-11 medium).

At the start of the experiment, 1 ml of diluted H$_2$O$_2$ was added to the incubation flasks to obtain H$_2$O$_2$ concentrations of 0 (control), 2.5, 5, 10, 15 and 20 mg L$^{-1}$. Population densities in the incubation flasks at the start of the experiment were 8.0 × 10$^6$ cells mL$^{-1}$ for *Microcystis* and 4.2 × 10$^5$ cells mL$^{-1}$ for *Chlorella*.

Incubation flasks were put on top of a laboratory bench at room temperature and mixed twice daily, as described above. Samples were taken prior to H$_2$O$_2$ addition (t = 0 h) and at t = 1 and 2 h after H$_2$O$_2$ addition for analysis of the H$_2$O$_2$ concentration. Population densities were counted 4 days after H$_2$O$_2$ addition.

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For the preparation of spent medium of Chlorella, 5 ml preculture of Chlorella and 15 ml BG-11 medium were loaded in 50 ml Falcon tubes and centrifuged at 4000 rpm at room temperature in a swing-out rotor (Rotanta 460R; Hettich, Tuttingen, Germany) for 15 min. The supernatant was separated from the pellet and 5 ml preculture of Microcystis was added to the supernatant to obtain mixtures of Microcystis with spent medium of Chlorella (treatment 4).

The pellets were harvested for disruption to obtain cellular lysate of Chlorella. For this purpose, pellets were transferred to 2 ml microcentrifuge tubes pre-loaded with 0.3 g of 0.1 mm zirconia/silica acid-washed beads, to which we added 1 ml of lysis buffer consisting of Tricine-NaOH pH 7.4, 10 mM MgCl\textsubscript{2}, 5 mM NaCl, 10 mM NaK phosphate buffer pH 7.4 and 1 mg of the protease inhibitor phenylmethylsulfonyl fluoride (all final concentrations). Cell preparations were disrupted in a mini beadbeater-8 (Biospec Products, Bartlesville, USA) during three alternating cycles of 1 min agitation and 2 min cooling. During the cooling phase, cells were kept at 4°C in a melting ice bath to avoid denaturation of enzymes. Subsequently, the supernatant was separated from the cellular debris and beads by centrifugation in a 4°C pre-cooled Eppendorf Microfuge for 1 min at 12 000g. The supernatant served as the crude extract from which dilute cellular lysate was made by adding BG-11 to a final volume of 5 ml. This cellular lysate was combined with 5 ml preculture of Microcystis and 15 ml BG-11 medium to obtain mixtures of Microcystis with cellular lysate of Chlorella (treatment 5).

**Experiment 3: Density dependence of interspecific protection**

In Experiment 3, we investigated whether the survival of Microcystis exposed to H\textsubscript{2}O\textsubscript{2} depends on the population density of Chlorella. First, we measured H\textsubscript{2}O\textsubscript{2} degradation by Chlorella monocultures at three different population densities: low Chlorella density (0.8 × 10\textsuperscript{6} cells ml\textsuperscript{-1}), intermediate Chlorella density (2.7 × 10\textsuperscript{6} cells ml\textsuperscript{-1}) and high Chlorella density (6.7 × 10\textsuperscript{6} cells ml\textsuperscript{-1}). The Chlorella monocultures were treated with different H\textsubscript{2}O\textsubscript{2} additions (10, 20 and 40 mg L\textsuperscript{-1}), in triplicate. The H\textsubscript{2}O\textsubscript{2} concentration was measured at t = 0, 1, 2, 3, 4 and 5 h after H\textsubscript{2}O\textsubscript{2} addition.

Next, we investigated mixtures of Microcystis and Chlorella, consisting of 13.6 × 10\textsuperscript{6} cells ml\textsuperscript{-1} of Microcystis and the three above-mentioned Chlorella densities, following the same procedures as in Experiment 2. These mixtures were exposed to six different H\textsubscript{2}O\textsubscript{2} concentrations (0, 5, 10, 15, 20 and 40 mg L\textsuperscript{-1}). After 5 days, population densities of Microcystis and Chlorella were counted.

