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Sensitivity to hydrogen peroxide of the bloom-forming cyanobacterium *Microcystis* PCC 7806 depends on nutrient availability

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ABSTRACT

Application of low concentrations of hydrogen peroxide (H₂O₂) is a relatively new and promising method to selectively suppress harmful cyanobacterial blooms, while minimizing effects on eukaryotic organisms. However, it is still unknown how nutrient limitation affects the sensitivity of cyanobacteria to H₂O₂. In this study, we compare effects of H₂O₂ on the microcystin-producing cyanobacterium *Microcystis* PCC 7806 under light-limited but nutrient-replete conditions, nitrogen (N) limitation and phosphorus (P) limitation. *Microcystis* was first grown in chemostats to acclimate to these different experimental conditions, and subsequently transferred to batch cultures where they were treated with a range of H₂O₂ concentrations (0-10 mg L⁻¹) while exposed to high light (100 µmol photons m⁻² s⁻¹) or low light (15 µmol photons m⁻² s⁻¹). Our results show that, at low light, N- and P-limited *Microcystis* were less sensitive to H₂O₂ than light-limited but nutrient-replete *Microcystis*. A significantly higher expression of the genes encoding for anti-oxidative stress enzymes (2-cys-peroxiredoxin, thioredoxin A and type II peroxiredoxin) was observed prior to and after the H₂O₂ treatment for both N- and P-limited *Microcystis*, which may explain their increased resistance against H₂O₂. At high light, *Microcystis* was more sensitive to H₂O₂ than at low light, and differences in the decline of the photosynthetic yield between nutrient-replete and nutrient-limited *Microcystis* exposed to H₂O₂ were less pronounced. Leakage of microcystin was stronger and faster from nutrient-replete than from N- and P-limited *Microcystis*. Overall, this study provides insight in the sensitivity of harmful cyanobacteria to H₂O₂ under various environmental conditions.

1. Introduction

Harmful cyanobacterial blooms threaten the water quality of drinking water reservoirs and recreational waters, and can cause severe ecological and economic damage (Qin et al., 2010; Michalak et al., 2013; Huisman et al., 2018). Freshwater cyanobacteria can produce a variety of toxins which may lead to poisonings in birds and mammals, including humans (Carmichael, 2001; Codd et al., 2005). One of the most ubiquitous cyanotoxins is the hepatotoxin microcystin (MC), which is produced in many variants by a wide range of bloom-forming cyanobacteria (Meriluoto et al., 2017). Many cyanobacterial species grow well in nutrient-rich waters and high temperatures, and the expansion of cyanobacterial blooms in lakes and reservoirs across the globe is therefore commonly attributed to eutrophication and climate change (Paerl and Huisman, 2008; O’Neil et al., 2012; Visser et al., 2016a; Ho et al., 2019). Biomass production of cyanobacterial blooms is often limited by phosphorus (P) and/or nitrogen (N) (Paerl, 2009) or, in highly eutrophied conditions, by light (Huisman et al., 1999; Verspagen et al., 2014).

Reduction of nutrient inputs into lakes is widely considered to be the most sustainable method to suppress cyanobacterial blooms (Fastner et al., 2016) and therefore preferred by water managers. However, effective nutrient reduction is often not feasible in a short time span. For example, nutrients often enter the water body via non-point sources or are internally released from the sediment, which hampers nutrient reduction efforts (Hamilton et al. 2016). Consequently, there is an increased interest in other methods to prevent, control or mitigate cyanobacterial blooms (Guo et al., 2007; Bullerjahn et al., 2016). Various methods to suppress cyanobacterial blooms have been developed over the last decades, each with their own advantages and
disadvantages (Ibelings et al., 2016; Matthijs et al., 2016). Examples of these methods are the use of phosphate-adsorptive materials (Douglas et al., 2016), biomanipulation with fish or mussels (Triest et al., 2016), use of water hyacinths (Qin et al., 2016), or the addition of chemical compounds such as copper sulfate or hydrogen peroxide (H₂O₂) (Jančula and Maršálek, 2011; Matthijs et al., 2016). In comparison to other chemical methods, the use of H₂O₂ to suppress cyanobacterial blooms has several advantages: H₂O₂ can selectively suppress cyanobacteria, it degrades rapidly to water and oxygen without leaving chemical traces in the environment, and it comes at a low economic cost compared to many other methods (Matthijs et al., 2016).

Application of low concentrations of 2-10 mg L⁻¹ H₂O₂ generates sufficient oxidative stress to suppress cyanobacterial blooms, while eukaryotic algae such as green algae and diatoms are much less affected (Barroin and Feuillade, 1986; Drábková et al. 2007a; Matthijs et al., 2012; Weenink et al., 2015). This difference in sensitivity to oxidative stress between cyanobacteria and eukaryotic algae is thought to originate from differences in photosynthesis when cells are exposed to excess light energy. While chloroplasts of eukaryotic organisms produce H₂O₂ in the Mehler reaction, cyanobacteria use a different ‘Mehler-like’ reaction in which the flavodiiron proteins Flv1 and Flv3 produce water directly without H₂O₂ formation (Helman et al., 2003; Allahverdiyeva et al., 2015). Consequently, cyanobacteria do not have to deal with similar levels of intracellular H₂O₂ as eukaryotic organisms. Indeed, many cyanobacteria, including Microcystis, lack major antioxidant enzymes such as catalases which are able to rapidly degrade large quantities of H₂O₂ (Bernroitner et al., 2009; Dietz et al., 2011; Schuurmans et al., 2018), although they still possess peroxiredoxins and peroxidases to degrade low levels of H₂O₂. These physiological differences may explain why cyanobacteria have a lower defense mechanism against H₂O₂ compared to eukaryotic organisms and as a result are more sensitive to H₂O₂.

