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# Selective suppression of harmful cyanobacteria in an entire lake with hydrogen peroxide

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## ABSTRACT

Although harmful cyanobacteria form a major threat to water quality, few methods exist for the rapid suppression of cyanobacterial blooms. Since laboratory studies indicated that cyanobacteria are more sensitive to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) than eukaryotic phytoplankton, we tested the application of H<sub>2</sub>O<sub>2</sub> in natural waters. First, we exposed water samples from a recreational lake dominated by the toxic cyanobacterium *Planktothrix agardhii* to dilute H<sub>2</sub>O<sub>2</sub>. This reduced the photosynthetic vitality by more than 70% within a few hours. Next, we installed experimental enclosures in the lake, which revealed that H<sub>2</sub>O<sub>2</sub> selectively killed the cyanobacteria without major impacts on eukaryotic phytoplankton, zooplankton, or macrofauna. Based on these tests, we introduced 2 mg L<sup>-1</sup> (60 μM) of H<sub>2</sub>O<sub>2</sub> homogeneously into the entire water volume of the lake with a special dispersal device, called the water harrow. The cyanobacterial population as well as the microcystin concentration collapsed by 99% within a few days. Eukaryotic phytoplankton (including green algae, cryptophytes, chrysophytes and diatoms), zooplankton and macrofauna remained largely unaffected. Following the treatment, cyanobacterial abundances remained low for 7 weeks. Based on these results, we propose the use of dilute H<sub>2</sub>O<sub>2</sub> for the selective elimination of harmful cyanobacteria from recreational lakes and drinking water reservoirs, especially when immediate action is urgent and/or cyanobacterial control by reduction of eutrophication is currently not feasible. A key advantage of this method is that the added H<sub>2</sub>O<sub>2</sub> degrades to water and oxygen within a few days, and thus leaves no long-term chemical traces in the environment.

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## 1. Introduction

Cyanobacterial blooms are favoured by high temperatures and nutrient load, and have increasingly become a major

nuisance in many freshwater and brackish ecosystems (Chorus and Bartram, 1999; Jöhnk et al., 2008; Paerl and Huisman, 2008). Dense cyanobacterial blooms shade away light for other phytoplankton (Mur et al., 1999; Huisman et al.,

Abbreviations: H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; ROS, reactive oxygen species.

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2004), and generally offer low food quality for herbivorous zooplankton compared to most eukaryotic phytoplankton species (Ghadouani et al., 2003; Wilson et al., 2006). The high turbidity of cyanobacterial blooms may also smother the growth of aquatic macrophytes, suppressing important underwater habitat for invertebrates and fish (Scheffer et al., 1993; Gulati and Van Donk, 2002). Furthermore, some cyanobacteria produce toxins, which can cause serious and sometimes fatal liver, digestive and neurological diseases (Carmichael, 2001; Codd et al., 2005). Toxic cyanobacterial blooms pose a significant threat to birds, mammals and human health, and make the water less suitable for drinking water, agricultural irrigation, fishing and recreation (Chorus and Bartram, 1999; Huisman et al., 2005).

Nutrient reduction is arguably the best strategy to reduce the incidence of harmful cyanobacterial blooms (Dokulil and Teubner, 2000; Conley et al., 2009; Smith and Schindler, 2009). Additional measures, such as artificial mixing (Visser et al., 1996; Huisman et al., 2004) and flushing (Verspagen et al., 2006; Mitrovic et al., 2011) of lakes, may also suppress cyanobacterial populations. Some lakes have been treated with clays that bind phosphate and coagulate with cyanobacterial cells, causing their sedimentation (Robb et al., 2003; Van Oosterhout and Lürling, 2011). Chemicals such as aluminium and copper have been used as cyanobacterial algicides (Griffiths and Saker, 2003). Each of these strategies has its drawbacks. Artificial mixing of lakes is costly. Flushing of lakes is not always feasible, and may cause water deficits in upstream areas during dry summer periods. Algicides can induce massive release of cyanotoxins by lysing cyanobacterial cells, thus engraving rather than resolving water-quality problems (Kenefick et al., 1993; Griffiths and Saker, 2003). The results of nutrient reduction programs often become effectively visible only after several years due to, e.g., sustained nutrient input from diffuse sources or internal nutrient loading from the sediment (Gulati and Van Donk, 2002; Søndergaard et al., 2003). This contrasts with societal demands, as bans on recreation and the economic damage caused by the closure of recreational waters or diminished access to irrigation and drinking water often request for immediate results (Verspagen et al., 2006; Guo, 2007). Hence, there is a clear need for effective intervention techniques that can rapidly suppress the proliferation of upcoming cyanobacterial blooms without negative side-effects on overall water quality.

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is a reactive oxygen species produced in natural waters mostly by the photolysis of dissolved organic matter exposed to UV radiation (Cooper and Zika, 1983).  $\text{H}_2\text{O}_2$  is also produced biologically, as by-product of photosynthesis, respiration and other metabolic processes (Apel and Hirt, 2004; Asada, 2006) and as signalling molecule (Veal et al., 2007).  $\text{H}_2\text{O}_2$  decays to water and oxygen by chemical and biological oxidation–reduction reactions, with decay rates in the order of a few hours to a few days depending on biological activity and the presence of redox-sensitive metals such as iron and manganese (Cooper and Zepp, 1990; Häkkinen et al., 2004).  $\text{H}_2\text{O}_2$  concentrations in surface waters of lakes range from 1 to 30  $\mu\text{g L}^{-1}$  (30–900 nM) (Cooper et al., 1989; Häkkinen et al., 2004). Light exposure of  $\text{H}_2\text{O}_2$  in the presence of iron or manganese may produce trace amounts of the highly reactive hydroxyl radical (Zepp et al., 1992). These

radicals cause damage to cells by the oxidation of proteins, lipids and DNA, resulting in severe oxidative stress (Mittler, 2002; Apel and Hirt, 2004; Latifi et al., 2009).

Several laboratory studies have indicated that cyanobacteria are more sensitive to hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) than green algae and diatoms. Barroin and Feuillade (1986) showed that as little as 1.75 ppm (corresponding to 1.75  $\text{mg L}^{-1}$ ) of  $\text{H}_2\text{O}_2$  had a deleterious effect on laboratory cultures of the cyanobacterium *Planktothrix rubescens* (formerly known as *Oscillatoria rubescens*), while a 10 times higher concentration proved totally harmless to the green alga *Pandorina morum*. Subsequently, Drábková et al. (2007a,b) investigated several more species, and showed that  $\text{H}_2\text{O}_2$  had generally a much stronger inhibitory effect on the photosynthesis of cyanobacteria than of eukaryotic phytoplankton. Barrington and Ghadouani (2008) found that cyanobacteria declined twice as fast as green algae and diatoms after  $\text{H}_2\text{O}_2$  addition to wastewater samples, and their recent work shows that application of  $\text{H}_2\text{O}_2$  to wastewater treatment ponds removed more than 50% of the cyanobacterial biomass within 48 h (Barrington et al., 2011).

Hence, these studies suggested the use of low concentrations of  $\text{H}_2\text{O}_2$  for the selective removal of cyanobacteria in lakes. Although adding chemicals to natural waters appears a somewhat strange management strategy,  $\text{H}_2\text{O}_2$  addition might not be as bad as it seems.  $\text{H}_2\text{O}_2$  occurs naturally in small concentrations in all surface waters (Cooper and Zika, 1983), and many organisms produce  $\text{H}_2\text{O}_2$  (Asada, 2006; Veal et al., 2007). Furthermore, since low  $\text{H}_2\text{O}_2$  concentrations are intended to work selectively against cyanobacteria, this method, if successful, may have the advantage that other aquatic organisms will remain largely unharmed. Finally,  $\text{H}_2\text{O}_2$  rapidly breaks down into water and oxygen, such that the added  $\text{H}_2\text{O}_2$  is unlikely to stay in the ecosystem for long. Yet, until now, the idea of adding  $\text{H}_2\text{O}_2$  to suppress cyanobacterial blooms has never been tested in natural waters.

