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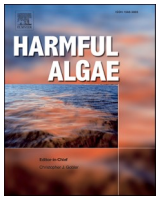

Shifts in phytoplankton and zooplankton communities in three cyanobacteria-dominated lakes after treatment with hydrogen peroxide

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ABSTRACT

Cyanobacteria can reach high densities in eutrophic lakes, which may cause problems due to their potential toxin production. Several methods are in use to prevent, control or mitigate harmful cyanobacterial blooms. Treatment of blooms with low concentrations of hydrogen peroxide (H_2O_2) is a promising emergency method. However, effects of H_2O_2 on cyanobacteria, eukaryotic phytoplankton and zooplankton have mainly been studied in controlled cultures and mesocosm experiments, while much less is known about the effectiveness and potential side effects of H_2O_2 treatments on entire lake ecosystems. In this study, we report on three different lakes in the Netherlands that were treated with average H_2O_2 concentrations ranging from 2 to 5 mg L^{-1} to suppress cyanobacterial blooms. Effects on phytoplankton and zooplankton communities, on cyanotoxin concentrations, and on nutrient availability in the lakes were assessed. After every H_2O_2 treatment, cyanobacteria drastically declined, sometimes by more than 99%, although blooms of *Dolichospermum* sp., *Aphanizomenon* sp., and *Planktothrix rubescens* were more strongly suppressed than a *Planktothrix agardhii* bloom. Eukaryotic phytoplankton were not significantly affected by the H_2O_2 additions and had an initial advantage over cyanobacteria after the treatment, when ample nutrients and light were available. In all three lakes, a new cyanobacterial bloom developed within several weeks after the first H_2O_2 treatment, and in two lakes a second H_2O_2 treatment was therefore applied to again suppress the cyanobacterial population. Rotifers strongly declined after most H_2O_2 treatments except when the H_2O_2 concentration was $\leq$ 2 mg L^{-1}, whereas cladocerans were only mildly affected and copepods were least impacted by the added H_2O_2. In response to the treatments, the cyanotoxins microcystins and anabaenopeptins were released from the cells into the water column, but disappeared after a few days. We conclude that lake treatments with low concentrations of H_2O_2 can be a successful tool to suppress harmful cyanobacterial blooms, but may negatively affect some of the zooplankton taxa in lakes. We advise pre-tests prior to the treatment of lakes to define optimal treatment concentrations that kill the majority of the cyanobacteria and to minimize potential side effects on non-target organisms. In some cases, the pre-tests may discourage treatment of the lake.

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1. Introduction

Cyanobacterial blooms appear to have increased in many freshwater bodies across the globe over the past decades (Michalak *et al.*, 2013; Taranu *et al.*, 2015; Ho *et al.*, 2019), most likely fueled by anthropogenic eutrophication and climate change (Paerl and Huisman, 2008; O'Neil *et al.*, 2012; Visser *et al.*, 2016). Cyanobacterial blooms often deteriorate 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). In particular, toxins produced by cyanobacteria (cyanotoxins) can be directly harmful to many organisms, including wildlife, pets, livestock, and humans, upon ingestion of contaminated water or can lead to indirect poisoning via the food chain (Carmichael, 2001; Codd *et al.*, 2005; Poste *et al.*, 2011; Bukaveckas *et al.*, 2017; Breinlinger *et al.*, 2021). Since cyanobacterial blooms often consist of mixtures of toxic and non-toxic strains, and seasonal population dynamics continuously alter the cyanobacterial community composition, the overall toxicity of a cyanobacterial blooms may vary strongly in time and space (Kardinaal *et al.*, 2007; Sabart *et al.*, 2010; Mantzouki *et al.*, 2018).

The most well-known cyanotoxins are microcystins, a class of cyclic heptapeptides that can be produced by many bloom-forming genera such as *Microcystis, Planktothrix, Dolichospermum, Anabaena, Gloeotrichia* and *Nostoc* (Meriluoto *et al.* 2017; Huisman *et al.*, 2018). Currently, at least 329 different microcystin (MC) variants have been identified (Meriluoto *et al.*, 2017; Bouaïcha *et al.*, 2019; Jones *et al.*, 2021; Janssen *et al.*, 2023), of which MC-LR, demethylated (dm) MC-LR, MC-RR and MC-YR are the most abundant (Faassen and Lürling, 2013; Mantzouki *et al.*, 2018). Other well-known cyanotoxins include nodularins, cylindrospermopsins, saxitoxins and anatoxins (Meriluoto *et al.*, 2017; Huisman *et al.*, 2018; Chorus and Welker, 2021), while less studied cyanopeptides include anabaenopeptins and oscillamide (Janssen, 2019; Monteiro *et al.*, 2021). This diversity of cyanotoxins can cause a wide range of toxicological effects, including liver and kidney damage caused by microcystins and nodularins as well as severe neurotoxic symptoms caused by, e.g., anatoxins-a and saxitoxins (Meriluoto *et al.*, 2017; Huisman *et al.*, 2018).

Reducing the nutrient loading of surface waters is an effective way to prevent cyanobacterial blooms (Vollenweider, 1968; Schindler, 1974; Conley *et al.*, 2009; Fastner *et al.*, 2016). However, controlling internal nutrient release from sediments (Søndergaard *et al.*, 2003) and reducing nutrient loads in catchment areas (Hamilton *et al.*, 2016) is not always easily achieved. It often takes many years before cyanobacterial blooms decline after nutrient reduction efforts (Jeppesen *et al.*, 2005). Hence, in addition to the development of long-term strategies to reduce nutrient inputs, there is a growing demand for short-term methods that can be used as an emergency measure to rapidly suppress toxic cyanobacterial blooms in recreational lakes and drinking water reservoirs, preferably with the least possible impact on the ecosystem (Ibelings *et al.*, 2016; Matthijs *et al.*, 2016; Lürling and Mucci, 2020). Several emergency methods have been developed to rapidly suppress cyanobacterial blooms. Examples include precipitation of cyanobacteria and removal of phosphate with phosphate-binding clays (Lürling et al., 2020a), or chemical treatment with e.g. copper sulfate or low concentrations of hydrogen peroxide (H_2O_2) (Drábková *et al.*, 2007a; Barrington and Ghadouani, 2008; Matthijs *et al.*, 2012, 2016).

Application of H_2O_2 has several advantages over other chemical treatments. H_2O_2 is naturally present in very low concentrations in freshwater ecosystems, as it is constantly produced and degraded by abiotic processes as well as enzymatically by a variety of organisms (Cooper and Zepp, 1990; Häkkinen *et al.*, 2004; Richard *et al.*, 2007; Mishra and Imlay, 2012). H_2O_2 has a short life-span, which allows controlled H_2O_2 treatments for a duration of several hours to days, depending on the environmental conditions, after which the added H_2O_2 degrades to water and oxygen by biotic and abiotic processes (Cooper and Zepp, 1990; Richard *et al.*, 2007). Another advantage of the use of low H_2O_2 concentrations is that it works selectively against cyanobacteria, while eukaryotic phytoplankton are less affected (Drábková *et al.*, 2007a,b; Matthijs *et al.*, 2012; Weenink *et al.*, 2015; Lusty and Gobler, 2020; Weenink *et al.*, 2022). The high H_2O_2 sensitivity of cyanobacteria can be attributed to a lack of important antioxidant enzymes such as monofunctional catalases and ascorbate peroxidase in many cyanobacteria (Passardi *et al.*, 2007; Bernroitner *et al.*, 2009). Therefore, cyanobacteria tend to exhibit much lower H_2O_2 degradation rates, and hence they are less protected against H_2O_2, than most eukaryotic phytoplankton (Weenink *et al.*, 2021).

So far, many studies have looked at effects of H_2O_2 on specific cyanobacterial species in the laboratory (Drábková *et al.*, 2007a,b; Weenink *et al.*, 2015; Piel *et al.*, 2020; Sandrini *et al.*, 2020) or in small-scale field incubations with lake water (Lin *et al.*, 2018; Sinha *et al.*, 2018; Yang *et al.*, 2018; Lusty and Gobler, 2020). Studies on H_2O_2 treatments of entire lakes, however, are scarce (Matthijs *et al.*, 2012; Barrington *et al.*, 2013; Huang and Zimba, 2020; Weenink *et al.*, 2021; Lusty and Gobler, 2023), and our understanding of the impacts of lake treatments on other aquatic organisms and ecosystem processes is limited (see e.g. Piel *et al.*, 2021; Weenink *et al.*, 2022). Hence, a thorough understanding of the effectivity of H_2O_2 treatments of cyanobacterial blooms under various conditions and potential side effects of the treatments on the lake ecosystem is still in its infancy.

To address this knowledge gap, our study investigates the impact of H_2O_2 addition on the community composition of phytoplankton and zooplankton, concentrations of extracellular and intracellular cyanotoxins, and nutrient availability during five lake treatments in three different lakes with various dominant cyanobacterial species. Our results contribute to a better understanding of the effectiveness of lake treatments with H_2O_2 and their potential side effects at the ecosystem level.

2. Materials and methods

2.1. The lakes

The three lakes investigated in this study are located in the area between Amsterdam and Rotterdam, the Netherlands (Fig. 1), under the governance of water authority Schieland and Krimpenerwaard (Lake Kralingse Plas) and water authority Rijnland (Lake Klinkenbergerplas and Lake Oosterduinse Meer). All three lakes suffer from severe cyanobacterial blooms during the summer months, although the lakes have different characteristics in terms of origin, depth, degree of isolation, and nutrient load.