**Experiment 4: H\textsubscript{2}O\textsubscript{2} degradation by spent medium and cellular lysate**

In Experiment 4 we investigated whether spent medium or cellular lysate of the organisms could degrade H\textsubscript{2}O\textsubscript{2}, e.g. by extracellular enzymes released in the spent medium or intracellular enzymes in the lysate. For this purpose, we prepared spent medium and cellular lysate of Chlorella monocultures and Microcystis monocultures, following the procedures described for Experiment 2. Population densities remaining in the spent medium were 0.8 × 10\textsuperscript{6} cells ml\textsuperscript{-1} for Microcystis and 0.2 × 10\textsuperscript{6} cells ml\textsuperscript{-1} for Chlorella, which is <10% of the population densities in the original samples. The cellular lysates did not contain intact cells, as confirmed by flow cytometry. At the start of the experiment, H\textsubscript{2}O\textsubscript{2} was added to the incubation flasks at six different initial concentrations (0, 2.5, 5, 10, 15 and 20 mg L\textsuperscript{-1}), and subsequent changes in H\textsubscript{2}O\textsubscript{2} concentration were monitored.

**H\textsubscript{2}O\textsubscript{2} analysis, cell counts and biovolumes**

In all laboratory experiments, H\textsubscript{2}O\textsubscript{2} concentrations were measured in triplicate using p-nitrophenyl boronic acid as a reagent according to Lu et al. (2011). The H\textsubscript{2}O\textsubscript{2} dependent formation of di-nitrophenol was quantified by the absorbance at 405 nm measured with a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Biovolumes were quantified with a Casy 1 TTT cell counter (OLS OMNI Life Science, Bremen, Germany) with a 60 μm capillary.

A BD Accuri C6 flow cytometer (BD Biosciences, San Jose, USA) was used to distinguish Microcystis and Chlorella cells and count their population densities. The flow cytometer was equipped with a blue argon laser (488 nm) with green (533 nm, FL1), orange (585 nm, FL2) and red diode lasers (640 nm) with red fluorescence detectors, a red diode laser (640 nm) with red fluorescence detector (675 nm, FL4), and photodiodes for forward scatter (FSC) and side scatter (SSC). A gating strategy was applied by plotting FSC versus log FL4 to distinguish cells from noise due to cellular debris. Subsequently, plots of FSC versus log FL3 were used to discriminate between the two species.

**Photosynthetic yield**

The photosynthetic yield was used as a proxy for the vitality of phytoplankton and was determined with a portable Mini-PAM-2 fluorometer according to the manufacturer’s instructions (Walz, Effeltrich, Germany). The 12-well plates containing phytoplankton cultures were dark adapted for 10 min before the photosynthetic yield was measured with the sensor of the Mini-PAM-2.
flourescence in the dark following a saturating light pulse (Maxwell and Johnson, 2000).

Lake treatments

To investigate how the presence of green algae affected the mitigation of harmful cyanobacterial blooms in lakes, we compared H2O2 treatments in two lakes in The Netherlands. The lake treatments followed the methods and procedures described in Matthijs et al. (2012). Lake Oosterduinse Meer (52° 16’ 55” N, 4° 30’ 28” E) had a surface area of 0.3 km² and an average depth of 7 m. This lake was treated on 16 June 2016 with ~5 mg L⁻¹ H2O2 to suppress the potentially toxin-producing cyanobacterium *Aphanizomenon flos-aquae*. In Lake Krabbeplas (51° 54’ 53” N, 4° 18’ 10” E), an isolated partition with a surface area of 0.03 km² and an average depth of 1.75 m was treated on 28 June 2016 with ~4.6 mg L⁻¹ H2O2 to suppress the potentially toxin-producing cyanobacteria *Pseudanabaena limnetica, Planktothrix agardhii* and *Coelomorom pusillum*. Both lakes had been closed for recreation by the local water management due to public health risks. During and after the treatment, H2O2 concentrations in the lakes were measured at multiple time points using Quantofix indicator sticks (Macherey-Nagel, Düren, Germany).