For effective suppression of cyanobacteria, it is important to know which environmental factors affect the sensitivity of the cyanobacteria to H₂O₂. Several studies have shown that high light strongly increases the H₂O₂ sensitivity of cyanobacterial cells (e.g. Drábková et al., 2007b; Mikula et al., 2012; Piel et al., 2020). This is likely caused by direct damage of H₂O₂ to photosystem II (PSII) and interference of H₂O₂ with the PSII repair mechanism (Nishiyama et al., 2006; Kojima et al., 2007; Blot et al., 2011; Wang et al., 2015), making cyanobacteria much more sensitive to photoinhibition. Whether nutrient availability also affects the sensitivity of cyanobacteria to H₂O₂ is not yet known. On the one hand, heterotrophic bacteria are often more resistant to oxidative stress under nutrient starvation (e.g., McDougald et al., 2002). On the other hand, nutrient limitation causes oxidative stress in phototrophic organisms including cyanobacteria, if they cannot process the excess light energy absorbed by photosynthesis (Kumar Saha et al., 2003; Schwarz and Forchhammer, 2005; Latifi et al., 2009). Therefore, one might expect that cyanobacteria will be more sensitive to H₂O₂ under nutrient-limited than under nutrient-replete conditions. Although cyanobacterial blooms often occur in lakes with high nitrogen (N) and phosphorus (P) loads, nutrients can temporarily limit the growth of cyanobacterial blooms (Davis et al., 2009; Xu et al., 2010). Hence, investigating effects of nutrient limitation on the H₂O₂ sensitivity of cyanobacteria is very relevant, not only for an improved fundamental understanding of the response of cyanobacteria to oxidative stress but also for practical applications such as the use of H₂O₂ in lake treatments.

In this study, we investigate how nutrient limitation affects oxidative stress in Microcystis, a harmful cyanobacterium that forms dense...
blooms in many eutrophic lakes across the globe (Harke et al., 2016; Huisman et al., 2018). In particular, we investigate the hypothesis that Microctis grown under N or P limitation will be more sensitive to H2O2 than Microcystis grown under nutrient-replete conditions. For this purpose, Microcystis aeruginosa PCC 7806 (from now on referred to as Microcystis) was cultured in chemostats under light-limited but nutrient-replete, N-limited or P-limited conditions (Fig. 1). Samples of the chemostats were transferred to batch cultures where they were treated with a range of H2O2 concentrations and exposed to low or high light intensities. Sensitivity to H2O2 of these batch cultures was assessed by measuring the photosynthetic vitality, which is defined as the photosynthetic yield of a treated culture expressed as percentage of the photosynthetic yield of an untreated control culture (Matthijs et al., 2012; Piel et al., 2020). Furthermore, we measured intra- and extracellular MC concentrations as well as changes in the expression of genes involved in oxidative stress responses, photoprotection and microcystin production. The results of this study will show whether the effectiveness of H2O2 treatments of cyanobacterial blooms may depend on the nutrient status of lakes.

2. Materials and methods

2.1. Chemostat cultures

The axenic strain Microcystis aeruginosa PCC 7806 was kindly provided by the Pasteur Culture Collection (Paris, France). The Microcystis strain was cultured in chemostats optimized for phytoplankton growth (Huisman et al., 2002) to enable acclimation of the cells to stable growth conditions before the batch experiments with H2O2 were conducted (Fig. 1). All chemostats consisted of flat vessels with an optical path length of 5 cm and an effective working volume of ~1.8 L. Nutrient and light conditions were adjusted to obtain three different limiting factors: light, nitrogen (N) and phosphorus (P) limitation (Table 1). For each of these three limitations, Microcystis was grown in 4 chemostats to ensure enough culture material was available for the batch experiments. One of the chemostat cultures collapsed, and in the end we therefore had only 3 biological replicates (chemostats) for N limitation. All chemostats were supplied with BG-11 mineral medium (Rippka et al., 1979). Light-limited but nutrient-replete chemostats were supplied with full BG-11 medium containing 10 mmol L−1 NaNO3 and 230 µmol L−1 K2HPO4, N-limited chemostats were supplied with a reduced NaNO3 concentration of 0.40 mmol L−1 and P-limited chemostats were supplied with a reduced K2HPO4 concentration of 17 µmol L−1.

Light was supplied to the chemostats from one side by white fluorescent tubes (Philips Master TL-D 90 De Luxe 18 W/865, Philips Lighting, Eindhoven, The Netherlands). The incident light intensity was set by grey neutral density filters (LEE filters, Andover, UK). Light-limited chemostats were exposed to 50 µmol photons m−2 s−1 while N- and P-limited chemostats were exposed to 15 µmol photons m−2 s−1 (Table 1). While Microcystis in lakes can be exposed to much higher light intensities at the water surface, scum layer formation in lakes creates self-shading, lowering the light intensity experienced by the cells. The applied light intensities and nutrient concentrations of the mineral medium ensured that the biovolumes in steady state of the light-, N- and P-limited chemostats were similar, thereby avoiding effects caused by large differences in biovolume on the sensitivity of cells to H2O2.

The dilution rate of all chemostats was set at 0.01 h−1, and hence at steady state the specific growth rate was identical for all applied conditions. The temperature in all chemostats was maintained at 25°C for optimal growth of Microcystis, using stainless steel cooling fingers connected to a water bath (Haake A28F/AC200; Thermo Fisher Scientific, Pittsburgh, PA, USA). This temperature is commonly found in surface waters during summer, when cyanobacteria proliferate in lakes (Verspagen et al., 2006; Diao et al., 2017). All chemostats were bubbled with CO2-enriched air at a flow rate of 25 L h−1. The CO2 concentration in the pressurized air was adjusted to 400 ppm CO2 as described before (Sandrini et al., 2015). The gas mixture was moistened with water at 25°C to suppress evaporation and led through a 0.20 µm Midisart 2000 filter (Sartorius Stedim Biotech GmbH, Göttlingen, Germany) to sterilize the air before entering the chemostats. The CO2 concentration in the gas mixture was checked regularly with an Environmental Gas Monitor for CO2 (EGM-4, PP Systems, Amesbury, MA, USA).