In this study, we investigate whether  $\text{H}_2\text{O}_2$  addition is able to selectively suppress cyanobacteria in an entire lake without affecting other biota. Our lake experiment was carried out in Lake Koetshuis, a small lake in the Netherlands. This lake suffered from frequent closure for recreation due to dense blooms of the nuisance cyanobacterium *Planktothrix agardhii*, which produced high concentrations of the hepatotoxin microcystin. The nearby Lake Langebosch served as control. The lake experiment was carried out in three steps. First, we ran laboratory tests with water samples taken from the lake to test the  $\text{H}_2\text{O}_2$  sensitivity of *P. agardhii*. Next, we used enclosures placed in the lake to estimate which range of  $\text{H}_2\text{O}_2$  concentrations would specifically hit the cyanobacteria while leaving other phytoplankton and zooplankton largely unaffected. Finally, we mixed the desired  $\text{H}_2\text{O}_2$  concentration homogeneously into the entire lake. For this purpose, we designed a small boat with a special ‘water harrow’, injecting dilute  $\text{H}_2\text{O}_2$  at different depths in the water column till just above the sediment. The entire operation was sized to accomplish treatment of the entire lake within a single day. After treatment of the lake, we monitored the  $\text{H}_2\text{O}_2$  concentration, the photosynthetic vitality of the cyanobacteria, and the population abundances of the cyanobacteria and other biota during several weeks.

## 2. Materials and methods

### 2.1. Description of the lake

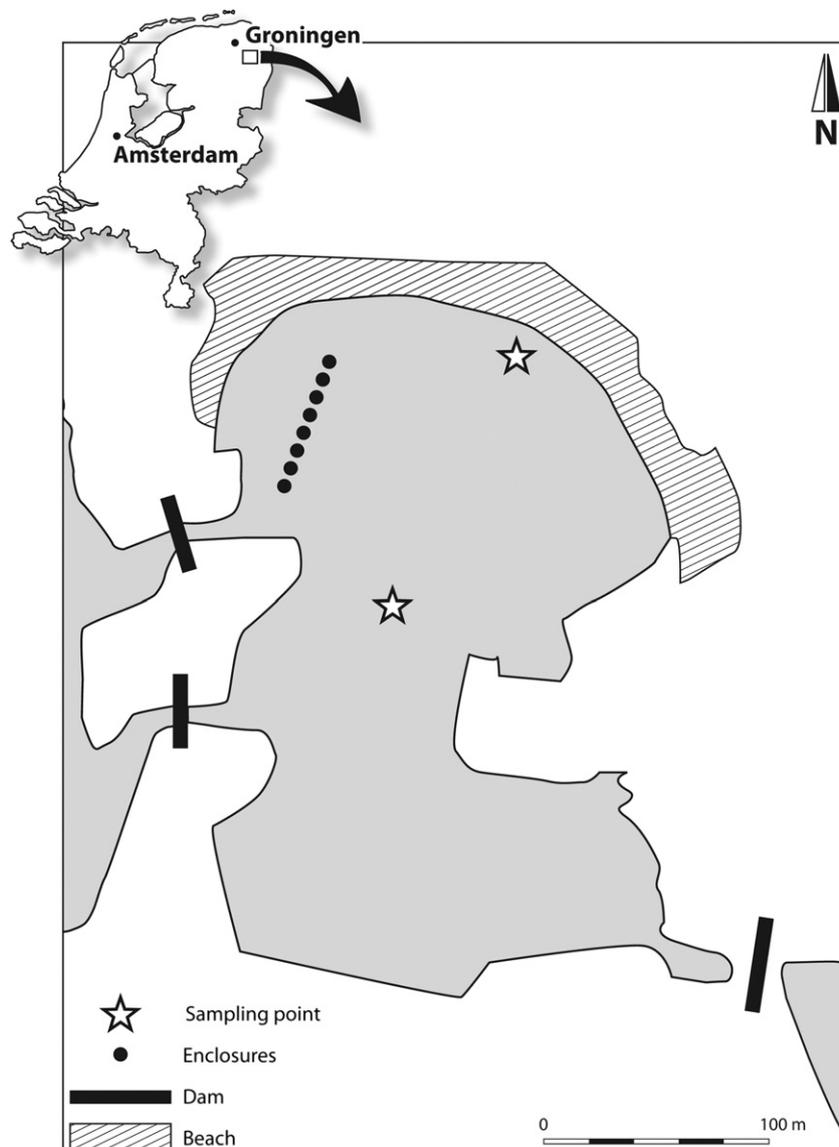
Lake Koetshuis (Fig. 1) is a shallow lake with a maximum depth of 2 m and a surface area of  $\sim 0.12 \text{ km}^2$ . The lake is located in the recreation area Borgerswold ( $53^\circ 6' \text{N}$ ,  $6^\circ 52' \text{E}$ ), which belongs to the municipality of Veendam, in the province of Groningen, the Netherlands. Over the summers of 2000–2010, total phosphorus concentrations in the lake averaged  $0.114 \text{ mg L}^{-1}$ , including  $0.031 \text{ mg L}^{-1}$  soluble reactive phosphorus. Total nitrogen concentrations averaged  $2.20 \text{ mg L}^{-1}$ , including  $0.11 \text{ mg L}^{-1}$  ammonium,  $0.02 \text{ mg L}^{-1}$  nitrite, and  $0.03 \text{ mg L}^{-1}$  nitrate. After works to adapt the lake for water skiing in 2007, which caused a temporary increase in turbidity, the lake became dominated by the harmful cyanobacterium *P. agardhii*, reaching population abundances of  $200\text{--}800 \times 10^3 \text{ cells mL}^{-1}$  during blooms. Since then, transparency of the lake has been

low, with Secchi depths between 0.3 and 0.4 m during the summer season. To limit input of nutrients and cyanobacteria from nearby waters, Lake Koetshuis was hydrologically isolated from other lakes in the Borgerswold area by the placement of dams in early 2009 (Fig. 1). However, isolation did not prevent the development of a *Planktothrix* bloom in late spring and early summer of 2009, and it was therefore decided to investigate whether the cyanobacterial blooms in Lake Koetshuis could be controlled by  $\text{H}_2\text{O}_2$  addition. One of the other lakes in Borgerswold, Lake Langebosch, served as a control. Lake Langebosch is also a *Planktothrix*-dominated lake, with a Secchi depth of 0.3–0.4 m, a maximum depth of 6 m and a surface area of  $0.46 \text{ km}^2$ .

### 2.2. Experiments

#### 2.2.1. Short-term laboratory incubations

To assess the  $\text{H}_2\text{O}_2$  sensitivity of field material dominated by *P. agardhii*, 10 water samples of 500 mL each were taken from



**Fig. 1** – Map of Lake Koetshuis with the location of the enclosures and the two sampling points. All inlets are closed by dams since early 2009, but the inlet in the south-east can be opened during periods of draught.

Lake Koetshuis on June 15, 2009, and incubated in the laboratory at room temperature and ambient daylight. Earlier laboratory studies by Barroin and Feuillade (1986) and Drábková et al. (2007a,b) had shown that cyanobacteria were sensitive to a few  $\text{mg L}^{-1}$  of  $\text{H}_2\text{O}_2$ . Accordingly, we added different volumes of a  $30 \text{ g L}^{-1}$  (3% w/v) stock solution of  $\text{H}_2\text{O}_2$  to reach final concentrations of 0, 0.5, 1, 2 and  $4 \text{ mg L}^{-1}$   $\text{H}_2\text{O}_2$  in the incubation bottles. Each of these five treatments was carried out in duplicate. Subsequent changes in  $\text{H}_2\text{O}_2$  concentration and in photosynthetic vitality estimated by pulse amplitude modulation fluorescence (see Section 2.3.3) were monitored for several hours.