Lake Kralingse Plas (51° 56' 9" N, 4° 30' 54" E) is the major recreational lake of the city of Rotterdam, with ~3.5 million visitors per year (Fig. 1A; Table 1). It is a shallow non-stratified lake, with an average depth of only 2 m, and a lake surface of ca. 1.0 km^2, that was artificially made by peat mining in the 17th century. The lake has suffered from a low water quality with severe algal blooms for many decades, until several measures were taken in 2011 to reduce nutrient inflow, to increase water transparency, and to stimulate growth of macrophytes. Despite these implementations, cyanobacteria did not disappear from the lake, although the dominant cyanobacterial taxa changed from *Planktothrix agardhii* and *Microcystis* sp. to *Dolichospermum* sp. and *Gloeotrichia* sp.

Lake Klinkenbergerplas (52° 11' 48" N, 4° 29' 20" E) (Fig. 1B) is a deep lake (average depth of 19 m) that is stratified during the summer months, with a lake surface of ca. 0.28 km^2 (Table 1). The lake was created by sand mining in the 1960s and 1970s, and was transformed into a recreational lake in the 1990s. The lake is hydrologically relatively isolated, i.e., there is no major influx of surface water from nearby canals. However, nutrient concentrations are influenced by nutrient-rich ground water infiltrating the lake and by nitrogen deposition from nearby urban and agricultural areas. The lake has high phosphate concentrations in summer (> 200 μg L^{-1}), fueling the dominance of various

cyanobacteria.

Lake Oosterduinse Meer (52° 16' 55" N, 4° 30' 28" E) (Fig. 1C) has an intermediate depth (average depth of 7 m), is stratified during the summer months, and has a lake surface of ca. 0.35 km^2 (Table 1). The lake originated from sand mining of an old dune area in the 1950s and 1960s, and is now only used for recreation. In addition to the influx of nutrient-rich ground water, the lake is also directly connected to a channel system that drains the surrounding flower fields. As a result, the lake is hypertrophic and regularly dominated by cyanobacteria during the summer months.

2.2. H_2O_2 treatments and monitoring

The lake treatments were performed with a boat equipped with a 'water harrow' (sensu Matthijs *et al.*, 2012) that was specially designed for homogeneous dosing of H_2O_2 to the upper 5 m of the lake, as described in Piel *et al.* (2021). The boat slowly zigzagged across the entire lake while injecting H_2O_2 from a 50% (w/w) H_2O_2 stock solution with the water harrow, using a computer-controlled system integrating the GPS position, cruise track and cruise speed of the boat as well as the water column depth to be treated to dynamically calculate the required H_2O_2 injection rate.

The target H_2O_2 concentration for the treatments of Lake Kralingse Plas was 5 mg L^{-1}, while the target H_2O_2 concentration for the treatments of Lake Klinkenbergerplas and Lake Oosterduinse Meer was 2.5 mg L^{-1} (Table 1). The target H_2O_2 concentration in Lake Kralingse Plas was higher to compensate for the breakdown of H_2O_2 by the abundant macrophytes in the lake. The high rate of H_2O_2 breakdown by macrophytes was confirmed in a lake enclosure experiment in advance of the treatment (unpublished data).

During the treatments of Lake Kralingse Plas and Lake Klinkenbergerplas, the shallower parts of the lake were treated either by carefully navigating the boat close to the shore or by gently spraying the H_2O_2 stock solution on top of cyanobacterial scum layers with a hand sprinkler at shallow places that could not be reached by the water harrow. Spraying was not needed in Lake Oosterduinse Meer, because the water harrow could easily reach the shoreline. The two treatments in this lake slightly differed in the way the boat navigated the lake area (as described in Piel *et al.*, 2021).

The first H_2O_2 treatment of Lake Kralingse Plas was on July 4, 5 and

Table 1
Lake treatments and environmental conditions during the treatments.

Lake	Kralingse Plas		Klinkenberger Plas	Oosterduinse Meer	
Treatment					
Treatment date	July 4-6 (2016)	August 8-9 (2016)	June 15 (2017)	June 19 (2018)	August 7 (2018)
Target H_2O_2 dosage (mg L^{-1})*	5	5	2.5	2.5	2.5
Weather					
Daily mean temperature (°C)	15.8	16.0	19.8	18.5	26.2
Max. temperature (°C)	22.3	20.0	25.6	22.2	33.7
Daily sunshine (hours)	8.7	8.1	10.7	4.8	10.0
Daily mean windspeed (m s^{-1})	3.4	4.2	5.7	4.6	2.6
Daily precipitation (mm)	18	16	0	0	2.0
Lake characteristics					
Average lake depth (m)	2	2	19	7	7
Max. lake depth (m)	3.5	3.5	35	13	13
Lake size (km^2)	1.0	1.0	0.28	0.35	0.35
Water temperature (°C)#	19.6	20.2	20.9	18.6	24.6
Thermocline depth (m)§	not str.	not str.	6	6.5	6
Euphotic depth (m)	3	2.3	1.6	1.9	1.5

Water temperature was measured at 0.5 m depth, in the morning.
§ not str. = not stratified.

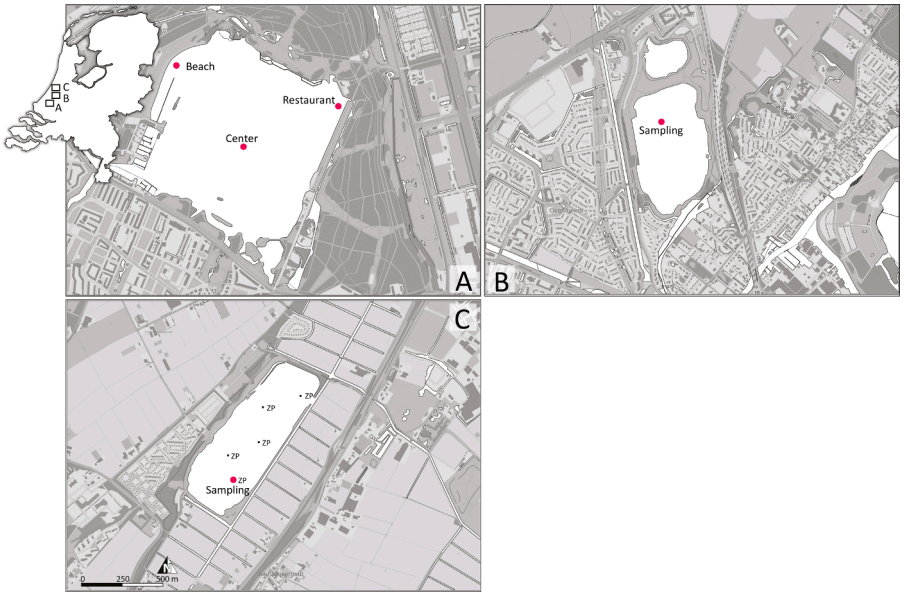

Fig. 1. Location of the lakes. (A) Lake Kralingse Plas, (B) Lake Klinkenbergerplas, and (C) Lake Oosterduinse Meer. The pink sampling points indicate the locations where the samples were collected. In Oosterduinse Meer, zooplankton samples (ZP) were collected at five locations and H_2O_2 concentrations were measured at many locations (not shown) spread randomly across the lake.

6, 2016 (July treatment), while the second treatment was on August 8 and 9, 2016 (August treatment). Due to the large surface area of Lake Kralingse Plas, the treatments took more than one day, while in the other lakes one day was sufficient. The treatment of Lake Klinkenberger Plas was on June 15, 2017, and of Lake Oosterduinse Meer on June 19, 2018 (June treatment) and August 7, 2018 (August treatment).

In Lake Kralingse Plas and Lake Oosterduinse Meer, H_2O_2 concentrations in the surface layer (0-1 m depth) were closely monitored at three or more locations in the lake during the first 24-30 h after the treatment, while in Lake Klinkenberger Plas triplicate samples were taken from one location. In Lake Kralingse Plas and Lake Oosterduinse Meer, we used Quantofix® peroxide test strips (sensitivity range: 0.5-25 mg L^{-1}) for rapid estimation of the H_2O_2 concentration (Macherey-Nagel GmbH & Co. KG Düren, Germany). In Lake Klinkenbergerplas, we applied a more sensitive colorimetric assay with *p*-nitrophenylboronic acid according to Lu *et al.* (2011) and Piel *et al.* (2020) to quantify the H_2O_2 concentration. For this assay, samples were collected from the surface layer of the lake and immediately brought to shore for processing. Subsamples of 55 mL were filtered in triplicate over a 1.2 μm pore size GF/C (Whatman GmbH, Dassel, Germany). Subsequently, 100 μL of each filtrate was added to 100 μl of 2 mM *p*-nitrophenylboronic acid reagent (Merck KGaA, Darmstadt, Germany) in 96-well plates, and incubated at complete darkness for 30-45 min. Thereafter, the absorption of *p*-nitrophenolate was measured at its absorption peak (405 nm) using a plate reader (Multiscan FC type 357, ThermoScientific™, Waltham, Massachusetts, USA).

In Lake Oosterduinse Meer, the H_2O_2 concentration was also monitored at various other locations and at various depths across the lake throughout the entire treatment day using Quantofix® peroxide test strips and a mobile strip reader (Quantofix®, Macherey-Nagel GmbH & Co., KG) for more accurate reads. The concentrations measured by the test strips were calibrated with the colorimetric assay described above. For each measurement, the time after H_2O_2 addition to the lake at that location was calculated by comparing the time and GPS coordinates of the measurement with the GPS track of the boat.

