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### A sea of change

*Impacts of reduced nitrogen and phosphorus loads on coastal phytoplankton communities*

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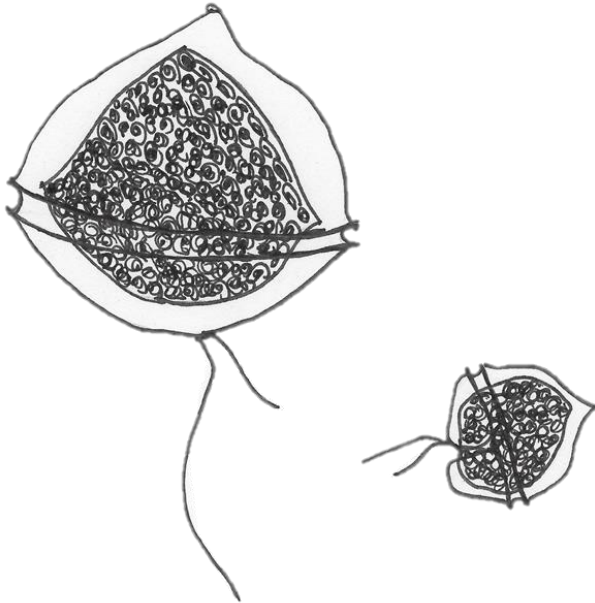
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## Chapter 6

### Termination of a toxic *Alexandrium* bloom with hydrogen peroxide



## **Termination of a toxic *Alexandrium* bloom with hydrogen peroxide**

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## Abstract

The dinoflagellate *Alexandrium ostenfeldii* is a well-known harmful algal species that can potentially cause paralytic shellfish poisoning (PSP). Usually *A. ostenfeldii* occurs in low background concentrations only, but in August of 2012 an exceptionally dense bloom of more than 1 million cells L<sup>-1</sup> occurred in the brackish Ouwerkerkse Kreek in The Netherlands. The *A. ostenfeldii* bloom produced both saxitoxins and spirolides, and is held responsible for the death of a dog with a high saxitoxin stomach content. The Ouwerkerkse Kreek routinely discharges its water into the adjacent Oosterschelde estuary, and an immediate reduction of the bloom was required to avoid contamination of extensive shellfish grounds. Previously, treatment of infected waters with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) successfully suppressed cyanobacterial blooms in lakes. Therefore, we adapted this treatment to eradicate the *Alexandrium* bloom using a three-step approach. First, we investigated the required H<sub>2</sub>O<sub>2</sub> dosage in laboratory experiments with *A. ostenfeldii*. Second, we tested the method in a small, isolated canal adjacent to the Ouwerkerkse Kreek. Finally, we brought 50 mg L<sup>-1</sup> of H<sub>2</sub>O<sub>2</sub> into the entire creek system with a special device, called a water harrow, for optimal dispersal of the added H<sub>2</sub>O<sub>2</sub>. Concentrations of both vegetative cells and pellicle cysts declined by 99.8% within 48 h, and PSP toxin concentrations in the water were reduced below local regulatory levels of 15 µg L<sup>-1</sup>. Zooplankton were strongly affected by the H<sub>2</sub>O<sub>2</sub> treatment, but impacts on macroinvertebrates and fish were minimal. A key advantage of this method is that the added H<sub>2</sub>O<sub>2</sub> decays to water and oxygen within a few days, which enables rapid recovery of the system after the treatment. This is the first successful field application of H<sub>2</sub>O<sub>2</sub> to suppress a marine harmful algal bloom, although *Alexandrium* spp. reoccurred at lower concentrations in the following year. The results show that H<sub>2</sub>O<sub>2</sub> treatment provides an effective emergency management option to mitigate toxic *Alexandrium* blooms, especially when immediate action is required.