Incident irradiance (Iin) and the irradiance penetrating through the chemostat vessel (Iw) were measured almost every day with a LI-COR LI-250 quantum photometer (LI-COR Biosciences, Lincoln, NE, USA) at ten positions on the front and back surface of the chemostat vessels, respectively. The chemostats were sampled 2-3 times per week. Cell numbers, biovolumes and average cell size in samples were determined in triplicate using a Casy 1 TCC cell counter (Schärer System GmbH, Reutlingen, Germany) with a 60 µm capillary. The pH of the samples was measured immediately after sample collection with a Lab 860 pH meter in combination with a BlueLine 28 Gel pH electrode (SCHOTT Instruments GmbH, Mainz, Germany). For the analysis of dissolved inorganic nutrients, samples were filtered over 47 mm GF/C glass filters (Whatman GmbH, Dassel, Germany). The filtrates were subsequently filtered over 0.2 µm pore size 25 mm Whatman polycarbonate membrane filters (GE Healthcare, Buckinghamshire, UK) and stored in plastic 15 mL tubes at -20°C until further analysis. Concentrations of dissolved nitrate, nitrite, ammonium and phosphate were measured using a San+ + Automated Wet Chemistry Analyzer (Skalar Analytical B.V., Breda, The Netherlands).

Regular microscopic inspections of chemostat samples did not reveal contamination with other photosynthetic organisms. Although all chemostats were started with axenic cultures of Microcystis, we could not prevent contamination by heterotrophic bacteria during the chemostat experiments. The number of heterotrophic bacteria in the cultures remained low (< <5% of the total cell counts) until the end of the experiments.

2.2. H2O2 sensitivity tests in batch cultures

To investigate the H2O2 sensitivity of Microcystis acclimated to the three different limiting factors, samples of 200-300 mL were taken from each of the chemostats as soon as a steady state (i.e., a stable population density) was reached. Samples from replicate chemostats with the same limiting factor were mixed and subsequently sterile BG-11 mineral medium was added to reach 3 L stock cultures with a final Microcystis biovolume of 100 mm3 L−1. We added full BG-11 medium for the light-limited stock culture, BG-11 with 10 mmol L−1 NaCl instead of NaNO3 and 230 µmol L−1 K2HPO4 for the N-limited stock culture, and BG-11 with 10 mmol L−1 NaNO3 but no K2HPO4 for the P-limited stock culture. These stock cultures were used for batch culture experiments in which the cells were exposed to different H2O2 concentrations (Fig. 1).

The batch culture experiments were carried out in Nalgene plastic
bottles (Thermo Fisher Scientific, Waltham, MA, USA) of 250 mL without lids. Each bottle was filled with 200 mL of stock culture. The temperature was maintained at 25°C using stainless steel cooling fingers connected to a water bath and aeration of the batch cultures was provided with compressed air. H₂O₂ was added to the cultures to final concentrations of 0, 1, 2, 4, 6 and 10 mg L⁻¹. The batch cultures were run in duplicate for each H₂O₂ concentration, resulting in a total of 12 batch cultures per limiting factor. As controls, H₂O₂ degradation without Microcystis cells was investigated by adding a final concentration of 10 mg L⁻¹ H₂O₂ to BG-11 medium and to Milli-Q water in Nalgene bottles, also both in duplicate.

The H₂O₂ sensitivity of Microcystis was investigated during exposure of the batch cultures to a low light intensity of 15 µmol photons m⁻² s⁻¹, and two weeks later the whole experiment was repeated using a higher light intensity of 100 µmol photons m⁻² s⁻¹ (Fig. 1). These two light intensities are representative of the range of light levels commonly found in the upper 0.5-2 meters of turbid lakes dominated by cyanobacterial blooms (Huismann et al., 2004; Verspagen et al., 2006). Light was provided by the same fluorescent tubes as for the chemostats, using grey neutral density filters to adjust the light intensity. The time interval between the low light and high light experiments was two weeks to ensure recovery of the chemostats to steady state after sampling.

In order to analyze the photosynthetic vitality and H₂O₂ concentration, 5 mL samples were taken from each batch culture just before adding H₂O₂, immediately after addition, then every half-hour after addition of H₂O₂ for the first 4 hours, and after 24 hours. MC was analyzed (as described in section 2.6) in samples taken before the addition of H₂O₂ as well as 1, 2, 4 and 24 hours after H₂O₂ addition. In addition, for biovolume analysis with the Casy cell counter (as described in section 2.1), a 1 mL sample of each batch culture was taken just before and 24 h after H₂O₂ addition.

2.3. Photosynthetic vitality

The photosynthetic vitality was determined as described by Piel et al. (2020). Microcystis cells from the 5 mL samples were collected on 25 mm GF/C filters (Whatman GmbH, Dassel, Germany) by filtration on a Millipore 1225 Sampling Manifold (Merck KGaA, Darmstadt Germany). Immediately after filtration, the cells were dark adapted for 5 minutes by covering the filters with rubber stoppers on the Millipore filtration unit. Subsequently, the rubber stoppers covering the filters were exchanged with a rubber stopper connected to a Mini-PAM fluorometer (Walz, Effeltrich, Germany), and the minimum fluorescence (F₀) and maximum fluorescence (Fₘ) were measured before and after a saturating light pulse, respectively. The maximum photosynthetic yield of dark adapted cells (Fₘ/F₀ also known as the maximum quantum yield of PSII electron transport) was calculated according to Maxwell and Johnson (2000):

\[
Fₘ/F₀ = (Fₘ - F₀)/Fₘ
\]

Photosynthetic vitality is defined as the photosynthetic yield of the H₂O₂-treated samples as percentage of the photosynthetic yield of the untreated control samples.