2.3. Lake monitoring

2.3.1. Environmental parameters

Depth profiles of Chl *a* fluorescence, oxygen saturation, light intensity, and temperature were collected using a Hydrolab Datasonde 5 (OTT Messtechnik GmbH & Co., Kempten, Germany) in Lake Oosterduinse Meer and Lake Klinkerbergerplas. From the light profiles, the euphotic depth (Z_{eu}, i.e., depth where the light intensity is 1% of the surface irradiance) was calculated. For Lake Kralingse Plas, data on water temperature and Secchi depth were kindly provided by water authority Schieland and Krimpenerwaard, and the euphotic depth was calculated from the Secchi depth (Z_s) according to the equation $Z_{eu} = 2.5\ Z_s$. Weather data from weather station Rotterdam (for Lake Kralingse Plas) and weather station Schiphol (for the other two lakes) were provided by the Royal Netherlands Meteorological Institute (KNMI) (www.knmi.nl).

2.3.2. Phytoplankton

Phytoplankton samples were collected approximately once or twice per week from a fixed location in the middle of the lake throughout the entire summer season, with more frequent sampling during the lake treatments. For the deeper lakes (Lake Oosterduinse Meer and Lake Klinkenbergerplas), integrated samples of the upper 6 m of the water column were taken with a flexible hosepipe (10 m length, 4 cm diameter). For the shallower lake (Lake Kralingse Plas), integrated samples were taken of the entire water column. The hosepipe was lowered into the water with its bottom end and top end both open, until it reached the specified depth. Once the hosepipe was in a vertical position and the water level in the hosepipe reached the surface of the lake, a stopper was inserted to seal the hosepipe at the top end. Subsequently, the bottom end of the hosepipe was brought to the surface, allowing the entire water column sample to be collected in a bucket. Subsamples were preserved with alkaline Lugol's iodine and stored at 4°C until analysis. Phytoplankton was identified to genus level, and if possible to species level, and counted with an inverted microscope using the Utermöhl method (Utermöhl, 1958) according to the European standard protocol (NEN-EN-15204). Biovolumes of the most abundant taxa were calculated based on size measurements of individuals according to Hillebrand *et al.* (1999), standard sizes were used to calculate biovolumes of rarer taxa.

Four depth profiles of Lake Klinkenbergerplas were taken with a Fluoroprobe (BBE Moldaenke, Kiel, Germany) on thetreatment day (15 June 2016), just before commencement of the treatment, to assess the vertical distributions of the two abundant cyanobacteria *Planktothrix rubescens* and *Dolichospermum* spp. before being affected by the H_2O_2 treatment. The striking difference in their photosynthetic pigments (phycoerythrobilin in *P. rubescens* versus phycocyanobilin in *Dolichospermum* spp.) was used to separate the signals of both taxa.

2.3.3. Zooplankton

Zooplankton samples were collected at the fixed sampling location in Lake Kralingse Plas and Lake Klinkenberger Plas and at five fixed locations spread over Lake Oosterduinse Meer (Fig. 1C). At each sampling location, a flexible hosepipe was used to collect 10 L integrated samples from the entire water column of Lake Kralingse Plas and from the top 9 m of the deeper Lake Klinkenbergerplas and Lake Oosterduinse Meer, in the same way as described above. The samples were concentrated over a 41 μm zooplankton mesh, preserved in Lugol's iodine and stored at 4°C until analysis with an inverted microscope. Zooplankton was counted with tubular and Bogorov counting chambers (Hydro-Bios, Kiel Germany), and classified in three different groups: cladocerans, copepods and rotifers.

2.3.4. Dissolved inorganic nutrients

Subsamples were taken from the integrated water samples used for phytoplankton analysis and were filtered over a 1.2 μm pore size GF/C glass fiber filter (Whatman GmbH, Dassel, Germany) before storage at –20°C. Prior to analysis, the samples were filtered over 0.2 μm pore size Whatman polycarbonate membrane filters (GE Healthcare, Buckinghamshire, UK). Concentrations of dissolved nitrate, nitrite, ammonium and phosphate were measured with colorimetric methods according to European standard protocols NEN-ISO-15923-1 and NEN-EN-ISO-15681-2, using a San++ Automated Wet Chemistry Analyzer (Skalar Analytical B.V., Breda, The Netherlands).

2.3.5. Cyanotoxins

In Lake Kralingse Plas, samples were taken from the middle of the lake to screen for the presence of intracellular and extracellular cyanotoxins before and after the treatment. Extraction and analysis were performed at Wageningen University Research. Extraction for intracellular toxins was based on existing methods (Lürling and Faassen, 2013; Faassen *et al.*, 2012; Rangel *et al.*, 2016). As the results had to be available as quickly as possible, the extraction procedure was simplified. Samples were concentrated on GF/C filters, which were subjected to three freeze/thaw cycles in liquid nitrogen. Next, the filters were extracted by adding 2.5 ml of 75% methanol (microcystins and nodularin), 0.1% formic acid (anatoxins and cylindrospermopsins) or 0.1 M HCl (saxitoxins). Extraction was performed for 15 minutes at 60°C. The extracts were dried in a TurboVap at 50°C and reconstituted in 800 μl methanol (microcystins and nodularin), 0.1% formic acid (anatoxins and cylindrospermopsins) or 20 mM HCl (saxitoxins). After centrifugation for 5 minutes at 1600 g, the filtrates were transferred to a vial for LC-MS/MS analysis.

Before samples were prepared for extracellular toxins, they were inspected by light microscopy and PhytoPAM (Walz, Germany) for the presence of cyanobacterial cells and cyanobacterial chlorophyll-*a*. When

these were not detected, the water sample was filtered over a GF/C filter and 1.5 ml subsamples were taken of the filtrate. The filtrates were dried in a TurboVap at 50°C and reconstituted in 150 µl methanol (microcystins and nodularin), 0.1% formic acid (anatoxins and cylindrospermopsins) or 20 mM HCl (saxitoxins). The reconstituted extracts were then transferred to vials with inserts for LC-MS/MS analysis.

For microcystins, LC-MS/MS analysis was performed as described in Lürling and Faassen (2013) using seven standards of microcystin variants (dmMC-RR, MC-RR, MC-YR, dmMC-LR, MC-LR, MC-LY, MC-LW and MC-LF). For anatoxins and cylindrospermopsins, LC-MS/MS analysis was performed as described in Faassen *et al.* (2012). LC-MS/MS analysis of saxitoxins was performed as described in Rangel *et al.* (2016).

In Lake Klinkenbergerplas and Lake Oosterduinse Meer, 55 ml subsamples were taken from 6 m integrated samples from the fixed sampling location at the lake center and filtered over 1.2 µm pore size GF/C glass fiber filters (Whatman GmbH, Dassel, Germany). Filters and filtrates were freeze-dried and subsequently stored at –20°C until analysis. Extraction and analysis of cyanotoxins (microcystins, nodularin, cylindrospermopsin and anatoxin-a) and anabaenopeptins were performed at NCSR "Demokritos", Athens, Greece. Analysis of the intracellular fraction was performed based on previously developed methods (Christophoridis *et al.*, 2018; Zervou *et al.*, 2021). Briefly, cyanotoxins were extracted from the filter samples with 9 mL of MeOH: H_2O (75:25 v/v) and subsequent sonication in an ultrasound bath for 15 min in order to cause cell lysis and release of toxins. The mixture was centrifuged with 2957 *g* for 10 min (20°C) and 3 mL of supernatant was evaporated to dryness under a gentle steam of nitrogen at 25°C. The residue was re-dissolved with 500 µL MeOH: H_2O (5:95 v/v) and sonicated for 5 min.

Lyophilized water samples were reconstituted with 500 µL MeOH: H_2O (5:95 v/v), vortexed thoroughly and sonicated for 5 min. Finally, reconstituted extracts of both the filter samples and water samples were centrifuged at 1588 g for 10 min (20°C) prior to LC-MS/MS analysis. The LC-MS/MS analysis was performed in a Finnigan Surveyor LC system, equipped with a Finnigan Surveyor AS autosampler (Thermo Fischer Scientific, USA), coupled with a Finnigan TSQ Quantum Discovery Max triple-stage quadrupole mass spectrometer (Thermo Fischer Scientific, USA), with electrospray ionization (ESI) in Multiple Reaction Monitoring (MRM) mode. The determination of cylindrospermopsin, anatoxin-a, nodularin and 12 microcystins (dmMC-RR, MC-RR, MC-YR, MC-HtyR, dmMC-LR, MC-LR, MC-HilR, MC-WR, MC-LA, MC-LY, MC-LW, MC-LF) was carried out according to the LC-MS/MS method described by Zervou *et al.* (2017). In addition, the following MRM transitions were monitored for the detection of three anabaenopeptin congeners: anabaenopeptin B (Anab. B) m/z 837.4 → m/z 84.0, m/z 201.1 and m/z 637.3, anabaenopeptin F (Anab. F) m/z 851.3 → m/z 140.0, m/z 201.0 and m/z 651.4 and oscillamide Y (Oscil. Y) m/z 858.4 → m/z 84.0, m/z 405.0 and m/z 681.4. A standard of Anab. B, purchased by ENZO Life Science (UK), was used for the quantification of anabaenopeptins. The concentration of all anabaenopeptins is expressed as Anab. B equivalents.

2.4. Statistical analysis

Two-sample *t*-tests were used to compare population abundances of each species during the seven days before and seven days after each lake treatment. We note that the number of datapoints per *t*-test was small (n=4-8). Although there is no principle objection against the use of *t*-tests with small sample sizes (De Winter, 2013), this implies that significant responses will be obtained only if the lake treatment has a large effect on the population abundance.