## Introduction

Harmful algal blooms (HABs) have been a consistent problem in coastal regions worldwide (Smayda, 1990; Anderson et al., 2002, 2008; Heisler et al., 2008), and seem to increase in terms of frequency, severity and the geographical expansion of toxic species (Hallegraeff, 1993; Paerl and Huisman, 2008). These

blooms can cause devastating impacts on public health, shellfisheries and local wildlife (Ayres, 1975; Paerl, 1988; Scholin et al., 2000; Hoagland et al., 2009).

The dinoflagellate *Alexandrium ostenfeldii* (Paulsen) Balech and Tangen is a HAB species known to produce saxitoxins associated with paralytic shellfish poisoning (PSP). Saxitoxin is a particularly acute paralytic shellfish toxin with as little as 1 mg fatal to humans (Bates and Rapoport, 1975). *A. ostenfeldii* can also produce spirolides, fast-acting neurotoxins which induce similar symptoms as saxitoxin but are not commonly produced by other species of the *Alexandrium* genus (Cembella et al., 2000; Otero et al., 2010). Usually, *A. ostenfeldii* strains produce either saxitoxins or spirolides (Suikkanen et al., 2013). In only a few strains, including European strains isolated from Danish and Scottish waters, both saxitoxins and spirolides were found simultaneously (Cembella et al., 2000; MacKinnon et al., 2006; Brown et al., 2010). These toxins can accumulate in shellfish and other filter-feeding organisms, often rendering them too toxic for consumption (Bates and Rapoport, 1975; Glibert et al., 2007; Etheridge, 2010). As is common with dinoflagellates, *A. ostenfeldii* has both vegetative and cyst life stages. A pellicle, or temporary cyst, forms directly from the vegetative stage as a highly sensitive response to environmental disturbances (Jensen and Moestrup, 1997; Bravo et al., 2010). This is a short-term cyst stage that allows for re-germination between 1 and 17 days (Bravo et al., 2010). In addition, *A. ostenfeldii* can also produce sexual cysts (also known as resting cysts), which form from sexual fission of two vegetative gametes when growth conditions decline and can persist in sediment for several years until optimal environmental conditions return (Jensen and Moestrup, 1997; McQuoid et al., 2002).

*Alexandrium ostenfeldii* is typically found in marine and brackish waters of temperate and cold regions (Balech and Tangen, 1985; Okolodkov and Dodge, 1996; Mackenzie et al., 1996; Maclean et al., 2003). It is relatively common in Europe, with reports from, e.g., Norway, Denmark, the Baltic Sea, Scotland, Italy and Spain (Jensen and Moestrup, 1997; Scatista et al., 2003; Gribble et al., 2005; Kremp et al., 2009; Otero et al., 2010). The species usually occurs in low background numbers, but during recent years high concentrations of 10,000–2 million cells L<sup>-1</sup> have been found in coastal waters of the Baltic Sea (Kremp et al., 2009; Häkanen et al., 2012). Although one of the earliest descriptions of a bloom attributed to this species comes from nearby Belgium (Woloszynska and

Conrad, 1939), there are no reported bloom events of *A. ostenfeldii* in The Netherlands.

In early August 2012, however, a toxic *Alexandrium ostenfeldii* bloom developed in the Ouwerkerkse Kreek, in the south-western region of The Netherlands (Fig. 6.1). The Ouwerkerkse Kreek is a brackish water creek. It functions as a natural recreational area and a drainage basin for agricultural fields and the local village Ouwerkerk. A pumping station regularly discharges water from the creek into the adjacent Oosterschelde estuary (Fig. 6.1) to prevent flooding of the surrounding lands. The Oosterschelde is a tidal estuary with extensive mussel, cockle and oyster beds (Nienhuis and Smaal, 1994; Troost et al., 2010). Because filter-feeding molluscs are important to the estuary, both financially as a fishery and ecologically to support the vast populations of water birds in the region, protecting these shellfish from harmful algal blooms is a top priority of the regional water management.