Additional water samples were filtered over  $0.45 \mu\text{m}$  membrane filters (Whatman, Maidstone, UK) to remove bacteria, phytoplankton and other organisms. The filtered water samples were incubated under similar conditions as described above to estimate degradation rates of  $\text{H}_2\text{O}_2$  by chemical processes.

### 2.2.2. Enclosure experiments

Eight circular enclosures of plexiglass, with a diameter of 1.1 m and a height of 1.5 m, were placed in the lake at a water depth of  $\sim 1.2 \text{ m}$  on June 14, 2009. The lower 10 cm of the enclosures was pressed into the sediment to stabilize the enclosures and prevent seeping in of surrounding water. Two days later, on June 16, different volumes of a  $30 \text{ g L}^{-1}$  stock solution of  $\text{H}_2\text{O}_2$  were mixed into the enclosures to reach final concentrations of 0, 2.5, 5 and  $8 \text{ mg L}^{-1}$   $\text{H}_2\text{O}_2$ . Each of these 4 treatments was carried out in duplicate. Subsequently, we monitored changes in photosynthetic vitality, phytoplankton and zooplankton abundances during several days.

### 2.2.3. Application to the entire lake

$\text{H}_2\text{O}_2$  was applied to Lake Koetshuis on July 14, 2009. The morning of this day was sunny, while the afternoon showed a mild thunderstorm and partly overcast sky with light intensities ranging from 100 to  $1200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . The water and air temperature were  $19.5$  and  $19^\circ\text{C}$ , respectively. The day after the sky was cloudy all day long.

We aimed at the homogeneous dispersal of a low concentration of  $\text{H}_2\text{O}_2$  throughout the entire body of the lake within a single day. To this end, we developed a special technique with a dispersal device that we have called the ‘water harrow’ (Matthijs et al., 2011). A container with one cubic meter of concentrated  $\text{H}_2\text{O}_2$  stock solution was mounted on a small boat (Fig. 2). These stock solutions were obtained from Solvay in 1000 L canisters as a sorbitol stabilised 10% w/v  $\text{H}_2\text{O}_2$  solution in water. On the vessel, computer-controlled pumps mixed the  $\text{H}_2\text{O}_2$  stock with lake water for an initial 500-fold dilution of the stock to arrive at a  $\text{H}_2\text{O}_2$  concentration of  $200 \text{ mg L}^{-1}$ . This pre-diluted solution was subsequently dispersed throughout the water body to reach an effective concentration of  $2 \text{ mg L}^{-1}$  in the entire lake.

Homogeneous distribution of the dilute  $\text{H}_2\text{O}_2$  solution into the lake proceeded via injection with the water harrow, a manifold extending about 2 m on each side of the boat (Fig. 2). The water harrow carried a  $\text{H}_2\text{O}_2$  dispersal system branching into 3 tubes spaced 1 m apart on each side of the boat. Each tube contained a series of outlet valves every 20 cm that can be positioned vertically from 50 cm to a maximum of 6 m depth in the water column for the dispersal of  $\text{H}_2\text{O}_2$ . An

additional tubing system delivered a stream of compressed air in parallel with the dosing of  $\text{H}_2\text{O}_2$  to achieve optimal mixing.  $\text{H}_2\text{O}_2$  injection stopped at 40 cm above the lake sediment to leave an undisturbed protective layer of bottom water safeguarding benthic organisms living in the sediments from exposure to  $\text{H}_2\text{O}_2$ . Positioning of the tubing and pump rates were computer-controlled using custom made software. The software integrated the GPS position and cruise speed of the vessel, the water column depth to be treated, and the stock concentration available for injection to dynamically calculate the required pump speed for homogeneous dosing of  $\text{H}_2\text{O}_2$  at the desired final concentration.

We aimed to bring a  $\text{H}_2\text{O}_2$  concentration in the lake that would selectively suppress the cyanobacteria without affecting other biota. The desired  $\text{H}_2\text{O}_2$  concentration of  $2 \text{ mg L}^{-1}$  was estimated from the laboratory incubations and lake enclosure experiments, as will be reported in Results section (Section 3.3). The total lake volume was estimated at  $\sim 240,000 \text{ m}^3$ , and it took a full working day, from 9 am until 9 pm, to inject the entire lake with  $\text{H}_2\text{O}_2$ . Some shallow near-shore areas could not be reached by the water harrow, and were treated separately by manual addition of a calculated amount of properly pre-diluted  $\text{H}_2\text{O}_2$  and mixing of the water by gentle circulation. The  $\text{H}_2\text{O}_2$  concentration was subsequently monitored at 20 random sites in the lake 3 h after  $\text{H}_2\text{O}_2$  addition and once more the next day.

For the entire operation, permission was obtained from the responsible authorities, and safety measures were taken in accordance with legislation. Concentrated  $\text{H}_2\text{O}_2$  was delivered on site by a certified transport company on the morning of its usage. Prior to the day of treatment public announcements were made and warning signs indicated that the lake was closed for public access. Stocks of  $\text{H}_2\text{O}_2$  were stored in a restricted area with entrance on permission only. The application was carried out by professionals experienced in handling of hydrogen peroxide.

## 2.3. Analyses

### 2.3.1. Hydrogen peroxide

Two methods were used to determine the hydrogen peroxide concentration. For the laboratory experiments, a 3 mL sample was placed immediately in an oxygen electrode chamber to measure total oxygen evolution after addition of  $1.5 \mu\text{L}$  (corresponding to 1 mU of enzyme activity) of the enzyme catalase (bovine liver, Sigma), which converts  $\text{H}_2\text{O}_2$  into water and oxygen. Given the rapid degradation rate of  $\text{H}_2\text{O}_2$ , transportation of water samples from the lake to the laboratory would take too long to determine the  $\text{H}_2\text{O}_2$  concentration in the lake by the same method. In the field, we therefore used peroxide Quantofix test sticks (Macherey–Merck, Darmstadt, Germany). These ready-to-go test sticks are intended only for direct measurement of distinct  $\text{H}_2\text{O}_2$  concentrations of 1, 3, 10, 30 and  $100 \text{ mg L}^{-1}$ . However, we were able to refine the measurements by making photographs of each test stick and subsequent comparison to our own calibration series.

### 2.3.2. Phytoplankton, zooplankton and macrofauna

Phytoplankton, zooplankton and macrofauna were sampled from the enclosures and at two sampling points in the lake:



Fig. 2 – The ‘water harrow’, the device that was used to bring hydrogen peroxide into Lake Koetshuis.

one in the middle of the lake and the other at a distance of 5 m from the recreational beach (Fig. 1).

Phytoplankton was sampled by taking 500 mL of water at 50 cm depth for duplicate determination of the phytoplankton species composition and microcystin concentration, in accordance with the monitoring protocol of the Dutch bathing water directive. The phytoplankton samples were fixed with Lugol’s iodine. The phytoplankton was identified to the species level and counted using an inverted microscope.

For zooplankton, we monitored the abundances of *Daphnia* and *Diaphanosoma* spp., the two dominant genera of herbivorous zooplankton in the lake. For this purpose, 12 subsamples of 2 L each were taken at equidistant depths covering the whole water column. The subsamples were filtered over a plankton net (mesh size 55  $\mu\text{m}$ ), and the filtered material was pooled and fixed with Lugol’s iodine. For each sample, we counted at least 100 individuals of *Daphnia* and *Diaphanosoma* with an inverted microscope to obtain reliable estimates of their population abundances, or we counted the entire sample volume if it contained less than 100 individuals.

Macrofauna was sampled by drawing a hand net (mesh size 0.5 mm) over the lake sediment, covering an area of about 1 m<sup>2</sup>. Macrofauna was identified to the genus level.