3. Results

3.1. Lake Kralingse Plas

3.1.1. H_2O_2 treatment of Lake Kralingse Plas

The shallow Lake Kralingse Plas was not stratified, and abundantly populated by submerged macrophytes, especially *Potamogeton pusillus* and *Chara* sp. The macrophytes interfered with the H_2O_2 injection system of the boat, and, as a consequence, H_2O_2 was injected only in the upper layer up till a depth of 1.0 – 1.5 m.

The weather was partly cloudy, with some periods of sunshine and some rain, during both the July and August treatment (Table 1). The H_2O_2 degradation dynamics after both treatments of Lake Kralingse Plas were quite similar. The highest H_2O_2 concentrations were measured at the sampling location 'Beach', with up to 5 mg L^{-1} and 9 mg L^{-1} H_2O_2 during the first few hours of the 1st and 2nd treatment, respectively, due to spraying of H_2O_2 at the shallow parts of the lake. Subsequently, H_2O_2 concentrations slowly declined, even though 1-3 mg L^{-1} of H_2O_2 was still detected after 24 h in both treatments (Fig. 2A and 2B).

3.1.2. Phytoplankton in Lake Kralingse Plas

Throughout the entire summer season, *Dolichospermum* sp. was by far the most abundant cyanobacterium detected in the phytoplankton samples of Lake Kralingse Plas (Fig. 3A; Fig. S1). At the time of the August treatment, a few large colonies of *Gloeotrichia* sp. were also present in the lake, as could be seen with the bare eye, but due to their large colony size and low number they were not detected during cell counting with the microscope. After the August treatment, *Gloeotrichia* colonies were not observed anymore in the water phase.

The July treatment significantly reduced the biovolume of *Dolichospermum* sp., from 1.3 mm^3 L^{-1} to near zero (Fig. 3A). Eukaryotic phytoplankton were not significantly affected by the lake treatment, and chlorophytes and diatoms increased to high abundances about one week after the July treatment (Fig. 3B). However, they could not maintain their dominance and *Dolichospermum* sp. slowly returned in the water column during the end of July and developed a large population in early August. During the August treatment, the biovolume of *Dolichospermum* sp. significantly declined from 10.7 mm^3 L^{-1} to less than 0.2 mm^3 L^{-1}, and remained low during the subsequent weeks. Eukaryotic phytoplankton again benefitted from the collapse of the cyanobacterial population, and especially chlorophytes and diatoms increased (Fig. 3B).

3.1.3. Zooplankton in Lake Kralingse plas

Before the July treatment, copepods and rotifers were the only zooplankton found in the water column. Rotifers showed a large but non-significant decline during the first few days after the July treatment, whereas copepods did not show a response (Fig. 3C; Fig. S1). The abundance of rotifers recovered within 10 days to similar quantities as before the July treatment and continued to increase in the following weeks. Also, cladocerans and copepods increased in the month after the July treatment. During the August treatment, both rotifers and cladocerans declined significantly and copepods showed a non-significant decline. The abundance of rotifers recovered again in ~2 weeks after the August treatment, whereas the abundance of cladocerans and copepods remained relatively low (Fig. 3C).

3.1.4. Nutrient availability in Lake Kralingse Plas

Concentrations of dissolved inorganic nutrients in Lake Kralingse Plas were relatively low in comparison to the other two lakes (Fig. 3D). The molar ratio between dissolved inorganic nitrogen and phosphorus (i.e., the DIN:DIP ratio) varied between 3 and 55 during the summer season. The concentrations of nitrate plus nitrite and phosphate remained stable and did not show a major response to the H_2O_2 treatments, whereas a substantial amount of ammonium was released after the *Dolichospermum* population collapsed during the August treatment.

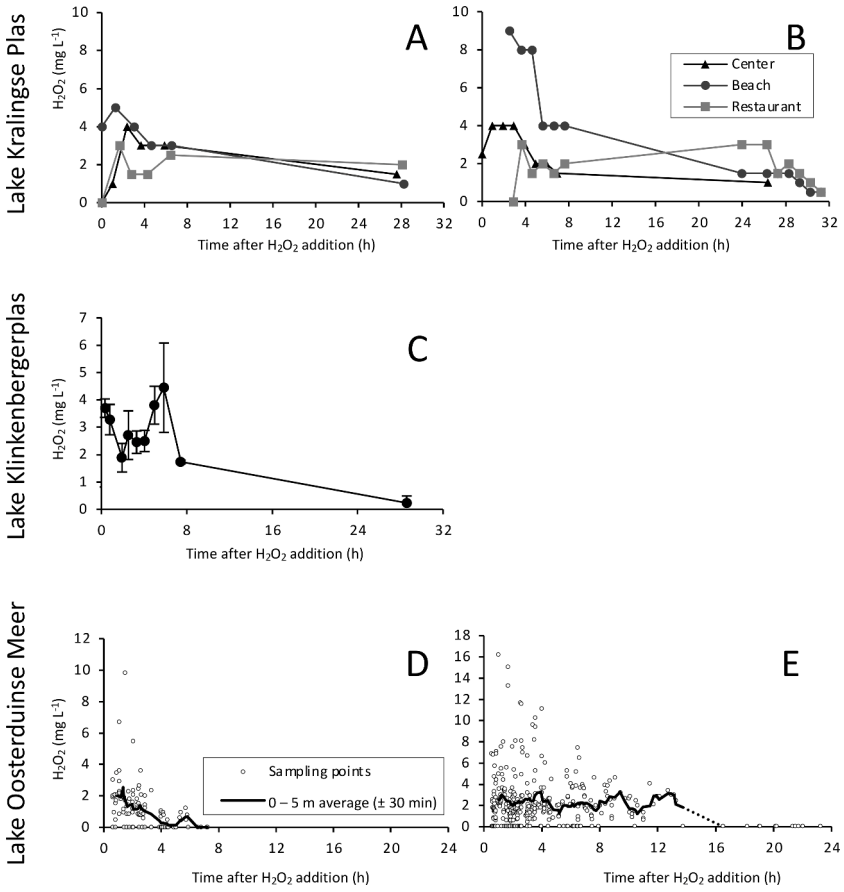

Fig. 2. H_2O_2 concentrations measured during the lake treatments. (A) The July treatment and (B) August treatment of Lake Kralingse Plas. (C) The treatment of Lake Klinkenbergerplas. (D) The June treatment and (E) August treatment of Lake Oosterduinse Meer. H_2O_2 concentrations were measured at three locations in Lake Kralingse Plas and at many locations spread across the lake in Lake Oosterduinse Meer. In Lake Klinkenbergerplas, H_2O_2 concentrations were measured in triplicate at a fixed sampling location in the middle of the lake. Data in (C) show mean ± SD (n=3). The line in panels (D) and (E) is a moving average of the measured H_2O_2 concentrations with a window size of 30 min.

3.1.5. *Cyanotoxins in Lake Kralingse Plas*

No intra- or extracellular cyanobacterial toxins were detected in the samples taken before and after each of the treatments.

3.2. *Lake Klinkenbergerplas*

3.2.1. *H_2O_2 treatment of Lake Klinkenbergerplas*

Lake Klinkenbergerplas was stratified, with a thermocline of ~6 m depth in mid-June (Table 1, Fig. S2). The weather during the treatment of Lake Klinkenbergerplas was mostly sunny and warm, with a moderate breeze and without rain (Table 1). The H_2O_2 concentration in the surface layer of the lake fluctuated between 1.7 and 4.4 mg L^{-1} H_2O_2, with an average of 2.7 mg L^{-1} H_2O_2, during the initial 8 h after H_2O_2 addition to the lake. Subsequently, the H_2O_2 concentration declined to ~0.2 mg L^{-1} on the day after the treatment (Fig. 2C).

3.2.2. *Phytoplankton in Lake Klinkenbergerplas*

The red cyanobacterium *Planktothrix rubescens* dominated the phytoplankton community in the lake during the first weeks of the monitoring period, with a biovolume of 54.6 mm^3 L^{-1} (integrated over the upper 6 m of the water column) at the end of May (Fig. 4A). While *P. rubescens* was also seen at the surface, fluorescence measurements indicated that the majority of the population was located between 2 and 5 m depth, around the thermocline, coinciding with an oxygen minimum (Fig. S2A). At the beginning of June, *P. rubescens* declined and subsequently maintained a stable biovolume of ~12 mm^3 L^{-1} (Fig. 4A). Shortly before the lake treatment, the cyanobacteria *Dolichospermum* spp. increased to 6.1 mm^3 L^{-1} in the upper layer of the water column, which reduced light availability for *P. rubescens* deeper down (Fig. S2C). Consequently, preceding the treatment, two cyanobacterial genera co-dominated the phytoplankton community, with *Dolichospermum* spp. prevalent in the upper 2 m of the water column and *P. rubescens* between 4 and 7 m depth (Fig. S2E, F and Fig. 4A). In addition, *P. rubescens* also floated in large clumps (with a diameter of a few cm) at the water surface (Fig. S3). Both species decreased after the H_2O_2 treatment, but in different ways. *Dolichospermum* spp. significantly declined immediately after the H_2O_2 treatment, while *P. rubescens* slowly decreased over a time span of 11 days (Fig. 4A; Fig. S4).

After the decline of *P. rubescens*, another filamentous cyanobacterium, here described as *Aphanizomenon* s.l. (i.e., one of the *Aphanizomenon*-like taxa in the order Nostocales), dominated the lake phytoplankton for ~4 weeks with a maximum biovolume of 13.1 mm^3 L^{-1}. After the decline of *Aphanizomenon* s.l., a mixture of *Limnothrix* sp. and *Pseudanabaena* sp. (both taxa were present, but difficult to distinguish morphologically) grew to 181 mm^3 L^{-1} and dominated the phytoplankton for ~3 weeks until the bloom collapsed mid August.