The bloom in Ouwerkerkse Kreek was first noticed when it was linked with the death of a dog through the ingestion of contaminated material. Post-mortem analysis of the stomach content of the dog revealed a saxitoxin concentration of 2–4 mg kg<sup>-1</sup>. Subsequent analysis of the creek revealed a dense bloom of *Alexandrium ostenfeldii*, with 1–2 million cells L<sup>-1</sup>, producing saxitoxin (STX), saxitoxin analogs (mainly gonyautoxins) and spirolides (SPX) at alarmingly high concentrations (STX and its analogs at 10–20 µg STXeq L<sup>-1</sup>; SPX at 20–40 µg L<sup>-1</sup>). The creek, a popular recreational area for nearby campgrounds (Fig. 6.1), was immediately closed to the public. To add to the crisis, an impending rain event would fill the creek to capacity. To avoid flooding of the agricultural land would therefore require pumping the toxic bloom into the Oosterschelde and directly onto active shellfish beds. Yet, to protect the shellfisheries, pumping of creek water into the Oosterschelde was restricted by local safety regulations, specifically defined for this crisis, to *Alexandrium* concentrations of less than 1000 cells L<sup>-1</sup> and saxitoxin concentrations less than 15 µg L<sup>-1</sup>. Thus, a quick and complete mitigation of the bloom was required to protect the Oosterschelde shellfisheries and the local community.

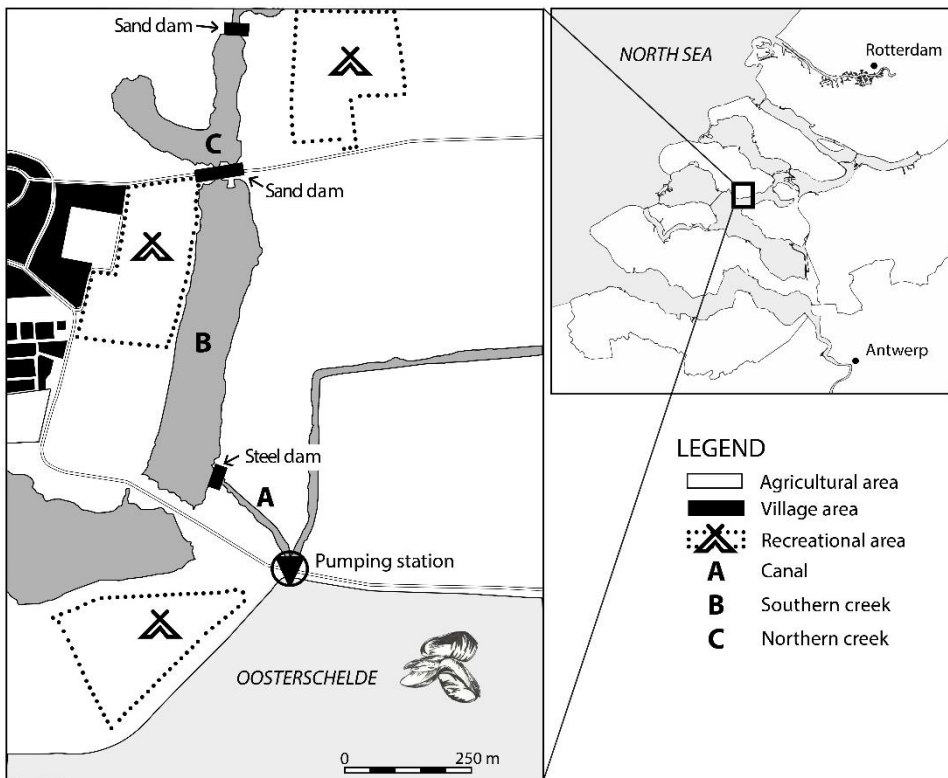
The ultimate prevention of harmful algal blooms in both freshwater and coastal waters is often an overall reduction of nutrient loads, as eutrophication is generally considered the primary cause for the increase in both occurrence and severity of harmful algal blooms (Heisler et al., 2008; Conley et al., 2009; Glibert