### 2.3.3. Photosynthetic yield

The photosynthetic yield is an indicator of the photosynthetic vitality of the phytoplankton. It was measured with a portable mini-PAM instrument (Walz, Effeltrich, Germany). Routinely, an aliquot of 20–50 mL of lake water was filtered over hydrophilic 2.5 cm diameter 0.45  $\mu\text{m}$  pore size glass fiber filters (Millipore) that were mounted on a vacuum filtration manifold with 12 slots for samples (Millipore). After filtration, the loaded filters were covered with rubber stoppers to provide darkness for 10 min, after which the photosynthetic yield was measured with the glass fiber optics of the mini-PAM fluorometer incorporated in a similar rubber stopper. The photosynthetic

yield ( $\Phi_{\text{PSII}}$ , also known as the quantum yield of PSII electron transport) was calculated according to Genty et al. (1989):

$$\Phi_{\text{PSII}} = (F_m - F_0)/F_m \quad (1)$$

where  $F_0$  and  $F_m$  are the minimum and maximum fluorescence, respectively. The photosynthetic yield of the control treatment without H<sub>2</sub>O<sub>2</sub> was used as reference value.

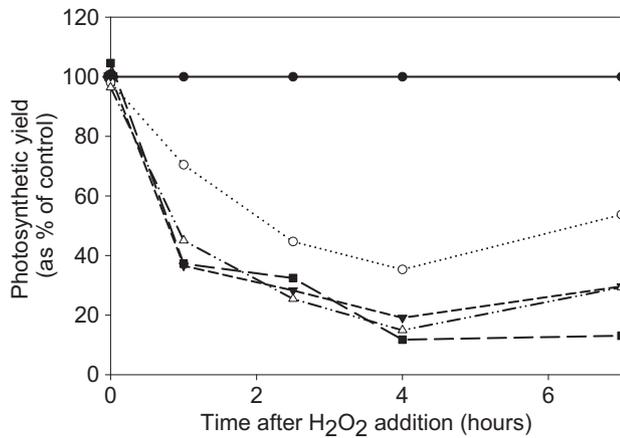
### 2.3.4. Microcystins

Microcystins were extracted from the cells by boiling water samples for 30 min in 50% methanol, thereby pooling the intracellular and extracellular microcystins. Subsequently, the solvent was evaporated under a stream of nitrogen and replaced by distilled water such that the final concentration of methanol did not exceed 5%. Total microcystin concentrations were determined using an enzyme-linked immunosorbent assay (ELISA; An and Carmichael, 1994; Metcalf et al., 2000) with a commercial ELISA kit (Envirogard of SDI). The cross-reactivity of the Envirogard plate kit, given at 50% B/B<sub>0</sub> (the concentration of microcystin causing 50% binding), is 0.31, 0.32, 0.38 and 0.47 ppb for microcystin-LR, -RR, -YR and nodularin, respectively (manufacturer’s specifications).

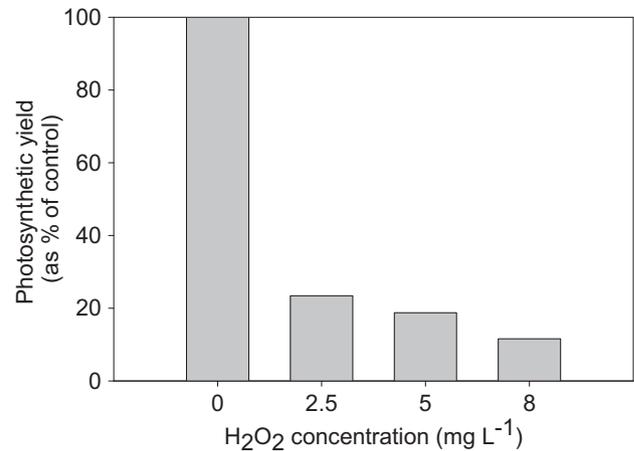
## 3. Results

### 3.1. Short-term laboratory incubations

Water samples with field material of *P. agardhii* incubated in the laboratory with different concentrations of H<sub>2</sub>O<sub>2</sub> showed a rapid decline of the photosynthetic vitality (Fig. 3). H<sub>2</sub>O<sub>2</sub> concentrations of 1, 2 and 4 mg L<sup>-1</sup> resulted in a suppression of the photosynthetic vitality to less than 30% of the control within 3 h, while the H<sub>2</sub>O<sub>2</sub> concentration of 0.5 mg L<sup>-1</sup> was less effective.



**Fig. 3 – Reduction of photosynthetic vitality in laboratory incubations exposed to different H<sub>2</sub>O<sub>2</sub> concentrations.** Photosynthetic vitality is expressed as percentage of the control (no H<sub>2</sub>O<sub>2</sub>) (closed circles 0 mg L<sup>-1</sup>; open circles 0.5 mg L<sup>-1</sup>; triangles down 1 mg L<sup>-1</sup>; triangles up 2 mg L<sup>-1</sup>; squares 4 mg L<sup>-1</sup>). Data show the mean of two duplicates per treatment.



**Fig. 4 – Reduction of photosynthetic vitality in lake enclosures exposed to different H<sub>2</sub>O<sub>2</sub> concentrations.** The photosynthetic vitality is expressed as percentage of the control (no H<sub>2</sub>O<sub>2</sub>), and was determined 3 h after H<sub>2</sub>O<sub>2</sub> had been added to the enclosures. Data show the mean of two duplicate enclosures per treatment.

Short-term incubation of filtered water samples without bacteria and phytoplankton showed that after 3 h of incubation about 0.3 mg L<sup>-1</sup> of H<sub>2</sub>O<sub>2</sub> was consumed by chemical oxidation–reduction processes occurring naturally in the water column. The H<sub>2</sub>O<sub>2</sub> concentration to be added to the lake enclosures and full lake treatment was sufficiently high to compensate for these degradation losses.

### 3.2. Enclosure experiments

The enclosure experiments in the lake showed similar changes in photosynthetic vitality as the earlier laboratory incubations. After 3 h, the photosynthetic vitality had decreased to ~20% of the control in the enclosure experiments with 2.5 mg L<sup>-1</sup> of added H<sub>2</sub>O<sub>2</sub> (Fig. 4). In the enclosures with higher concentrations of H<sub>2</sub>O<sub>2</sub> (5 and 8 mg L<sup>-1</sup>) the photosynthetic vitality was reduced only slightly more.