Eukaryotic phytoplankton had a very low abundance in the lake throughout the entire monitoring period, and counting errors due to low numbers resulted in high variability over time (Fig. 4B; Fig. S4). None of the eukaryotic phytoplankton were significantly affected by the H_2O_2 treatment. However, chrysophytes and diatoms temporarily increased several days after the treatment, followed by a more persistent increase of the cryptophytes that remained present throughout the second half of the monitoring period.

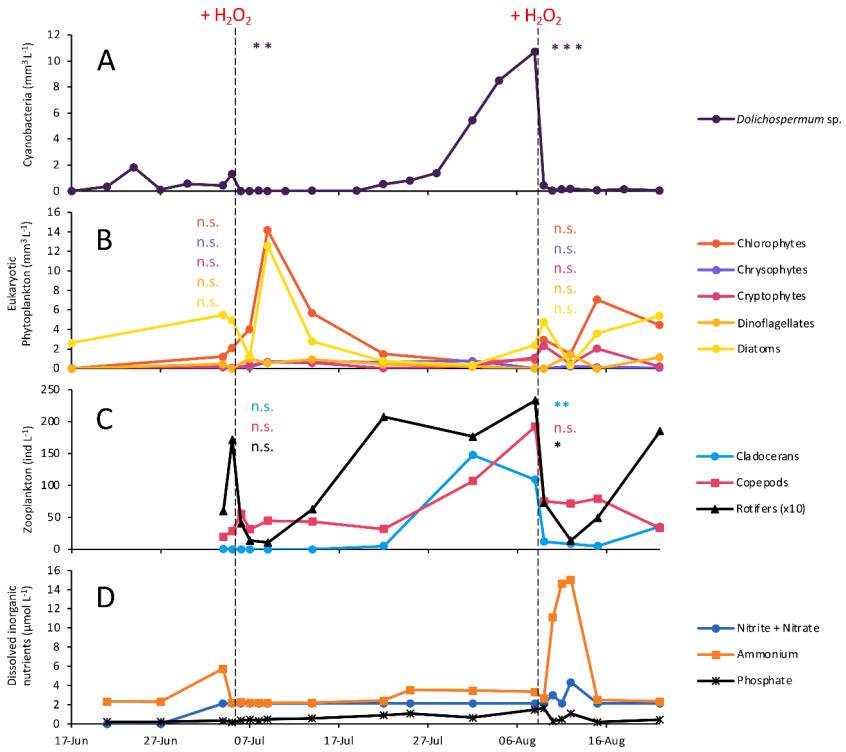

Fig. 3. Plankton community composition and nutrients in Lake Kralingse Plas throughout the summer season. (A) Cyanobacteria, (B) eukaryotic phytoplankton, (C) zooplankton, (D) dissolved inorganic nutrients. The two vertical dashed lines indicate the timing of the two H_2O_2 treatments of Lake Kralingse Plas, in early July and in August. Significant differences between species abundances before and after the lake treatments are indicated as * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$; n.s. = not significant.

3.2.3. *Zooplankton in Lake Klinkenbergerplas*

Before the H_2O_2 treatment, rotifers strongly decreased from 4762 to 813 individuals L^{-1} and they declined even more during the first few days after the H_2O_2 treatment to a minimum of 47 individuals L^{-1} (Fig. 4C; Fig. S4). Subsequently, rotifers remained low in numbers, with <500 individuals L^{-1}, until the end of the monitoring period. Cladocerans showed a non-significant decline after the treatment and subsequently remained stable, while copepods did not show major changes in response to the H_2O_2 treatment.

3.2.4. *Nutrient availability in Lake Klinkenbergerplas*

The concentration of nitrate plus nitrite was largely stable between 0.09 and 3 µmol L^{-1} throughout the entire monitoring period, while the phosphate concentration exceeded 12 µmol L^{-1} during the first weeks (Fig. 4D). Consequently, the molar DIN:DIP ratio was <1 prior to the H_2O_2 treatment. The ammonium concentration steeply increased immediately after the H_2O_2 treatment and showed a more prolonged period with high concentrations during the collapse of *Aphanizomenon* s. l. later in the season. A drastic decline of the dissolved phosphate concentration was observed directly after the addition of H_2O_2, followed in the next few days by a recovery to a similar phosphate concentration as before the treatment. Thereafter, the phosphate concentration slowly decreased until it was depleted by mid-August (Fig. 4D).

3.2.5. *Cyanotoxins in Lake Klinkenbergerplas*

Intracellular concentrations of dmMC-RR and dmMC-LR in *P. rubescens* were highest at the beginning of the monitoring period, in May, with concentrations of 84.7 µg L^{-1} and 2.7 µg L^{-1}, respectively (Fig. 4E). With the decline of *P. rubescens*, also the intracellular microcystin concentrations declined. Intracellular anabaenopeptins showed a similar pattern. While intracellular microcystins and anabaenopeptins did not show an immediate response to the H_2O_2 treatment, they declined by ~40% between 24 h and 48 h after H_2O_2 addition, concomitant with peaks in the extracellular dmMC-RR and anabaenopeptin concentrations (Fig. 4F). Subsequently, both intracellular and extracellular dmMC-RR remained present in the water at low concentrations for several weeks, whereas the anabaenopeptins disappeared a few days after the H_2O_2 treatment.

3.3. *Lake Oosterduinse Meer*

3.3.1. *H_2O_2 treatment of Lake Oosterduinse Meer*

Lake Oosterduinse Meer was stratified with a thermocline at approximately 6-6.5 m depth (Table 1). The weather was mostly cloudy without rain during the June treatment, whereas it was mostly sunny, humid and very warm during the August treatment (Table 1). The H_2O_2 exposure differed between the June and August treatments. During the June treatment, the average H_2O_2 concentration in the surface layer was ~2 mg L^{-1} during the initial 3 h and declined below the detection limit of 0.5 mg L^{-1} around 6 h after H_2O_2 addition to the lake (Fig. 2D). A much longer exposure was observed during the August treatment, when the average H_2O_2 concentration fluctuated between 2 and 3.5 mg L^{-1} H_2O_2 for more than 12 h and declined below the detection limit ~16 h after H_2O_2 addition to the lake (Fig. 2E).

3.3.2. *Phytoplankton in Lake Oosterduinse Meer*

The filamentous cyanobacterium *Aphanizomenon* sp. formed a late spring bloom with a maximum biovolume of 21.5 mm^3 L^{-1} in early June. However, it significantly declined by 90% within 96 h after the June treatment (Fig. 5A; Fig. S5). During the subsequent weeks cyanobacterial abundances remained low until *Dolichospermum* sp. slowly started to increase from mid-July onwards, while *Planktothrix agardhii* appeared in early August. Both species reached a maximum biovolume of ~6.0 mm^3 L^{-1} just before the August treatment. The two species responded differently to the H_2O_2 treatment in August. *Dolichospermum* sp. significantly declined to near zero within a few hours, while

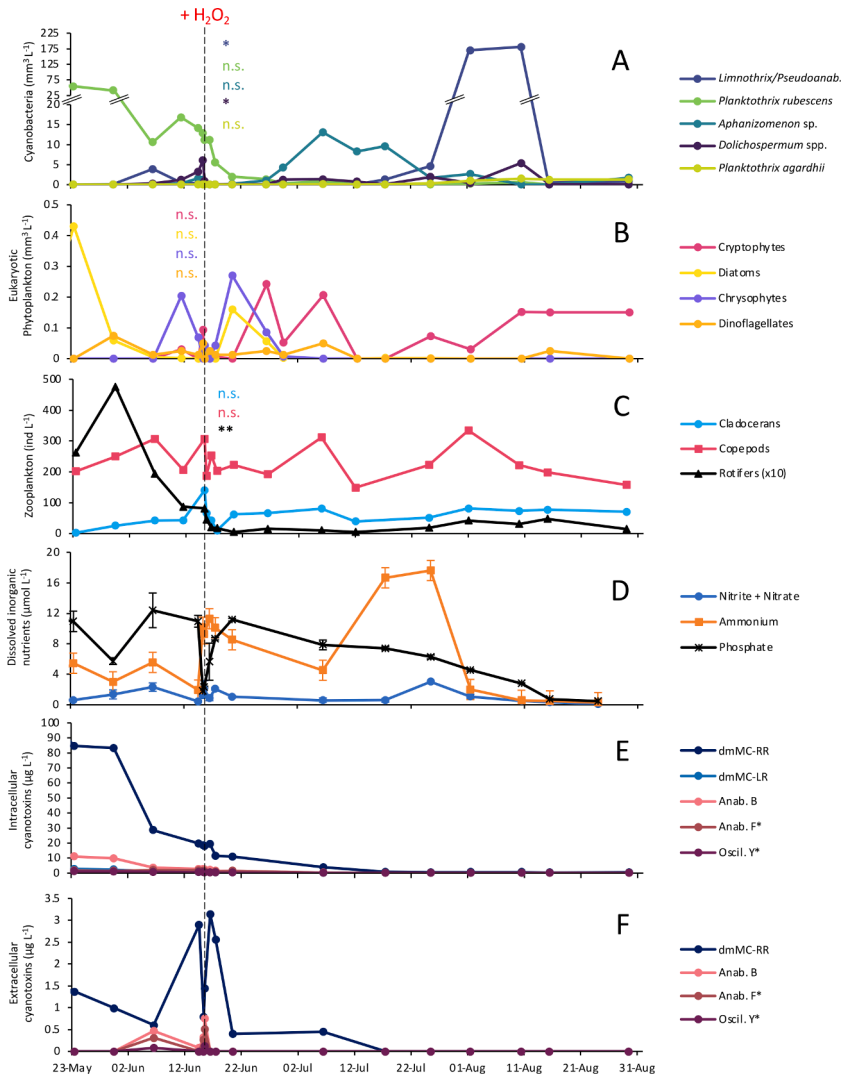

Fig. 4. Plankton community composition, nutrients and cyanotoxins in Lake Klinkenbergerplas throughout the summer season. (A) Cyanobacteria, (B) eukaryotic phytoplankton, (C) zooplankton, (D) dissolved inorganic nutrients, (E) intracellular cyanotoxins, (F) extracellular cyanotoxins. The vertical dashed line indicates the timing of the H_2O_2 treatment of Lake Klinkenbergerplas, in mid June. Anab. = anabaenopeptin; Oscil. = oscillamide. Significant differences between species abundances before and after the lake treatments are indicated as * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$; n.s. = not significant.