2.4. Dose-response models

Photosynthetic vitality at 4 hours after addition of H₂O₂ was described as a function PV of the added H₂O₂ concentration according to a logistic dose-response model (Piel et al., 2020):

\[
PV = \frac{C}{1 + e^{B(\text{EC}_{50} - \text{H}_2\text{O}_2) - \text{H}_2\text{O}_2)}}
\]

where A is the estimated EC₅₀ value (i.e. the H₂O₂ concentration at which PV is 50%), B is the exponential slope of the dose-response model, and C is the photosynthetic vitality of the control samples that were not exposed to H₂O₂ (i.e., C was set to 100%). The parameters A and B were estimated by fitting the dose-response model to the photosynthetic vitality data using nonlinear least-squares regression.

2.5. Hydrogen peroxide measurements

H₂O₂ concentrations were measured in filtrates of the 5 mL samples. Subsamples of 100 µL from these filtrates were immediately transferred to a 96-well microtiter plate and mixed with 100 µL of 2 mmol L⁻¹ p-nitrophenylboronic acid reagent (Merck KGaA, Darmstadt, Germany), according to Lu et al. (2011). After reacting with H₂O₂, the formation of the color reagent p-nitrophenolate at room temperature was complete in 30 to 45 min, and the color remained stable for several hours. For the quantification of the reagent, absorption of p-nitrophenolate was measured at its absorption peak (405 nm) using a SPECTROstar Nano plate reader (BMG Labtech GmbH, Ortenberg, Germany). A 33% (w/w) stock solution (VWR, Amsterdam, The Netherlands) was used to make a calibration curve (0.01-10 mg L⁻¹ H₂O₂) that was included on each microtiter plate. For each filtrate, the H₂O₂ concentration was measured in quadruplicate. Pilot experiments showed that losses of H₂O₂ by the filtration procedure were <1%.

2.6. Microcystin analysis

To assess the intracellular unbound MC content and the extracellular MC in the water phase, cells on the filters and the filtrates were stored at -20°C and subsequently freeze-dried. MC was extracted with 75% MeOH and analyzed with HPLC according to Van de Waal et al. (2011), using a Shimadzu LC-20AD HPLC system with a SPD-M20A photodiode array detector (Shimadzu, Kyoto, Japan). The two MC variants, [Asp³]MC-LR and MC-LR (Tonk et al., 2009), produced by Microcystis PCC 7806 were summed as separation of both peaks was not possible. The sum of the two variants is hereafter referred to as MC. Each sample was analyzed in duplicate. A heatmap to summarize the toxin data was made with R (version 3.6.2) using the heatmap.2 function of the package gplots (version 3.0.3) (www.R-project.org).

2.7. Gene expression analysis

Gene expression was analyzed with reverse transcription quantitative PCR (RT-qPCR), applied to a separate series of batch cultures using the same experimental design. A separate series of batch experiments was necessary, because (1) the volume of the batch cultures was too small to obtain sufficient material for both RT-qPCR and the previous measurements, and (2) it was not possible to process the RNA samples quickly enough when many other measurements needed to be done at the same time. In the gene expression experiments, batch cultures were treated with 0 mg L⁻¹ and 2 mg L⁻¹ H₂O₂ at a low light intensity of 15 µmol photons m⁻² s⁻¹. The batch cultures were run in quadruplicate for each H₂O₂ concentration, resulting in a total of 8 batch cultures per limiting factor. From each batch culture, 40 mL samples for gene expression analysis were taken just before and 2 h after the addition of H₂O₂. The 40 mL samples were centrifuged immediately in a pre-cooled Rotanta 460R centrifuge (Hettich GmbH & Co. KG, Tuttlingen, Germany) at 4°C and 2380 g for 5 minutes. Subsequently, the medium was decanted and the cells were resuspended in 1 mL TRIzol (Thermo Fisher Scientific, Waltham, MA, USA), frozen in liquid nitrogen, and stored in -80°C until further analysis.

We investigated the expression of a selected number of genes that had shown a strong response to H₂O₂ addition in the full transcriptome analysis of Schuurmans et al. (2018). Specifically, using primers designed in this study and previous studies (Sandrinii et al., 2014, Sandrinii et al., 2016a, Sandrinii et al., 2016b) (Table S1), transcripts of the following genes were targeted with RT-qPCR: 2-cys prx (peroxiredoxin), trxA (thioredoxin), G2prx (type-2 peroxiredoxin), isiA (iron stress-induced protein A), flv2 (photoprotective flavodiiron-2 protein), flv4 (photoprotective flavodiiron-4 protein), mcyB (microcystin-
synthetase, operon mcyABC), mcyE (microcystin synthetase, operon mcyDEFGHIJ) and 16S rRNA (reference gene).

RNA extraction and RT-qPCR were done as described previously (Sandrini et al., 2015). In short, RNA was extracted with TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) according to the supplier's instructions, using 0.5 mm BasingBeads (Zymo Research, Orange, CA, USA) to facilitate cell disruption. After the phase separation steps, the Direct-Zol™ RNA MiniPrep kit (Zymo Research, Orange, CA, USA) was used for RNA purification. The optional in-column DNase I digestion was included. RNA concentrations were quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and all RNA samples had A260/A280 Values above 1.7. Reverse transcription reactions were completed using the Superscript III enzyme (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the qPCR Maxima SYBR green master mix (Thermo Fisher Scientific, Waltham, MA, USA) was applied with our primers to the obtained cDNA samples to analyze PCR amplification in an ABI 7500 real-time PCR device (Applied Biosystems, Foster City, CA). Samples were analyzed in duplicate. The two-step cycling protocol was applied, with a denaturation temperature of 95°C (15 s) and a combined annealing/extension temperature of 60°C (60 s) during 40 cycles. Melting-curve analysis was performed on all samples to rule out non-specific PCR products. The passive reference dye ROX was used to correct for any well-to-well variation.