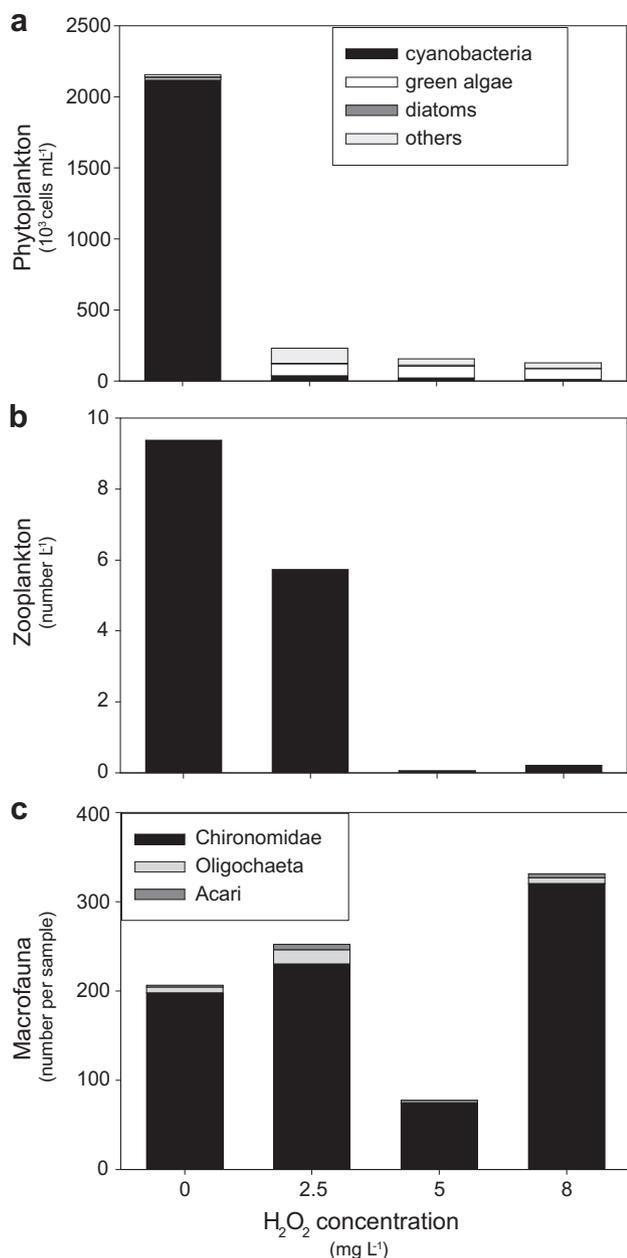
At the onset of the experiments, all enclosures were strongly dominated by the cyanobacterium *P. agardhii* with an average concentration of  $1199 \times 10^3$  cells mL<sup>-1</sup>, while the summed concentration of all other phytoplankton species contributed only  $29 \times 10^3$  cells mL<sup>-1</sup>. In the control treatment (0 mg L<sup>-1</sup> of H<sub>2</sub>O<sub>2</sub>), *P. agardhii* increased to  $2088 \times 10^3$  cells mL<sup>-1</sup> at day 9 of the experiment, while the other phytoplankton species remained below  $40 \times 10^3$  cells mL<sup>-1</sup> (Fig. 5a). This was in marked contrast to the enclosures with added H<sub>2</sub>O<sub>2</sub>, where *P. agardhii* was reduced to  $\sim 35 \times 10^3$  cells mL<sup>-1</sup> at day 9, which is a reduction of >98% compared to the control. Conversely, the eukaryotic phytoplankton species (particularly green algae, but also diatoms, cryptophytes, chrysophytes, and euglenophytes) had increased to  $190 \times 10^3$  cells mL<sup>-1</sup> in the enclosures with 2.5 mg L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> (Fig. 5a). In the enclosures with 5 and 8 mg L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>, the eukaryotic phytoplankton species had also increased compared to the control, but to a slightly lesser extent, and the green algae showed damage of their external cell wall surface under the microscope.

The population abundance of herbivorous zooplankton (*Daphnia* and *Diaphanosoma* spp.) had decreased by about 35% in the enclosures with 2.5 mg L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>, although the daphnids in these enclosures appeared vital and many of them carried eggs. Herbivorous zooplankton was strongly affected at higher H<sub>2</sub>O<sub>2</sub> concentrations, where they had disappeared nearly completely (Fig. 5b).

Macrofauna in the enclosures was dominated by chironomids, and to a lesser extent by oligochaete worms and water mites (Acari). The abundance of macrofauna did not show a clear relationship with the H<sub>2</sub>O<sub>2</sub> addition and was quite variable among enclosures, with lowest numbers in the enclosures at 5 mg L<sup>-1</sup> (Fig. 5c). Overall, macrofauna was more abundant in the enclosures than in the lake itself, which might be related to reduced wind mixing or protection from predators (e.g., fish) in the enclosures. Indeed, one of the two enclosures at 5 mg L<sup>-1</sup> was inhabited by two small perch and the other enclosure was inhabited by two mysids (*Neomysis integer*) for the entire duration of the experiment, while we did not observe these predators in the other enclosures. Both species are known to feed on chironomids, and it is likely that they were responsible for the low chironomid abundance in the 5 mg L<sup>-1</sup> enclosures, a phenomenon that has also been observed in other enclosure studies (Diehl, 1995).

### 3.3. Application to the entire lake

The laboratory incubations indicated that at least 1.0 mg L<sup>-1</sup> of H<sub>2</sub>O<sub>2</sub> would be required to effectively suppress the cyanobacteria (Fig. 3), while the lake enclosure experiments indicated that more than 2.5 mg L<sup>-1</sup> would harm herbivorous zooplankton (Fig. 4b). Furthermore, laboratory incubation of filtered water samples indicated that  $\sim 0.3$  mg L<sup>-1</sup> of H<sub>2</sub>O<sub>2</sub> would be rapidly consumed by chemical oxidation–reduction processes. This so-called matrix consumption was taken into account in our calculation of the desired dosing. Accordingly,



**Fig. 5 – Abundance of (a) phytoplankton, (b) zooplankton (*Daphnia* and *Diaphanosoma* spp.), and (c) macrofauna in lake enclosures exposed to different H<sub>2</sub>O<sub>2</sub> concentrations. The abundances were determined 9 days after H<sub>2</sub>O<sub>2</sub> had been added to the enclosures. Data show the mean of two duplicate enclosures per treatment. Phytoplankton and zooplankton abundances differed by < 5% between duplicate enclosures. Macrofauna was more variable, and differed by ~30% between duplicate enclosures.**

we decided to apply 23 mL of the 10% H<sub>2</sub>O<sub>2</sub> stock solution per m<sup>3</sup> of lake water using the water harrow, thereby aiming at a desired H<sub>2</sub>O<sub>2</sub> concentration of 2.0 mg L<sup>-1</sup> throughout the entire lake.

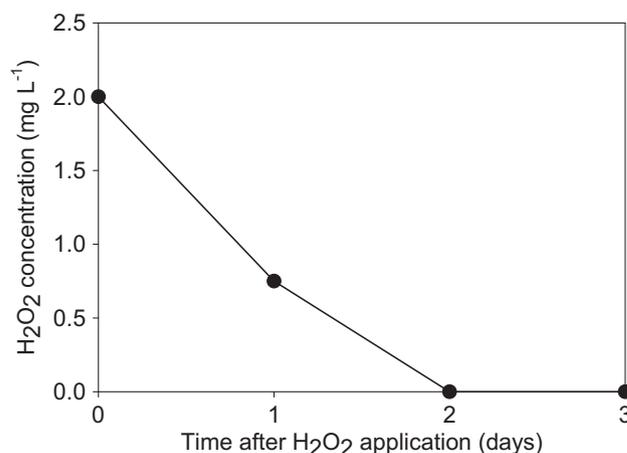
The H<sub>2</sub>O<sub>2</sub> concentration was measured before, during and after the addition at 20 random sites in the lake. Before addition, the H<sub>2</sub>O<sub>2</sub> concentration was below the detection limit of

~0.1 mg L<sup>-1</sup> of the calibrated peroxide Quantofix test sticks at all sites. Within 3 h after addition, we measured H<sub>2</sub>O<sub>2</sub> concentrations of 1.0 mg L<sup>-1</sup> at 2 sites, 2.0 mg L<sup>-1</sup> at 17 sites, and 2.5 mg L<sup>-1</sup> at 1 site. The added H<sub>2</sub>O<sub>2</sub> concentration was rapidly degraded, to 0.7 mg L<sup>-1</sup> after one day and to below the detection limit after two days (Fig. 6).