P. agardhii was still present with a biovolume of 4.3 mm^3 L^{-1} at 24 h after the treatment. The change in cyanobacterial composition was also visible as a striking decrease of the 'greenness' of the lake after the treatment (Fig. S6). Although *P. agardhii* declined during the next few days, it recovered within 10 days after the treatment and formed a late summer bloom (Fig. 5A).

Dinoflagellates were not significantly affected by the short H_2O_2 exposure of the June treatment, and subsequently increased to a high biovolume during the absence of cyanobacteria (Fig. 5A,B). The large dinoflagellate population strongly decreased just prior to the August treatment (see Fig. S5 for a detailed view), but persisted after the treatment and remained an important component of the phytoplankton community until the end of summer. Diatoms appeared to show a similar pattern as the dinoflagellates, although at lower abundance, and did not respond significantly to the two H_2O_2 treatments either. The biovolume of chlorophytes remained low during the entire summer season.

3.3.3. Zooplankton in Lake Oosterduinse Meer

The short H_2O_2 exposure of the June treatment did not have a significant impact on the zooplankton numbers (Fig. 5; Fig. S5). In contrast, rotifers were strongly and significantly affected by the August treatment, declining from 11,660 individuals L^{-1} one day before the treatment to 770 individuals L^{-1} four days after the treatment. Subsequently, rotifer abundances remained quite low until the end of the monitoring period. Cladocerans and copepods showed a small non-significant decline immediately after the August treatment (see Fig. S5 for a detailed view). The number of copepods increased during the next several days, while the number of cladocerans remained stable.

3.3.4. Nutrient availability in Lake Oosterduinse Meer

The nitrate plus nitrite concentration was not affected by the two H_2O_2 treatments and remained low and stable throughout the entire monitoring period (Fig. 4D). Strong but temporary increases of the ammonium concentration coincided with declines of cyanobacteria after both H_2O_2 treatments and also with the natural decline of *P. agardhii* at the end of summer. The phosphate concentration in Lake Oosterduinse Meer was higher than in the two other lakes, remained high throughout the summer season, and was not affected by the two H_2O_2 treatments. The molar DIN:DIP ratio was <1 throughout almost the entire monitoring period, but temporarily increased to slightly above 1 during the first few days after both H_2O_2 treatments.

3.3.5. Cyanotoxins in Lake Oosterduinse Meer

Intracellular dmMC-RR and Anab. B were absent or detected only in

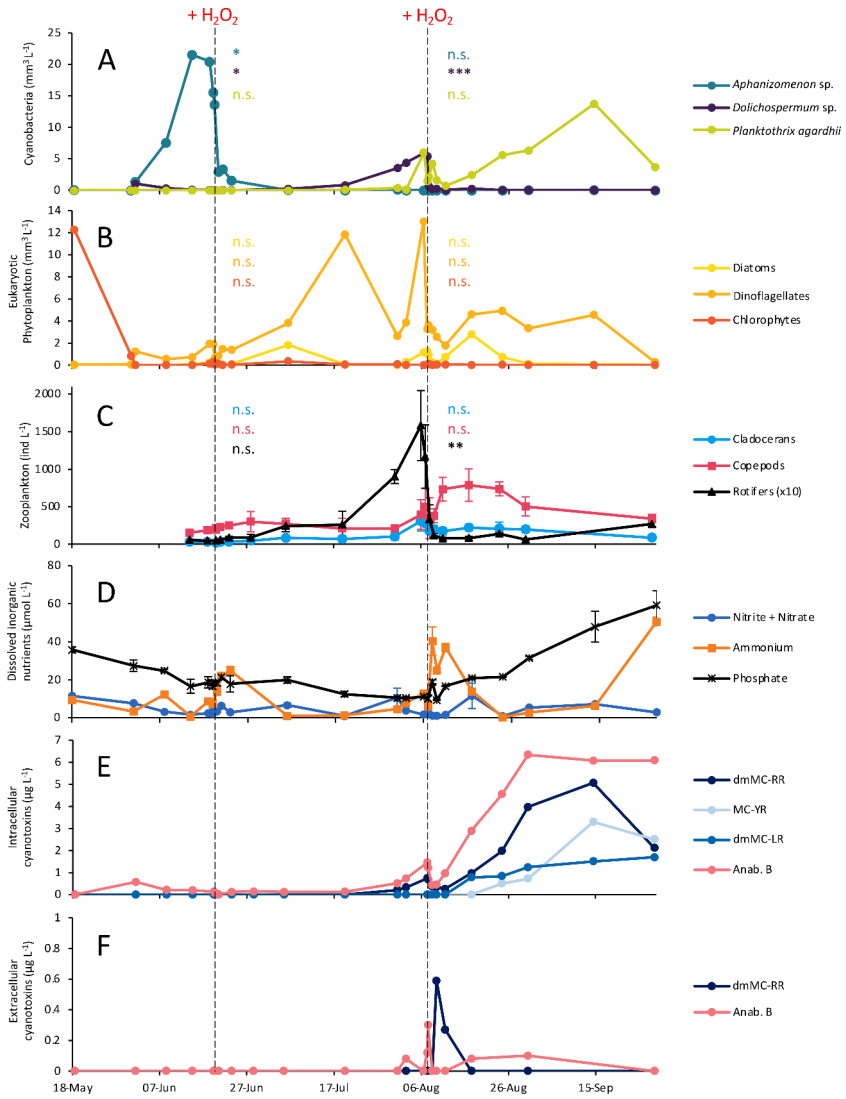

Fig. 5. Plankton community composition, nutrients and cyanotoxins in Lake Oosterduinse Meer throughout the summer season. (A) Cyanobacteria, (B) eukaryotic phytoplankton, (C) zooplankton (data points show mean ± SD at n=5 sampling locations across the lake), (D) dissolved inorganic nutrients, (E) intracellular cyanotoxins, (F) extracellular cyanotoxins. The two vertical dashed lines indicate the timing of the two H_2O_2 treatments of Lake Oosterduinse Meer, in June and in August. Anab. = anabaenopeptin. Significant differences between species abundances before and after the lake treatments are indicated as * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$; n.s. = not significant.

low concentrations during June and July, but their concentrations slightly increased during the first week of August (Fig. 5E). Both intracellular dmMC-RR and Anab. B temporarily declined after the H_2O_2 treatment in August, but subsequently intracellular dmMC-RR, dmMC-LR, MC-YR and Anab. B rapidly increased to high concentrations as *P. agardhii* thrived in late summer.

The temporary decline of the intracellular dmMC-RR and Anab. B concentrations after the H_2O_2 treatment in August coincided with peaks of extracellular dmMC-RR and Anab. B in the lake water (Fig. 5F). The slightly delayed peak of extracellular dmMC-RR disappeared again after a few days, while extracellular Anab. B rapidly declined after the lake treatment, but was also detected in low concentrations during the late summer bloom of *P. agardhii*.

4. Discussion

Although application of H_2O_2 is considered a promising method to suppress cyanobacterial blooms, most studies have been limited to small-scale incubations while only a few studies have treated entire lakes (Matthijs et al., 2012; Barrington *et al.*, 2013; Huang and Zimba, 2020; Weenink *et al.*, 2021; Piel *et al.*, 2021; Weenink *et al.*, 2022; Lusty and Gobler, 2023). This study investigated the effects of H_2O_2 on phytoplankton, zooplankton, nutrient concentrations, and cyanotoxin concentrations in five whole-lake treatments of three different lakes. The results confirm that the addition of low concentrations of H_2O_2 (2-5 mg L^{-1}) can rapidly suppress cyanobacterial blooms, but also show effects on the zooplankton community.

4.1. H_2O_2 dynamics during lake treatments

Our results illustrate that applying H_2O_2 in lakes and subsequent monitoring of the H_2O_2 concentrations during lake treatments is much more challenging than running controlled H_2O_2 experiments in a laboratory Erlenmeyer flask. Unlike the stable and homogeneous conditions created in the laboratory, every lake shows spatial and temporal variation in abiotic and biotic characteristics. Consequently, we observed substantial variation in H_2O_2 concentrations and degradation rates between the five lake treatments, and even between two different treatments in the same lake. In Lake Oosterduinse Meer, for example, the two lake treatments received a similar H_2O_2 dosage, yet this resulted in a

substantially higher average H_2O_2 concentration and in a longer H_2O_2 exposure time during the second treatment. It is likely that these differences in H_2O_2 exposure between the lake treatments have led to differences in the responses of the plankton communities.