Relative changes in gene expression for all samples were calculated using the 16S rRNA gene as reference gene and batch cultures that originated from the light-limited chemostats before addition of H2O2 (t=0 h) as reference sample. The LinRegPCR software tool version 2017.1 (Ramakers et al., 2003; Ruijter et al., 2009) was used for baseline correction, calculation of quantification cycle (Cq) values and calculation of the amplification efficiency (E) of each individual run using linear regression (Table S1). Amplification efficiencies of individual samples calculated with LinRegPCR were between 1.8 and 2.0. In addition, standard curves were made using 10-fold dilutions of cDNA samples. These standard curves were used to calculate amplification efficiencies for each of the applied primer sets. Amplification efficiencies of the primer sets calculated from standard curves were between 1.92 and 2.06 (Table S1). The relative changes in gene expression were calculated with the comparative cycle threshold (Ct) method (Livak and Schmittgen, 2001). Negative controls of the qPCR and negative controls of the reverse transcriptase reaction did not result in significant amplification. Two-way ANOVA tests were applied to identify effects of the limiting factor and of the addition of H2O2 (n=4 biological replicates per treatment). Post-hoc comparisons of the means were based on Tukey's HSD test, using a significance level α of 0.05. A heatmap to summarize the gene expression data was made with R (version 3.6.2) using the heatmap.2 function of the package gplots (version 3.0.3) (www.R-project.org).

3. Results

3.1. Chemostats in steady state

At steady state, the light intensity Iout transmitted through the chemostat vessels was high in N- and P-limited chemostats, but significantly lower in light-limited chemostats (one-way ANOVA: \( F_{2,8} = 43.54, p < 0.001 \); Table 2). Dissolved inorganic nitrogen (DIN) concentrations differed between conditions, with high nitrate concentrations in P-limited and light-limited chemostats but significantly lower nitrate concentrations in N-limited chemostats (one-way ANOVA: \( F_{2,8} = 6507, p < 0.001 \); Table 2). Dissolved inorganic phosphorus (DIP) was high in light-limited and N-limited chemostats, but significantly lower in P-limited chemostats (one-way ANOVA: \( F_{2,8} = 2224, p < 0.001 \); Table 2). This confirms that the applied chemical composition of the mineral media indeed led to light-limited, N-limited and P-limited conditions.

N-limited Microcystis had a slightly but significantly larger cell diameter than light-limited and P-limited Microcystis (one-way ANOVA: \( F_{2,8} = 7.338, p = 0.015 \); Table 2). Extracellular MC concentrations (expressed per liter of culture) were significantly lower in N-limited chemostats (one-way ANOVA: \( F_{2,7} = 74.3, p = 0.001 \); Table 2). Intracellular MC contents (expressed per cell) also tended to be lower under N-limited conditions, but did not differ significantly between the three limitations (one-way ANOVA: \( F_{2,7} = 2.40, p = 0.161 \); Table 2).

3.2. Photosynthetic vitality

The nutrient and light conditions influenced the decline of the photosynthetic vitality after H2O2 addition. The photosynthetic vitality declined faster in batch cultures exposed to high light than in those exposed to low light (Fig. 2). At low light, Microcystis pre-cultured under light-limited but nutrient-replete conditions was more sensitive to H2O2 concentrations \( \geq 2 \text{mg L}^{-1} \) than N- and P-limited Microcystis (Fig. 2B-E). At high light, Microcystis was more sensitive to H2O2 than at low light, but the difference in H2O2 sensitivity between the three limiting factors was less pronounced (Fig. 2A-E). The decline in photosynthetic vitality was accompanied by strong bleaching of the cultures (see Fig. 1).

Dose-response models of the photosynthetic vitality after 4 h as function of the H2O2 concentration confirmed that nutrient limitation affected the sensitivity of Microcystis to H2O2 (Fig. 3). At low light, the estimated EC50 values ranged from 2.0 mg L\(^{-1}\) H2O2 for light-limited Microcystis to 2.9 and 3.0 mg L\(^{-1}\) H2O2 for N-limited and P-limited Microcystis, respectively (Fig. 3A; Table S2). At high light, the estimated EC50 was -1.1 mg L\(^{-1}\) H2O2 for all three limitations (Fig. 3B; Table S2).

3.3. Hydrogen peroxide degradation

H2O2 in the batch cultures disappeared within 1-1.5 h after addition of 1 mg L\(^{-1}\) H2O2 (Fig. 4A), while H2O2 degradation took longer after addition of higher H2O2 concentrations (Fig. 4B). At 4, 6 and 10 mg L\(^{-1}\) H2O2, degradation of H2O2 was faster for N-limited Microcystis than for light-limited and P-limited Microcystis (Fig. 4C,D,E). Furthermore, in N-limited cultures, H2O2 degradation was faster in low light than in high light. As control, H2O2 degradation was tested in Milli-Q water and BG-11 mineral medium without cells (Fig. S1). No H2O2 degradation was observed in Milli-Q water, while the H2O2 concentration in BG-11 mineral medium decreased only slightly, from 10 to 9.7 mg L\(^{-1}\) after four hours and to 8.8 mg L\(^{-1}\) after 24 hours.