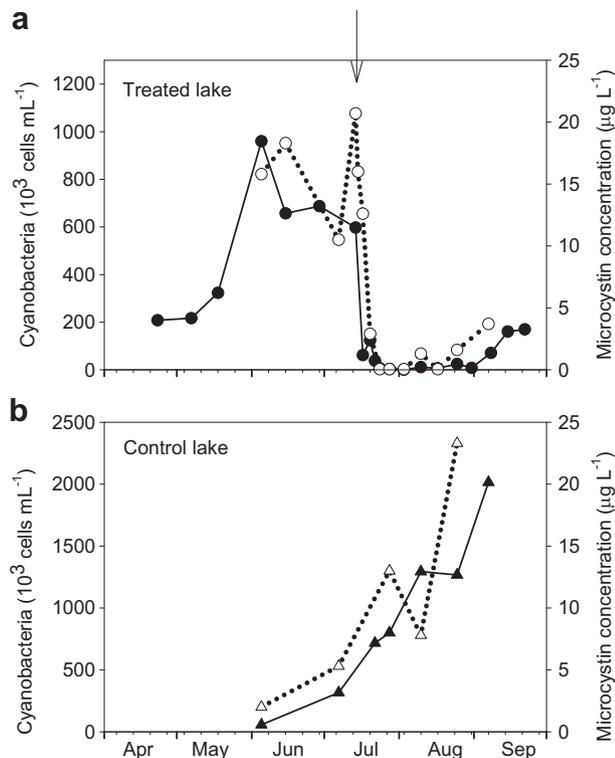
Within 3 h after H<sub>2</sub>O<sub>2</sub> addition, the photosynthetic vitality of the phytoplankton community measured at 7 random locations in the lake declined to only 18–30% of the photosynthetic vitality measured prior to the H<sub>2</sub>O<sub>2</sub> addition. This was followed by a sharp decrease of the cyanobacterial abundance, from 600 × 10<sup>3</sup> cells mL<sup>-1</sup> the day before H<sub>2</sub>O<sub>2</sub> injection to less than 10 × 10<sup>3</sup> cells mL<sup>-1</sup> within 10 days after H<sub>2</sub>O<sub>2</sub> injection (Fig. 7a). The microcystin concentration showed a similar sharp decline with a time lag of 2 days. For comparison, we also collected data from the untreated Lake Langebosch, which served as a control (cf. [Materials and methods](#)). Both the cyanobacterial abundance and microcystin concentration in Lake Langebosch continued to rise (Fig. 7b), in marked contrast to their strong decline in the treated Lake Koetshuis. Note that the *Planktothrix* bloom started later in Lake Langebosch than in Lake Koetshuis. This might be associated with the greater water depth (4–6 m) of Lake Langebosch, which may have resulted in less favourable light conditions for phytoplankton growth and possibly also a slower rise in water temperature during spring.

Cyanobacterial abundance remained low for 7 weeks after H<sub>2</sub>O<sub>2</sub> addition (Fig. 7a). However, in early September a sudden increase of cyanobacteria was observed. Although cyanobacterial abundance remained far below the high levels seen before the treatment, it reached almost 200 × 10<sup>3</sup> cells mL<sup>-1</sup> by the end of September. The cyanobacteria in this autumn period consisted of a mixture of *P. agardhii* and the newly arrived species *Woronichinia naegeliana*. An invasion of *W. naegeliana* was also found in the untreated Lake Langebosch at the same time.

The total abundance of eukaryotic phytoplankton varied between 8 × 10<sup>3</sup> and 26 × 10<sup>3</sup> cells mL<sup>-1</sup>, and apparently was not strongly affected by the H<sub>2</sub>O<sub>2</sub> addition (Fig. 8a). Green algae, consisting of a mixture of many species with highest



**Fig. 6 – Decline of H<sub>2</sub>O<sub>2</sub> concentration in Lake Koetshuis after the H<sub>2</sub>O<sub>2</sub> addition. Data show the mean of 20 H<sub>2</sub>O<sub>2</sub> measurements at different locations within the lake.**

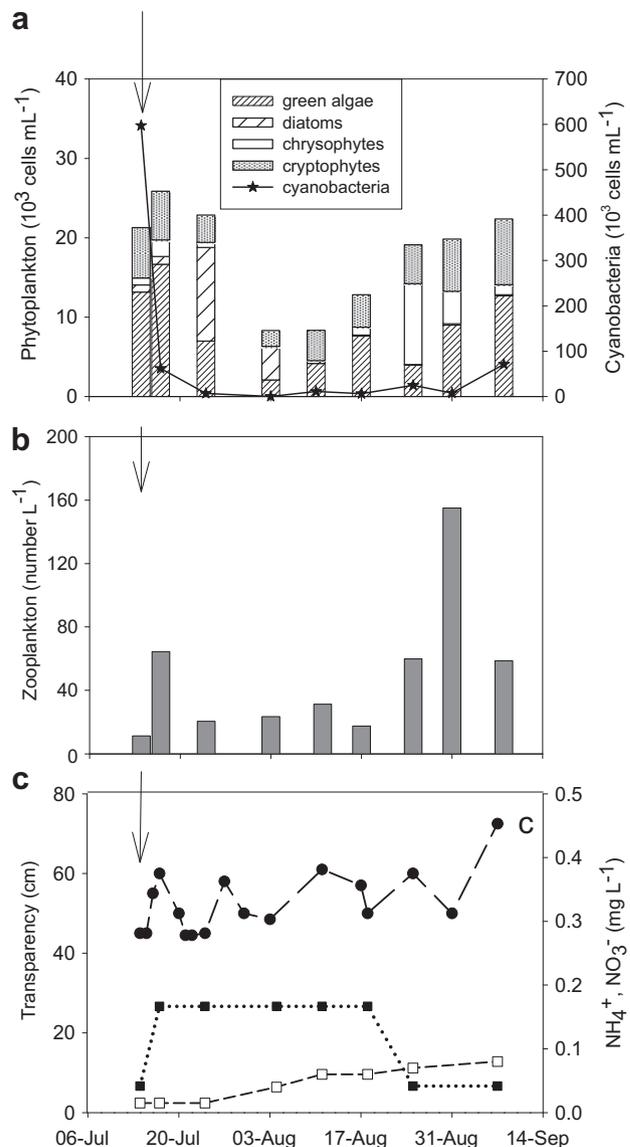


**Fig. 7** – Changes in cyanobacterial abundance (closed symbols) and microcystin concentration (open symbols) after the  $\text{H}_2\text{O}_2$  application (arrow). (a) The treated Lake Koetshuis; (b) the untreated Lake Langebosch. The sample on the day of the application was taken 1 h in advance of the treatment. For both lakes, data are shown for the sampling point near the recreational beach. Cyanobacterial abundances at the sampling point in the middle of the lake were  $\sim 12\%$  higher, and showed similar temporal changes as near the recreational beach.

abundances of *Ankyra judayi*, *Coelastrum* spp., and *Closterium acutum* var. *acutum*, decreased during the first 3 weeks, followed by an increase during the subsequent weeks. Diatoms peaked about 10 days after the  $\text{H}_2\text{O}_2$  application and decreased thereafter. Chrysophytes remained rather stable in the weeks after the application with a clear peak at the end of August. Cryptophytes (mainly *Plagioselmis nannoplantica* and *Cryptomonas* spp.) remained rather stable throughout the period of investigation (Fig. 8a).

Herbivorous zooplankton (*Daphnia* and *Diaphanosoma* spp.) was approximately three times more abundant at the time of the full lake experiment than in the enclosure experiments carried out one month earlier. Their abundance remained relatively stable during the first 5 weeks after  $\text{H}_2\text{O}_2$  addition (Fig. 8b), indicating that the applied dosage of  $2 \text{ mg L}^{-1} \text{ H}_2\text{O}_2$  did not have a major negative effect on herbivorous zooplankton.

Despite the strong decline in cyanobacterial abundance, the transparency of the lake remained stable at 45–60 cm during almost the entire period of investigation (Fig. 8c). After the  $\text{H}_2\text{O}_2$  addition, the ammonium concentration increased rapidly, and decreased a couple of weeks later. The initial



**Fig. 8** – (a) Abundances of eukaryotic phytoplankton (bars) after  $\text{H}_2\text{O}_2$  addition (arrow) to Lake Koetshuis. For comparison, the graph also shows the collapse of the cyanobacterial population (line and stars); note the difference in scale between the two y-axes. (b) Abundance of herbivorous zooplankton after  $\text{H}_2\text{O}_2$  addition (arrow) to the lake. (c) Transparency (closed circles), ammonium (closed squares), and nitrate (open squares) after  $\text{H}_2\text{O}_2$  addition (arrow) to the lake. The sample on the day of the application was taken 1 h in advance of the treatment. Data are shown for the sampling point near the recreational beach; the sampling point in the middle of the lake showed very similar patterns.

increase of ammonium might be associated with massive lysis of cyanobacterial cells after the  $\text{H}_2\text{O}_2$  addition, releasing their intracellular nitrogen into the water column. The nitrate concentration remained low during the first 10 days after  $\text{H}_2\text{O}_2$  addition, and then gradually increased (Fig. 8c). This indicates that the microbially-driven nitrification process, oxidizing ammonium to nitrate, was still functional after  $\text{H}_2\text{O}_2$  addition.