It is not straightforward to pinpoint the exact causes underlying the differences in H_2O_2 degradation rates, since several variables may have played a role simultaneously. For example, mixed phytoplankton populations, including cyanobacteria, can degrade H_2O_2 faster when present in larger densities (Weenink *et al.*, 2015, 2021), as was clearly the case during the first treatment in Lake Oosterduinse Meer compared to the second treatment. Also the presence of compounds that can react with H_2O_2, such as dissolved and particulate organic matter, may have partly degraded H_2O_2 during the first treatment, thereby reducing the concentration and exposure time. Another variable influencing the exposure time may be the presence of submerged macrophytes. Photosynthesizing plants and green algae share the same potency to rapidly degrade H_2O_2 using a variety of antioxidant enzymes such as ascorbate peroxidases and catalases (Ishikawa and Shigeoka, 2008). In a pilot experiment with lake enclosures in Lake Kralingse Plas, a substantially higher H_2O_2 degradation rate was observed in the vicinity of macrophytes than in lake water without macrophytes. To compensate for the expected H_2O_2 degradation by these aquatic plants, the target H_2O_2 concentration in Lake Kralingse Plas was set higher (5 mg L^{-1} H_2O_2) than the target H_2O_2 concentration in the other lakes (2.5 mg L^{-1} H_2O_2) where the abundance of submerged macrophytes was negligible. However, as indicated by the recent toxicity study of Weenink *et al.* (2022), this higher H_2O_2 dosage could potentially have adverse effects on zooplankton. Indeed, several zooplankton groups declined in abundance after the July and August treatment of Lake Kralingse Plas.

A complicating factor in assessing actual H_2O_2 concentrations during lake treatments appears to be the extent to which concentrated H_2O_2 is mixed within the water column. Ideally, H_2O_2 will mix homogeneously within the water column to the target concentration immediately after the water harrow injects H_2O_2. Our study shows, however, that this was not always the case. Incomplete or slow mixing caused spatial variation in H_2O_2 concentrations in the lake water, both in the surface layers of the lakes as well as vertically. This was clearly demonstrated by the large spatial variability of the H_2O_2 concentrations measured during the first few hours of the treatments of Lake Kralingse Plas and Lake Oosterduinse Meer, while the spatial variability gradually diminished several hours after the H_2O_2 addition (Fig. 2).

Given incomplete mixing of the injected H_2O_2 as well as variation in H_2O_2 degradation rates, it is difficult to accurately predict the H_2O_2 concentrations during lake treatments in advance (even with a specialized boat). For example, a similar H_2O_2 dosage may still lead to different concentrations and exposure times in the lake. However, the experience gained from the lake treatments helped us to improve the H_2O_2 injection from the boat and the H_2O_2 monitoring. Efficient feedback between H_2O_2 injection rates from the boat and H_2O_2 measurements in the lake is important in order to adjust the injection rate and target the desired concentrations.

4.2. How do H_2O_2 treatments affect the phytoplankton community?

All dominant cyanobacterial species declined by 84% to 100% within a few days after the H_2O_2 treatment compared to their biovolume one day before the treatment. The dominant cyanobacterial species *Aphanizomenon* sp. and *P. rubescens* were completely eliminated by the H_2O_2 treatments in Lake Oosterduinse Meer and Lake Klinkenbergerplas, respectively, and did not return during the growing season. However, new cyanobacterial blooms dominated by other taxa developed in these lakes later in summer (*Dolichospermum* spp. and *P. agardhii* in Lake Oosterduinse Meer; *Aphanizomenon* sp. in Lake Klinkenbergerplas). In two cases, the same cyanobacteria returned. *Dolichospermum* spp. were largely eliminated during the July treatment in Lake Kralingse Plas, but returned after approximately one month. Sediment samples showed high abundances of *Dolichospermum* spp. akinetes in the sediment of the lake during the summer period (M.J. van Herk, unpublished data), which may have provided the inoculum for the return of *Dolichospermum* spp. In Lake Oosterduinse Meer, *P. agardhii* was suppressed after the August treatment, but formed a new cyanobacterial bloom approximately three weeks later, possibly from remnants of the previous pelagic population or by recolonization from the adjacent ditch.

Despite the proposed defenses against reactive oxygen species in diazotrophic cyanobacteria (Bernroitner *et al.*, 2009; Banerjee *et al.*, 2013), *Aphanizomenon* sp. and *Dolichospermum* spp. were very sensitive to the applied H_2O_2 concentrations and declined strongly during the lake treatments. *Aphanizomenon* sp. has also previously been shown to be sensitive to H_2O_2 treatments (Lusty and Gobler, 2020). In contrast, the two non-diazotrophic *Planktothrix* species declined slowly and *P. agardhii* even recovered within several days after the August treatment in Lake Oosterduinse Meer. The lower sensitivity of both *Planktothrix* species in this study contrasts with previous observations in which *P. agardhii* was very sensitive to H_2O_2 (Barroin and Feuillade, 1986; Matthijs *et al.*, 2012; Sinha *et al.*, 2018; Yang *et al.*, 2018). Several reasons might have caused this discrepancy between the studies. First, different strains of the same species may vary in H_2O_2 sensitivity, as has previously been shown for *Microcystis aeruginosa* (Schuurmans *et al.*, 2018; Latour *et al.*, 2022) and *P. rubescens* (Lürling et al., 2020b). Hence, differences in H_2O_2 sensitivity between lakes may be attributed to variation in the genetic composition of the blooms. Second, differences in environmental conditions in the lakes could also have affected the H_2O_2 treatment. For example, it is known that light availability strongly influences the effectiveness of H_2O_2 to suppress cyanobacteria (Drábková et al., 2007a; Mikula *et al.*, 2012; Piel *et al.*, 2020). Compared to *Dolichospermum* spp. and *Aphanizomenon* sp., who are known to be able to position themselves close to the surface where ample light is available (Mantzouki *et al.*, 2016), *Planktothrix* species often thrive in deeper layers under lower light conditions (Kromkamp *et al.*, 2001). During both lake treatments that involved *Planktothrix* species, *Dolichospermum* spp. was blooming at the surface and prevented higher light intensities from penetrating to deeper layers in the water column. In particular, the lower light intensity to which *P. rubescens* in Lake Klinkenbergerplas was exposed (Fig. S2), will have decreased the effectivity of H_2O_2. Third, injection of H_2O_2 into the lakes was confined to the upper 5 m of the water column and hence may not have reached the deeper water layers, which may have provided a spatial refuge for part of the *Planktothrix* populations in the relatively deep Lake Oosterduinse Meer and Lake Klinkenbergerplas.

In contrast to the cyanobacteria, none of the eukaryotic phytoplankton groups showed a significant response to the lake treatments. Hence, our results clearly show that eukaryotic phytoplankton were less sensitive to the applied H_2O_2 than cyanobacteria, in agreement with many previous studies (e.g., Drábková *et al.*, 2007b; Matthijs *et al.*, 2012; Weenink *et al.*, 2015; Lusty and Gobler, 2020; Weenink *et al.*, 2022). The Mehler reaction of eukaryotic phytoplankton species can produce high amounts of H_2O_2 during photosynthesis in excessive light (Asada, 1999), which is subsequently degraded by catalases and/or peroxidases. Thus, eukaryotic photosynthetic organisms need this enzymatic protection against oxidative stress, because their own photosynthetic activity produces H_2O_2. In contrast, cyanobacteria get rid of excess light energy using a Mehler-like reaction mediated by flavodiiron proteins without producing H_2O_2 (Helman *et al.*, 2003; Allahverdiyeva *et al.*, 2015), and hence they have a lower need for protective enzymes than eukaryotic phytoplankton. As a consequence, most eukaryotic phytoplankton are capable to degrade H_2O_2 at a much faster rate than cyanobacteria (Weenink *et al.*, 2021). This difference in photophysiology likely explains why eukaryotic phytoplankton are generally much less affected by H_2O_2 treatments than cyanobacteria.

Our results indicate that eukaryotic phytoplankton even profited from the H_2O_2 treatments, as shown by the increase of chlorophytes and diatoms after the treatments of Lake Kralingse Plas and the increase of

dinoflagellates and diatoms after the first treatment of Lake Oosterduinse Meer. These observations align with previous findings in multispecies laboratory studies and microcosm experiments (Weenink *et al.*, 2015; Yang *et al.*, 2018; Lusty and Gobler, 2020). The lake treatments disrupted cyanobacterial dominance. This disruption facilitated eukaryotic phytoplankton, sometimes after a small initial decline, to fill the open niches that became available after the treatment, when both nutrients and light were available in ample supply. Although cyanobacteria are often strong competitors (Huisman *et al.*, 2018), their elimination by H_2O_2 allowed eukaryotic phytoplankton to temporarily dominate the plankton community for at least several weeks in both Lake Kralingse Plas and Lake Oosterduinse Meer, until ultimately cyanobacterial populations took over again and developed a new bloom. The only exception was Lake Klinkenbergerplas, where eukaryotic phytoplankton had an extremely low abundance prior to the treatment, which created an opportunity for another cyanobacterium (*Aphanizomenon* sp.) to rapidly establish itself after the disruption of the *P. rubescens* bloom by the lake treatment. In total, these results indicate that the presence of eukaryotic phytoplankton can delay the re-establishment of a new cyanobacterial population after a lake treatment but cannot prevent new cyanobacterial blooms later in the season.