3.4. Biovolume changes

The biomass development of Microcystis (expressed as the 24-hour change in total biovolume) differed between the experimental conditions (Fig. 5). In the control treatment (no H2O2 addition) and after addition of only 1 mg L\(^{-1}\) H2O2 the light-limited Microcystis population showed growth, whereas N-limited and P-limited Microcystis showed very little or no change in total biovolume. At low light, the biovolume of light-limited Microcystis declined after addition of \( \geq 2 \text{mg L}^{-1} \text{H}_2\text{O}_2 \) (Fig. 5A), but a higher H2O2 dosage of \( \geq 4 \text{mg L}^{-1} \text{H}_2\text{O}_2 \) was required to suppress the biovolume of N- and P-limited Microcystis (Fig. 5C,D,E). At high light, the biovolume declined for all three limiting factors after addition of \( \geq 2 \text{mg L}^{-1} \text{H}_2\text{O}_2 \) (Fig. 5B,E). Cell counts reported in Fig. S2 showed less pronounced differences between the treatments than the total biovolumes, indicating that the cells affected by H2O2 shrank in size.

3.5. Intra- and extracellular microcystins

Intracellular MC of light-limited Microcystis decreased two to four hours after addition of \( \geq 2 \text{mg L}^{-1} \text{H}_2\text{O}_2 \) (Fig. 6A,B). In contrast, intracellular MC in N- and P-limited Microcystis decreased only after more
than four hours after addition of ≥4 mg L\(^{-1}\) H\(_2\)O\(_2\) (Fig. 6C-F). Parallel to the decrease in intracellular MC, extracellular MC increased. However, the extracellular MC concentration never exceeded 79% of the intracellular MC concentration at the beginning of the experiment.

### 3.6. Gene expression of selected genes

H\(_2\)O\(_2\) addition and the imposed nutrient or light limitation both affected expression of the investigated *Microcystis* genes (Fig. 7; see Tables S3 and S4 for the underlying statistics).

Prior to H\(_2\)O\(_2\) addition, three genes encoding for anti-oxidant enzymes, 2-cys-prx (2-cys-peroxiredoxin), trxA (thioredoxin A) and t2prx (type II peroxiredoxin), were expressed significantly higher in N- and P-limited *Microcystis* than in light-limited *Microcystis* (Fig. 7; Tables S3 and S4). Expression of trxA and t2prx strongly increased after addition of H\(_2\)O\(_2\) in all cultures, with higher expression levels in N- and P-limited *Microcystis* than in light-limited *Microcystis*.

Expression of the genes flv2 and flv4, encoding for photoprotective flavodiiron proteins, was slightly but significantly higher prior to H\(_2\)O\(_2\) addition for P-limited *Microcystis* (in case of flv2) or for both N- and P-limited *Microcystis* (in case of flv4) than for light-limited *Microcystis* (Fig. 7; Tables S3 and S4). Addition of H\(_2\)O\(_2\) slightly reduced the expression of flv2 and flv4 in all cultures. Conversely, expression of the iron-stress-induced gene isA was lower prior to H\(_2\)O\(_2\) addition in N- and P-limited *Microcystis* than in light-limited *Microcystis*. Addition of H\(_2\)O\(_2\) increased the expression of isA to similar levels as in light-limited *Microcystis* prior to H\(_2\)O\(_2\) addition.

Expression of MC synthetase genes mcyB and mcyE prior to H\(_2\)O\(_2\) addition was significantly higher in the N- and P-limited cultures than in light-limited *Microcystis*. Addition of H\(_2\)O\(_2\) resulted in a reduction in expression levels of mcyB and mcyE in all cultures (Fig. 7; Tables S3 and S4).

### 4. Discussion

#### 4.1. Microcystis is more resistant to H\(_2\)O\(_2\) under nutrient limitation and low light intensity

This study aimed to investigate whether nutrient limitation of cyanobacteria is a factor that needs to be considered when treating cyanobacterial blooms in lakes with H\(_2\)O\(_2\). Since nutrient limitation is known to induce oxidative stress in cyanobacteria (Kumar Saha et al., 2003; Schwarz and Forchhammer, 2005; Latifi et al., 2009), we hypothesized that *Microcystis* grown under N or P limitation will be more sensitive to H\(_2\)O\(_2\) than *Microcystis* grown under replete conditions. In contrast to this hypothesis, our results indicate an increased resistance to H\(_2\)O\(_2\) of N- and P-limited *Microcystis* compared to light-limited but nutrient-replete *Microcystis*. This difference in H\(_2\)O\(_2\) sensitivity can most likely be explained by the observed higher expression of genes encoding for anti-oxidative stress enzymes in N- and P-limited *Microcystis* than in light-limited *Microcystis* prior to H\(_2\)O\(_2\) treatment (Fig. 7). Consequently, H\(_2\)O\(_2\) degradation by *Microcystis* was faster under N limitation than under light-limited but nutrient-replete conditions (Fig. 4). However, despite being similarly resistant to H\(_2\)O\(_2\) as N-limited *Microcystis*, P-limited *Microcystis* showed a slower H\(_2\)O\(_2\) degradation rate quite similar to that of light-limited *Microcystis*.

Our photosynthetic vitality measurements confirm previous findings that H\(_2\)O\(_2\) suppresses cyanobacteria more effectively at high light than at low light (Piel et al., 2020). H\(_2\)O\(_2\) directly damages the D1 protein of PSII (Wang et al., 2015) and also suppresses the repair mechanism of PSII (Nishiyama et al., 2006; Kojima et al., 2007; Blot et al., 2011), which likely explains why H\(_2\)O\(_2\) causes greater damage to cyanobacteria at high light intensities (Nishiyama et al., 2006; Piel et al., 2020). Consequently, sensitivity differences between light-limited and nutrient-limited *Microcystis* were minor when exposed to high oxidative stress induced by H\(_2\)O\(_2\) at high light intensity, whereas these sensitivity differences were much more pronounced at low light intensity (Figs. 2 and 3).