Finally, the biodiversity of the macrofauna remained more or less constant at 25 identified genera during the lake treatment, including all species groups commonly observed in eutrophic shallow lakes (e.g., snails, amphipods, water beetles, dragonfly larvae, mayflies, caddisflies).

#### 4. Discussion

The present study was inspired by the proposal advanced by several laboratory studies (Barroin and Feuillade, 1986; Drábková et al., 2007a,b; Barrington and Ghadouani, 2008) that dilute concentrations of hydrogen peroxide might enable selective elimination of cyanobacteria from mixed phytoplankton communities. To test the feasibility of this innovative approach in natural waters, we applied  $\text{H}_2\text{O}_2$  to a recreational lake to suppress dense blooms of the harmful cyanobacterium *P. agardhii*. The  $\text{H}_2\text{O}_2$  treatment was very successful. The cyanobacterial population collapsed within a few days and stayed low for seven weeks, while the remaining plankton community appeared much less affected.

Finding the right dosage is a critical issue for the application of  $\text{H}_2\text{O}_2$  to lakes. Laboratory incubations of water from Lake Koetshuis showed that a concentration of  $1 \text{ mg L}^{-1}$  was already effective to decrease the photosynthetic vitality of *P. agardhii* drastically. However, the laboratory incubations also pointed at a fast degradation rate of  $\text{H}_2\text{O}_2$ , which necessitated the usage of a slightly higher  $\text{H}_2\text{O}_2$  concentration in field applications. The enclosure experiments showed that eukaryotic phytoplankton and macrofauna thrived at all  $\text{H}_2\text{O}_2$  concentrations applied. However,  $\text{H}_2\text{O}_2$  addition in a concentration higher than  $2.5 \text{ mg L}^{-1}$  would not be wise, as zooplankton appeared rather sensitive. On the basis of this information, we decided to apply a dosage of  $2 \text{ mg L}^{-1} \text{H}_2\text{O}_2$  to Lake Koetshuis to ensure optimal suppression of the cyanobacteria without affecting the zooplankton too much.

A second critical issue is the need for a homogeneous distribution of  $\text{H}_2\text{O}_2$  throughout the entire lake. Local areas of higher concentration would harm the zooplankton population. Conversely, local areas of lower concentration would enable the survival of a small population of cyanobacteria, which could act as a potential source for rapid reinvasion of the lake. Moreover, the fast degradation rate of  $\text{H}_2\text{O}_2$  implied that the entire lake should be treated with  $\text{H}_2\text{O}_2$  within a single day, to avoid reinvasion of sanitized areas by cyanobacteria from not yet treated areas. As a precautionary step, the original stock solution with 10%  $\text{H}_2\text{O}_2$  was pre-diluted with lake water to a concentration of  $200 \text{ mg L}^{-1}$  before the actual addition to the body of the lake took place. Rapid mixing and dilution of the injected  $\text{H}_2\text{O}_2$  was facilitated by simultaneous injection of air using the 'water harrow'. This approach resulted in a highly homogeneous distribution of  $\text{H}_2\text{O}_2$  across the entire lake, as the great majority of measuring points revealed the desired concentration of  $2 \text{ mg L}^{-1}$  and local spots with much higher concentrations of  $\text{H}_2\text{O}_2$  were not detected. The added  $\text{H}_2\text{O}_2$  disappeared naturally within two days.

Following the treatment, the cyanobacterial population collapsed by 90% in three days to more than 99% in ten days. A potential risk of such massive cell lysis is that intracellular toxins (e.g., microcystins) of the cyanobacterial population are

released into the water column, a phenomenon that is commonly observed when cyanobacterial blooms are treated with copper sulphate (Kenefick et al., 1993; Jones and Orr, 1994). However, an important difference between copper sulphate and hydrogen peroxide is that the latter is a strong oxidant enhancing degradation of microcystins (Cornish et al., 2000; Bandala et al., 2004). Our results show that the total microcystin concentration decreased at a similar rate as the cyanobacterial population (Fig. 7a), which is indeed consistent with the idea that microcystins have been oxidized by  $\text{H}_2\text{O}_2$  during or soon after cell lysis. Oxidation by  $\text{H}_2\text{O}_2$  is stimulated by light and causes breakdown of microcystin into peptide residues by modification of the Adda-moiety and/or breakage of the amino-acid ring structure of microcystin (Liu et al., 2003). The release of photosynthetic pigments by the lysing cells may have further contributed to the photocatalytic oxidation of microcystins (Tsuji et al., 1994; Robertson et al., 1999). Products of the oxidation of microcystin-LR and -RR are non-toxic (Lawton et al., 1999; Liu et al., 2002; Rodríguez et al., 2007, 2008), and are thus no longer a danger for public health.

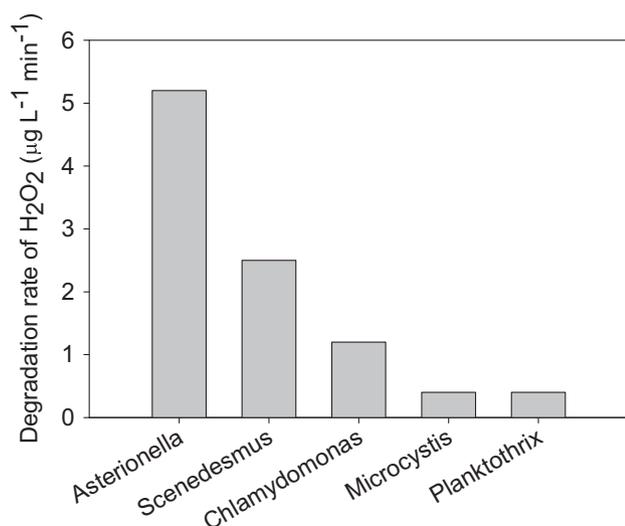
After the  $\text{H}_2\text{O}_2$  application and subsequent cyanobacterial collapse, the concentration of cyanobacteria in the lake remained low for seven weeks. In contrast, eukaryotic phytoplankton species did not show an immediate response to the  $\text{H}_2\text{O}_2$  addition, but displayed gradual changes in species composition over timescales of several weeks. Such changes in species composition are very common in plankton communities (Reynolds, 2006; Benincà et al., 2008), and are probably not treatment related. Apparently, eukaryotic phytoplankton species did not take advantage of the collapse of the cyanobacteria, as the eukaryotes did not show a conspicuous rise in numbers (Fig. 8a). This observation differed from the lake enclosure experiments carried out one month earlier, where eukaryotic phytoplankton strongly increased within 9 days after  $\text{H}_2\text{O}_2$  addition. These contrasting results might be attributed to differences in grazing intensity. Herbivorous zooplankton was three times more abundant during the lake treatment than in the enclosure experiments. Abundant zooplankton could potentially suppress population growth of eukaryotic phytoplankton, many of which are more edible than filamentous cyanobacteria (DeMott et al., 2001; Wilson et al., 2006).

After 7 weeks, the cyanobacteria increased again. *P. agardhii* and a colonial cyanobacterium, *W. naegeliana*, jointly increased to nuisance levels by the end of September. The return of cyanobacterial dominance might have a natural cause, since the added  $\text{H}_2\text{O}_2$  concentrations had long been degraded. However, in early September, some water had been supplied into the lake from adjacent waters (via the inlet at the south-east side of the lake; Fig. 1), to compensate for water losses by evaporation after a dry and warm summer period. Lake Langebosch was at that time also dominated by a mixture of *P. agardhii* and *W. naegeliana*, and it seems likely that this water inlet provided the inoculum for the renewed cyanobacterial dominance.