4.3. How do H_2O_2 treatments affect zooplankton?

The three main zooplankton groups in our study - copepods, cladocerans and rotifers - differed in their response to the H_2O_2 treatments. Copepods were hardly affected by most lake treatments but showed a pronounced although non-significant decline during the August treatment of Lake Kralingse Plas, which was one of the most severe treatments in our dataset with exposure to relatively high H_2O_2 concentrations during more than 24 h. Cladocerans showed a large and significant decrease in abundance after the August treatment of Lake Kralingse Plas, and subsequently their abundance in this lake remained persistently low. Conversely, cladoceran populations showed smaller non-significant declines after the treatment of Lake Klinkenbergerplas and the August treatment of Lake Oosterduinse Meer, and thereafter their abundances in these two lakes stabilized. In contrast, the smaller rotifers strongly declined after each lake treatment, except after the June treatment of Lake Oosterduinse Meer, which was the mildest treatment in our dataset during which the exposure to concentrations of 2 mg L^{-1} H_2O_2 lasted only ~3 h.

Similar results were observed in other studies. Matthijs *et al.* (2012) found that population abundances of the cladocerans *Daphnia* and *Diaphanosoma* spp. were not affected by the lake treatment of 2 mg L^{-1} H_2O_2, but were almost completely eliminated in lake enclosures exposed to a higher H_2O_2 dosage of 5 mg L^{-1}. Sinha *et al.* (2018) reported that rotifers (*Brachionus* sp.) and cladocerans (*Daphnia* sp.) declined in experimental ponds treated with 4 mg L^{-1} H_2O_2, whereas calanoid and cyclopoid copepods were not affected. Yang *et al.* (2018) studied lake enclosures in an aquaculture pond and found no effect on zooplankton populations exposed to 1.3 mg L^{-1} H_2O_2, suppression of rotifers and cladocerans in treatments with 6.7 mg L^{-1} and 20 mg L^{-1} H_2O_2, and suppression of copepods only in treatments with 20 mg L^{-1} H_2O_2. Weenink *et al.* (2022) performed a multifaceted study, in which EC_{50} and LC_{50} values were estimated from laboratory toxicity tests with 26 species of phytoplankton, zooplankton and macroinvertebrates, and compared the responses of these organisms to two whole-lake treatments with H_2O_2. Their results showed that the species that were most sensitive to H_2O_2 included not only cyanobacterial taxa, but also rotifers and the small cladocerans *Ceriodaphnia dubia* and *Daphnia pulex*, whereas copepods and ostracods were the least H_2O_2 sensitive zooplankton taxa (Weenink *et al.*, 2022). Hence, combining the results of these previous studies and our own findings, we conclude that rotifers appear the most sensitive zooplankton group, cladocerans are somewhat intermediate, whereas copepods are least sensitive to H_2O_2.

4.4. Cyanotoxin dynamics

The impact of the H_2O_2 treatments on cyanotoxin concentrations varied among the investigated lakes. In Lake Klinkenbergerplas, the concentrations of microcystins, anabaenopeptins and oscillamide were strongly reduced after treatment and subsequently remained low throughout the summer. In contrast, in Lake Oosterduinse Meer microcystins and anabaenopeptins concentrations showed only a temporary decline and then increased again a few days after the August treatment, in parallel with the recovery of the *P. agardhii* population. These findings show that H_2O_2 treatments vary in their effectiveness, and sometimes only have a short-term temporary effect. It is thus important to continue the monitoring of potentially toxic cyanobacteria and their cyanotoxins after lake treatments.

Our results indicate that intracellular cyanotoxins were released into the water column due to lysis of the cyanobacterial bloom, as shown by the decrease of intracellular microcystins and anabaenopeptins concentrations and concomitant increase of their extracellular concentrations after the lake treatments. A similar release of cyanotoxins is also commonly observed in laboratory cultures of cyanobacteria exposed to H_2O_2 (e.g., Ross *et al.*, 2006; Schuurmans *et al.*, 2018; Sandrini *et al.*, 2020), and is caused by the damaging effects of high oxidative stress on cell membrane integrity (Mikula *et al.*, 2012; Huo *et al.*, 2015).

Interestingly, the extracellular anabaenopeptins concentration peaked within a few hours after the H_2O_2 treatment, whereas the extracellular microcystins concentration peaked about two days later, both in Lake Oosterduinse Meer and Lake Klinkenbergerplas. This delayed appearance of extracellular microcystins is also known from laboratory studies (Schuurmans *et al.*, 2018), and is in line with previous findings that extracellular microcystins may at first be undetectable because they can bind to proteins under oxidative stress (Zilliges *et al.*, 2011), while they may detach from these proteins and thus be released into the water column when the proteins are being degraded or after the oxidative stress is relieved (Miles *et al.*, 2016; Schuurmans *et al.*, 2018). These observations warn that cyanotoxins can still be present in lakes after the cyanobacterial blooms have been effectively removed by H_2O_2 treatments.

Our results show, however, that extracellular microcystin concentrations declined a few days after the H_2O_2 treatments, although dmMC-RR subsequently maintained low extracellular concentrations (<0.5 μg L^{-1}) in Lake Klinkenbergerplas as long as *P. rubescens* was present. Similarly, other studies also observed a rapid decline of the extracellular microcystin concentration a few days after the lake was treated with H_2O_2 (Matthijs *et al.*, 2012; Yang *et al.*, 2018). Contrary to earlier suggestions by Matthijs *et al.* (2012), it is unlikely that chemical oxidation by H_2O_2 is directly responsible for the degradation of microcystins (He *et al.*, 2012; Huo *et al.*, 2015; Schuurmans *et al.*, 2018; Piel *et al.*, 2020), because the H_2O_2 concentration applied to the lakes seems too low for rapid chemical oxidation of microcystins (Huo et al., 2015) and the added H_2O_2 had already disappeared from the lakes by the time that the microcystins were degraded (compare Fig. 2C and Fig. 4E and F). More likely, microcystin-degrading bacteria were actively involved in the pronounced decline of the extracellular microcystin concentration a few days after the treatments (Jones *et al.*, 1994; Dziga et al., 2019a; Massey and Yang, 2020). Recent studies have further shown that addition of microcystin-degrading enzymes (Dziga et al., 2019b) and modified clays (Lürling et al., 2020a) after H_2O_2 treatments can strongly reduce extracellular microcystin concentrations in the water column.

4.5. Nutrient dynamics

After treatment, the ammonium concentration temporarily increased in the water column of all three lakes. A similar increase of the ammonium concentration was also observed in previous lake treatments with H_2O_2 (Matthijs *et al.*, 2012; Sinha *et al.*, 2018), and can be attributed to the degradation of nitrogen-rich molecules such as proteins released by

the lysing cyanobacterial bloom. What caused the steep temporary decline of the phosphate concentration in Lake Klinkenbergerplas and the more subtle decrease of phosphate in Lake Kralingse Plas is less clear. Possible explanations include chemical precipitation of phosphate with iron that has been oxidized by the H_2O_2 treatment (Li *et al.*, 2009), or phosphate uptake by a rapidly growing microbial population feasting on the organic molecules released by the lysing cyanobacteria (Piel *et al.*, 2021).

5. Conclusions and recommendations

Our results confirm that addition of H_2O_2 in low concentrations can be an effective method to rapidly suppress cyanobacterial blooms in eutrophic and hypertrophic lakes. In all cases, the treatment disrupted cyanobacterial dominance in the lakes, as the dominant cyanobacterial species declined by at least 84% within a few days. Cyanotoxins and ammonium were released from the lysing cyanobacterial blooms into the lake water, and the free extracellular cyanotoxins declined below detection after several days. We therefore recommend that cyanotoxins should be carefully monitored after H_2O_2 treatments and that lakes that contain highly toxic cyanobacterial blooms should remain closed for recreation and other services such as drinking water production until cyanotoxin concentrations have declined below guideline values.

Eukaryotic phytoplankton were much less affected by the applied H_2O_2 concentrations, and often temporarily increased in abundance after the collapse of the cyanobacterial bloom. Over time, however, a new cyanobacterial bloom may develop in the lake, which may instigate a second H_2O_2 treatment later in the season. Our results also showed major differences in zooplankton responses to the lake treatments. Rotifers were more sensitive to H_2O_2 than cladocerans, while copepods were hardly affected by most lake treatments. Thus, successful lake applications will require a careful balance between a sufficiently high H_2O_2 dosage to effectively suppress the cyanobacterial bloom and a sufficiently low H_2O_2 dosage (preferably ≤ 2 mg L^{-1}) to minimize impacts on non-target species.

CRediT authorship contribution statement

Tim Piel: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing. **Giovanni Sandrini:** Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. **Erik F.J. Weenink:** Investigation, Methodology, Writing – review & editing. **Hongjie Qin:** Investigation, Methodology, Writing – review & editing. **Maria J. van Herk:** Investigation, Methodology, Writing – review & editing. **Mariël Léon Morales-Grooters:** Investigation, Methodology, Writing – review & editing. **J. Merijn Schuurmans:** Investigation, Methodology, Writing – review & editing. **Pieter C. Slot:** Investigation, Methodology, Writing – review & editing. **Geert Wijn:** Methodology, Writing – review & editing. **Jasper Arntz:** Investigation, Methodology, Writing – review & editing. **Sevasti-Kiriaki Zervou:** Investigation, Methodology, Writing – review & editing. **Triantafyllos Kaloudis:** Investigation, Methodology, Writing – review & editing. **Anastasia Hiskia:** Investigation, Methodology, Writing – review & editing. **Jef Huisman:** Conceptualization, Data curation, Supervision, Writing – review & editing. **Petra M. Visser:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Supplementary materials

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