#### 4.2. Anti-oxidative stress genes are higher expressed in nutrient-limited *Microcystis*

Genome sequencing has revealed that many cyanobacteria lack catalase but do contain other anti-ROS enzymes such as peroxiredoxins (Bernroitter et al., 2009; Dietz et al., 2011). This also applies to sequenced genomes of *Microcystis*, including *Microcystis* NIES-843 (Kaneko et al., 2007; Bernroitter et al., 2009), *Microcystis* PCC 7005 (Sandrini et al., 2014) and *Microcystis* PCC 7806 (Franqueul et al., 2008; Schuurmans et al., 2018) and the transcriptome of *Microcystis* LE-3 (Harke and Gobler, 2013). Schuurmans et al. (2018) investigated the full transcriptome of *Microcystis* PCC 7806, and found that of all anti-ROS genes, the peroxiredoxin genes 2-cys-prx and t2prx and thioredoxin gene trxA gave the strongest response to H\(_2\)O\(_2\) addition. For this reason, we investigated the expression of these three anti-ROS genes in our study.

Our results show that gene expression of 2-cys-prx, trxA and t2prx differed between nutrient-limited and light-limited *Microcystis*. Prior to H\(_2\)O\(_2\) addition, all three genes were significantly higher expressed in N- and P-limited *Microcystis* than in light-limited *Microcystis*. This observation is consistent with previous studies, which have shown that preconditioning of cyanobacteria to N- or P-starvation results in increased oxidative stress and prompts an elevated expression of anti-oxidative stress genes (Kumar Saha et al., 2003; Schwarz and Forchhammer, 2005; Latifi et al., 2009). Specifically, in line with our...
findings, N limitation also induced an increased expression of 2-cys-prx in Synechocystis sp. PCC 6803 and Synechococcus elongatus PCC 7942 (Stork et al., 2005). Our results show that expression of 2-cys-prx, trxA and t2prx in N-limited Microcystis and of trxA and t2prx in P-limited Microcystis increased even further during H₂O₂ exposure, to levels well above those in light-limited Microcystis. In a similar experiment, Schuurmans et al. (2018) measured higher expression levels of 2-cys-prx, trxA and t2prx in a non-toxic mutant strain of Microcystis PCC 7806 than in the wildtype, and showed that the non-toxic mutant was better prepared to oxidative stress and hence more resistant to H₂O₂ than the wildtype. Along the same lines, the observed higher expression levels of anti-oxidative stress genes prior to H₂O₂ addition implies that nutrient-
limited Microcystis are better prepared for high oxidative stress, which is likely responsible for their higher resistance to H₂O₂ in comparison to limited Microcystis.

Upregulation of anti-oxidative stress genes in N- and P-limited Microcystis is more likely caused by oxidative stress due to nutrient limitation, rather than by preconditioning of these cultures to a higher light intensity. Nutrient limitation has been shown to increase internal ROS formation in cyanobacteria by affecting photosynthesis and respiration, forcing a response of anti-ROS enzymes (Kumar Saha et al., 2003; Schwarz and Forchhammer, 2005). Drábková et al. (2007b) found that preconditioning of Microcystis PCC 7806 to higher light intensities did not influence the effectiveness of H₂O₂ to suppress cyanobacteria. In addition, Mikula et al. (2012) and Lürling et al. (2014) preconditioned nutrient-replete Microcystis to light intensities of 140 μmol photons m⁻² s⁻¹ and 100 μmol photons m⁻² s⁻¹, respectively, and found a comparable H₂O₂ sensitivity as for the light-limited Microcystis of our study which was supplied with only 15 μmol photons m⁻² s⁻¹.

Expression of photoprotective genes was also investigated in our study, since they may contribute to the defense of phototrophic organisms against oxidative stress. Flavodiiron proteins encoded by the genes flv2 and flv4 are known to play an important role in the photo-protection of cyanobacteria, by dissipating excess light energy at PSII (Zhang et al., 2012; Bersanini et al., 2014). While expression of flv2 in N-limited Microcystis and flv2 and flv4 in P-limited Microcystis was slightly higher in comparison to light-limited Microcystis prior to H₂O₂ addition, both genes were downregulated after H₂O₂ addition. Furthermore, the iron stress-induced gene, isiA, which can also contribute to the protection of cyanobacteria from high light stress by dissipating excess energy (Yeremenko et al., 2004; Havaux et al., 2005), did not show a consistent response to H₂O₂ exposure. These results indicate that the photoprotective proteins Flv2, Flv4 and isiA do not have an active involvement in the defense of cyanobacteria against H₂O₂.

4.3. Microcystin dynamics

Our results show that expression of the MC synthetase genes mcyB and mcyE was strongly downregulated after H₂O₂ treatment of Microcystis. This appears to contradict the previously suggested hypothesis that MC protects Microcystis against oxidative stress when cells are exposed to H₂O₂ (Dziallas and Grossart, 2011; Zilliges et al., 2011). We note that the studies of Dziallas and Grossart (2011) and Zilliges et al. (2011) used a very low H₂O₂ dosage representative of natural H₂O₂ concentrations in lakes (0.001-0.050 mg L⁻¹; Cooper and Lean, 1989; Häkkinen et al., 2004; Cory et al., 2017). Their H₂O₂ concentrations were much lower than the 2-10 mg L⁻¹ of H₂O₂ required to suppress photosynthesis and biomass development of blooming cyanobacteria in the laboratory experiments of, e.g., Drábková et al. (2007a), Schuurmans et al. (2018), Piel et al. (2020) and this study and in the lake treatments of Matthijs et al. (2012, 2016). In fact, contrary to Zilliges et al. (2011), Schuurmans et al. (2018) showed that MC-producing strains of Microcystis were more sensitive to H₂O₂ than non-MC-producing strains. They hypothesized that MC interferes with the activity of redox-sensitive proteins including peroxiredoxins and thioredoxin and thereby prevents these anti-ROS enzymes from effectively protecting the cells against H₂O₂. Similar to Schuurmans et al. (2018), in our study mcy genes were strongly downregulated whereas genes encoding peroxiredoxin and thioredoxin were strongly upregulated after H₂O₂ addition, supporting the view that MC does not offer protection to Microcystis when exposed to high levels of oxidative stress.