Why would cyanobacteria be more sensitive to  $\text{H}_2\text{O}_2$  than eukaryotic phytoplankton? It might be that cyanobacteria produce less enzymes eliminating reactive oxygen species (ROS). Indeed, one of the common ROS-eliminating enzymes

in eukaryotes, ascorbate peroxidase, is lacking in cyanobacteria (Passardi et al., 2007), and other enzymes of the haem peroxidase family have been found in only a few cyanobacteria (Bernroitner et al., 2009). However, their function can be substituted by thioredoxin-using peroxidases and other peroxidases ubiquitous in cyanobacteria (Dietz, 2003; Latifi et al., 2009). Another explanation for the  $\text{H}_2\text{O}_2$  sensitivity of cyanobacteria might be provided by the Mehler reaction. The Mehler reaction is a side activity of photosynthetic electron transfer, producing  $\text{H}_2\text{O}_2$  in eukaryotic phytoplankton and higher plants when exposed to high light (e.g., Asada, 2006). Interestingly, recent evidence indicates that the Mehler reaction does not produce  $\text{H}_2\text{O}_2$  or other ROS in cyanobacteria (Helman et al., 2003, 2005; Latifi et al., 2009; Allahverdiyeva et al., 2011). Hence, we suggest that the high  $\text{H}_2\text{O}_2$  sensitivity of cyanobacteria may stem from lower oxidative stress levels in cyanobacterial cells due to the apparent absence of  $\text{H}_2\text{O}_2$  formation during cyanobacterial photosynthesis. This may limit the need for induced expression of ROS-eliminating enzymes in cyanobacteria. This reasoning is supported by preliminary laboratory experiments, where we incubated similar biovolumes of different phytoplankton species with  $\text{H}_2\text{O}_2$ . The results show that the rate of  $\text{H}_2\text{O}_2$  degradation was much lower in cultures of cyanobacteria than in those of green algae and diatoms (Fig. 9). This intriguing hypothesis on the physiological mechanisms underlying the  $\text{H}_2\text{O}_2$  sensitivity of cyanobacteria may clearly warrant further investigation.

Several questions are still open before  $\text{H}_2\text{O}_2$  addition can be routinely applied for the selective elimination of cyanobacteria in lakes. For instance, our results do not tell whether a similar  $\text{H}_2\text{O}_2$  dosage of  $2 \text{ mg L}^{-1}$  would also be optimal for other lakes. The optimal dosage may depend on the population abundance and species composition of the



**Fig. 9 – Degradation rates of  $\text{H}_2\text{O}_2$  by different phytoplankton species. In all experiments an equal biovolume of organisms ( $60 \mu\text{L}$ ) was exposed to  $2 \text{ mg L}^{-1}$  of  $\text{H}_2\text{O}_2$ . Degradation rates were tested for two cyanobacteria (*Microcystis aeruginosa* PGC 7806 and *Planktothrix agardhii* CYA 126/3), two green algae (*Scenedesmus obliquus* CCAP 276/3a and *Chlamydomonas reinhardtii*) and one diatom (*Asterionella formosa*).**

cyanobacteria. Furthermore, the rate at which added  $\text{H}_2\text{O}_2$  will be consumed by biological and chemical oxidation–reduction processes will probably vary among lakes, depending upon, e.g., concentrations of dissolved organic carbon, iron and manganese, redox potential, light conditions, and biological activity (Cooper and Zepp, 1990; Häkkinen et al., 2004). Hence, we highly recommend further study of the  $\text{H}_2\text{O}_2$  sensitivity of defined laboratory cultures and field samples from a wide range of cyanobacteria-dominated lakes, to obtain a better understanding of the dose–response relationship.

Other important questions concern the frequency at which  $\text{H}_2\text{O}_2$  should be applied to lakes, and whether repeated treatments of the same lake system will remain effective on the long term. Cyanobacterial blooms often consist of mixtures of toxic and non-toxic strains (Kurmayer and Kutzenberger, 2003; Kardinaal et al., 2007; Briand et al., 2008), and these toxic and non-toxic strains may respond differently to changes in environmental conditions (Van de Waal et al., 2011). Recently, Zilliges et al. (2011) and Dziallas and Grossart (2011) reported that microcystin-producing strains are less sensitive to  $\text{H}_2\text{O}_2$  than non-microcystin-producing genotypes, which might suggest that  $\text{H}_2\text{O}_2$  addition to lakes could select for microcystin-producing strains. However, the applied  $60 \mu\text{M}$   $\text{H}_2\text{O}_2$  concentration in our lake study was at least two orders of magnitude higher than the nanomolar range of  $\text{H}_2\text{O}_2$  concentrations in the experiments of Zilliges et al. (2011) and Dziallas and Grossart (2011), because we aimed to eliminate the entire cyanobacterial population within a few hours. Yet, this raises the question whether microcystin-producing cyanobacteria might become more resistant to  $\text{H}_2\text{O}_2$  in waters that would be frequently exposed to  $\text{H}_2\text{O}_2$  treatments. We cannot immediately answer this concern, as this will require long-term monitoring of treated lakes. In the subsequent summers of 2010 and 2011, however, *P. agardhii* also dominated Lake Koetshuis, and we did not find an increase in the microcystin content of the *P. agardhii* bloom indicating that selection for toxic strains had not occurred. In both years, we applied  $\text{H}_2\text{O}_2$  to the lake again and this led to a similar collapse of the *P. agardhii* population and microcystin concentration as in 2009.

Reduction of nutrient loading is undoubtedly the most effective approach to control cyanobacterial blooms, and this management strategy is applied worldwide (Conley et al., 2009; Smith and Schindler, 2009). These lake restoration projects have shown that total phosphorus concentrations should be reduced below  $30\text{--}50 \mu\text{g/L}$  for effective control of cyanobacterial blooms (Cooke et al., 1993; Chorus and Mur, 1999; Downing et al., 2001). Although many re-oligotrophication programs have been successful, it may take several years before the first results of nutrient reduction become visible (Gulati and Van Donk, 2002; Jeppesen et al., 2005). Moreover, nutrient reduction may not always be feasible, for instance in densely populated urban areas, in watersheds with intense agriculture, or in systems that are highly eutrophic naturally. Selective removal of cyanobacteria by  $\text{H}_2\text{O}_2$  addition may accelerate the effectiveness of re-oligotrophication programs, and may push lakes from a turbid state into an alternative state without cyanobacterial dominance (Scheffer et al., 1993). Accordingly, based on our results, we propose that  $\text{H}_2\text{O}_2$  application offers an interesting

approach to suppress cyanobacterial blooms, especially for those lakes where immediate action is urgent and/or cyanobacterial control by reduction of eutrophication is currently not feasible.

## 5. Conclusions

Motivated by promising results from laboratory incubations and lake enclosure experiments, we applied 2 mg L<sup>-1</sup> of H<sub>2</sub>O<sub>2</sub> to a recreational lake to suppress dense blooms of the harmful cyanobacterium *P. agardhii*. The cyanobacterial population collapsed by 99% within a few days, which was followed by a similar decline of the microcystin concentration with a time lag of 2 days. Subsequently, cyanobacterial abundances remained low for 7 weeks. Negative impacts on eukaryotic phytoplankton species, zooplankton and macrofauna appeared mild. Although this application of H<sub>2</sub>O<sub>2</sub> is still in its infancy, and there are still many open questions, our results demonstrate that low concentrations of H<sub>2</sub>O<sub>2</sub> can be used for the selective elimination of harmful cyanobacteria in recreational waters. The approach has the key advantage that cyanobacteria are suppressed while other lake biota are much less affected. Moreover, the added H<sub>2</sub>O<sub>2</sub> degrades to water and oxygen within a few days, and thereby leaves no long-term chemical traces in the environment.

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