Phytoplankton was sampled from the top 30 cm of the lake, from 3 days prior up to 7 days after the H2O2 treatment in Lake Oosterduinse Meer, and from the day of treatment up to 9 days after the H2O2 treatment in Lake Krabbeplas. Phytoplankton samples were preserved with 0.4% Lugol’s iodine solution and stored in the dark at 4°C until microscopic analysis. Phytoplankton was identified to species level when possible and counted with an inverted microscope (Zeiss IM35, Oberkochen, Germany) using a 1 ml counting chamber following the Utermöhl method (Utermöhl, 1958). Biovolumes of the phytoplankton were calculated from cell numbers and cellular geometry according to Hillebrand et al. (1999).

Acknowledgements

We dedicate this manuscript to our late colleague and co-author Dr. Hans C.P. Matthijs, who inspired and initiated this study. This research was supported by two grants of the Dutch Research Council (NWO), grant number NWO 871 15020 and grant number NWO/TTW 14005. We thank Corné van Teulingen and Pieter Slot for their valuable assistance with Experiments 1 and 2, and Bas van Beusekom for his help with the flow cytometer analyses. We are most grateful to Johan Oosterbaan (water board Rijnland), Rob Hoefnagel and Ronald Bakkum (water board Delfland), and Geert Wijn, Jasper Amtz, Renée Talens, Reijer Hoijtink, Erik te Poele, Henk de Wilde and Bas Klein Goldewijk (ARCADIS Netherlands) for their contribution during the lake treatments. We thank AQUON for phytoplankton analysis of Lake Krabbeplas. We thank the anonymous referees for their thoughtful comments, which helped us to further improve the manuscript.

Author Contributions

E.F.J.W., H.C.P.M., P.M.V. and J.H. designed the study. E.F.J.W. performed the lab experiments with technical support by H.C.P.M., J.M.S. and C.A.M.S. E.F.J.W., T.P. and P.M.V. performed the sampling and measurements during the lake treatments. M.J.V.H. identified and quantified the phytoplankton in samples of Lake Oosterduinse Meer. E.F.J.W. and J.H. wrote the manuscript, and all authors except our late colleague H.C.P.M. commented on the final version.

References


Green algae protect cyanobacteria against H$_2$O$_2$  2419


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Minimum fluorescence (F$_{m}$) and maximum fluorescence (F$_{n}$) in monocultures of three species of freshwater cyanobacteria (Microcystis, Anabaena and Planktothrix) after addition of different H$_2$O$_2$ concentrations. Data show mean ± standard deviation (n = 3 per data point).

Fig. S2. Minimum fluorescence (F$_{m}$) and maximum fluorescence (F$_{n}$) in monocultures of six species of freshwater green algae (Chlorella, Desmodesmus, Kirchneriella, Ankistrodesmus, Monoraphidium and Chlamydomonas) after addition of different H$_2$O$_2$ concentrations. Data show mean ± standard deviation (n = 3 per data point).

Fig. S3. H$_2$O$_2$ degradation by the lysate and spent medium of both Microcystis and Planktothrix is slow in the absence of intact cells of Microcystis (Chlorella, Desmodesmus, Kirchneriella, Ankistrodesmus, Monoraphidium and Chlamydomonas) after addition of different H$_2$O$_2$ concentrations.

Table S1. Phytoplankton community before and after H$_2$O$_2$ treatment of Lake Oosterduinse Meer on 16 June 2016. Samples were collected 3 days prior to H$_2$O$_2$ addition, and after 1, 2, 4, and 7 days. At the treatment day, two samples were taken: one sample one hour before the H$_2$O$_2$ addition and another sample five hours after all H$_2$O$_2$ was added.

Table S2. Phytoplankton community before and after H$_2$O$_2$ treatment of Lake Krabbeplas on 28 June 2016. Samples were collected one hour before H$_2$O$_2$ addition started on the treatment day, and after 1, 2, 6, 7 and 9 days.