During H₂O₂ treatment of Microcystis, intracellular MC leaked out of the cells resulting in a subsequent increase of extracellular MC. Oxidative stress has been shown to damage cell membrane integrity of Microcystis, allowing MC and other cellular contents to exude from the cells (Ross et al., 2006; Mikula et al., 2012; Wang et al., 2015; Piel et al., 2020). Quantitatively, however, the decrease of intracellular MC exceeded the increase of extracellular MC concentration, such that the total MC (intracellular MC + extracellular MC) declined after H₂O₂ addition. This might be attributed to MC degradation, but it is more likely that MC was binding to the thiol groups of redox-sensitive proteins under oxidative conditions (Zilliges et al., 2011) and consequently became undetectable with standard extraction techniques (Meissner et al., 2013). Schuurmans et al. (2018) found that MC bound to proteins likely get converted back to detectable “unbound” MC after several days, consistent with other observations that thiol-conjugation of MC is reversible and hence free MC can be slowly released from the protein-bound fraction (Miles et al., 2016). Extracellular MC concentrations were highest 24 hours after the treatment, with a strong tendency to be higher in samples that were treated with a lower H₂O₂ concentration. A possible explanation for this observation is that higher H₂O₂ concentrations may cause more extensive binding of MC to proteins and hamper the subsequent release of MC from the proteins.
4.4. Implications for lake treatments

Our results show that, at low light intensities, N- and P-limited *Microcystis* were more resistant to H$_2$O$_2$ than light-limited *Microcystis*, probably due to a higher expression of anti-oxidative stress genes prior and also after H$_2$O$_2$ addition. At higher light intensities, suppression of cyanobacteria by H$_2$O$_2$ was more effective (Piel et al., 2020), and differences in H$_2$O$_2$ sensitivity between nutrient-replete and nutrient-limited *Microcystis* were negligible. These results indicate that, during cloudy days or in very turbid lakes, H$_2$O$_2$ resistance of *Microcystis* will be more pronounced in nutrient-limited than in nutrient-saturated waters. Furthermore, shaded *Microcystis* cells in layers within or below dense surface scums may be less affected by H$_2$O$_2$ than cells dwelling in full sunlight at or near the surface, especially if available nutrients have been depleted by the scum layer. In our experience, deliberate mixing of the upper meters of the water column during the H$_2$O$_2$ treatment of lakes not only disperses the added H$_2$O$_2$ but also disperses the scum layer, and thereby enhances H$_2$O$_2$ exposure and brings shaded cells from deeper layers below the scums into the sunlit surface. We conclude that for the treatment of nutrient-limited lakes, H$_2$O$_2$ concentrations

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**Figure 4.** Hydrogen peroxide degradation of *Microcystis* PCC 7806 under N-limited, P-limited and light-limited conditions after addition of different H$_2$O$_2$ concentrations. (A) 1 mg L$^{-1}$ H$_2$O$_2$, (B) 2 mg L$^{-1}$ H$_2$O$_2$, (C) 4 mg L$^{-1}$ H$_2$O$_2$, (D) 6 mg L$^{-1}$ H$_2$O$_2$, (E) 10 mg L$^{-1}$ H$_2$O$_2$. Triangles indicate batch cultures exposed to low light (LL, 15 µmol photons m$^{-2}$ s$^{-1}$), while open circles indicate cultures exposed to high light (HL, 100 µmol photons m$^{-2}$ s$^{-1}$). Each data point represents the average of two biological replicates. Please note that panels (A) and (B) use a different scale for the y-axis than panels (C)-(E).
Figure 5. Relative biovolumes of Microcystis PCC 7806 under light-limited, N-limited and P-limited conditions after addition of different H$_2$O$_2$ concentrations. (A,B) Light-limited Microcystis exposed to (A) low light and (B) high light. (C,D) N-limited Microcystis exposed to (C) low light and (D) high light. (E,F) P-limited Microcystis exposed to (E) low light and (F) high light. The biovolumes after 24 h are expressed as percentage of biovolume at the start of the experiment (t = 0). Dashed black lines indicate the biovolumes of the control cultures at the start of the experiment. The bars represent the average of two biological replicates.
Figure 6. MC concentrations of *Microcystis* PCC 7806 under light-limited, N-limited and P-limited conditions after addition of different H$_2$O$_2$ concentrations. The heatmaps show the amounts of total MC, intracellular MC, and extracellular MC of (A,B) light-limited *Microcystis*, at (A) low light intensity and (B) high light intensity. (C,D) N-limited *Microcystis*, at (C) low light intensity and (D) high light intensity. (E,F) P-limited *Microcystis* at (E) low light and (F) high light intensity. The color key indicates the relative amount of MC as percentage of total MC at the start of the experiment (t = 0 h). Different rows in the heatmaps show different H$_2$O$_2$ concentrations used in the batch cultures, while columns in the heatmaps indicate different time points. The values represent the average of two biological replicates.
may need to be adjusted during cloudy weather and in turbid waters. H$_2$O$_2$ treatments of cyanobacterial blooms are thus expected to be most effective in hypertrophic waters during sunny days, which coincides with the growth conditions that are usually considered to be ideal for cyanobacterial bloom development.

Author contributions

GS, TP, PMV and JH designed the experiments. GS, TP, TX, EW and HQ performed the lab experiments, assisted by PCS. GS and TP analyzed the data. GS, TP, PMV and JH wrote the manuscript. All authors have taken part in the manuscript revisions and agreed with the scientific content.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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