et al., 2010). Unfortunately, a measurable decline of HAB events from nutrient reduction initiatives may take years to materialize. Rapid HAB reduction options are minimal and with mixed effectiveness. These include artificial mixing or flushing of enclosed water bodies (Visser et al., 1996; Huisman et al., 2004; Verspagen et al., 2006). In more open water situations, clay dispersal or clay-bound algicides which aggregate cells and induce sedimentation have been the most common tools against marine harmful algal blooms (Pierce et al., 2004; Beaulieu et al., 2005; Lee et al., 2008; Pan et al., 2011). In the case of the Ouwkerkse bloom, flushing would still have transferred the toxic bloom to the Oosterschelde estuary, while clay dispersal and artificial mixing might not be sufficiently effective in this enclosed creek area.

In laboratory studies, low concentrations of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) have been shown to be effective against harmful cyanobacteria (Barroin and Feuillade, 1986; Drábková et al., 2007a,b; Barrington and Ghadouani, 2008) and the “brown tide” forming HAB species *Aureococcus anophagefferens* (Randhawa et al., 2012). Hydrogen peroxide is a naturally occurring reactive oxygen species (ROS) which can induce oxidative stress to cells causing physical damage and detrimentally impacting photosynthetic yield (Mittler, 2002). It is produced naturally by the photolysis of dissolved organic matter exposed to UV radiation (Cooper and Zika, 1983), and also by phytoplankton as a by-product of photosynthesis, respiration and other metabolic processes (Apel and Hirt, 2004; Asada, 2006).  $\text{H}_2\text{O}_2$  decays to water and oxygen through chemical and biological oxidation–reduction processes (Cooper and Zepp, 1990; Häkkinen et al., 2004). This decay occurs within hours or days depending on, e.g., biological activity, organic matter content, and the concentrations of redox-sensitive metals like iron and manganese (Cooper and Zepp, 1990; Häkkinen et al., 2004). Recent studies have applied  $\text{H}_2\text{O}_2$  to remove cyanobacteria from waste stabilization ponds (Barrington et al., 2011, 2013). In 2009,  $\text{H}_2\text{O}_2$  was applied for the first time to an entire lake, at a final concentration of  $2 \text{ mg L}^{-1}$ , to selectively suppress a cyanobacterial bloom (Matthijs et al., 2012). Hydrogen peroxide has also been explored as a treatment option against dinoflagellates in ballast water of ships (Ichikawa et al., 1992; Bolch and Hallegraeff, 1993; Gregg et al., 2009). The required  $\text{H}_2\text{O}_2$  dosage varied strongly among different species. For instance, Ichikawa et al. (1992) reported that cysts of *Alexandrium catenella* could be effectively removed at  $\text{H}_2\text{O}_2$  concentrations of  $30 \text{ mg L}^{-1}$ , whereas Bolch

and Hallegraef (1993) found that  $5000 \text{ mg L}^{-1}$  was required to remove cysts of *Gymnodinium catenatum* from ballast water.

This study reports on the application of  $\text{H}_2\text{O}_2$  to eradicate the *Alexandrium ostenfeldii* bloom from the Ouwerkerkse Kreek, which to our best knowledge represents the first case where  $\text{H}_2\text{O}_2$  was used to terminate a toxic dinoflagellate bloom. We developed the  $\text{H}_2\text{O}_2$  treatment in three steps. First, we investigated the required  $\text{H}_2\text{O}_2$  dosage in laboratory experiments with *A. ostenfeldii*. Second, we tested the method in a canal that was isolated from the Ouwerkerkse Kreek. Finally, we applied the optimized method to the entire creek system, and monitored subsequent changes in the concentrations of *A. ostenfeldii* and toxins as well as effects on other organisms in the creek.

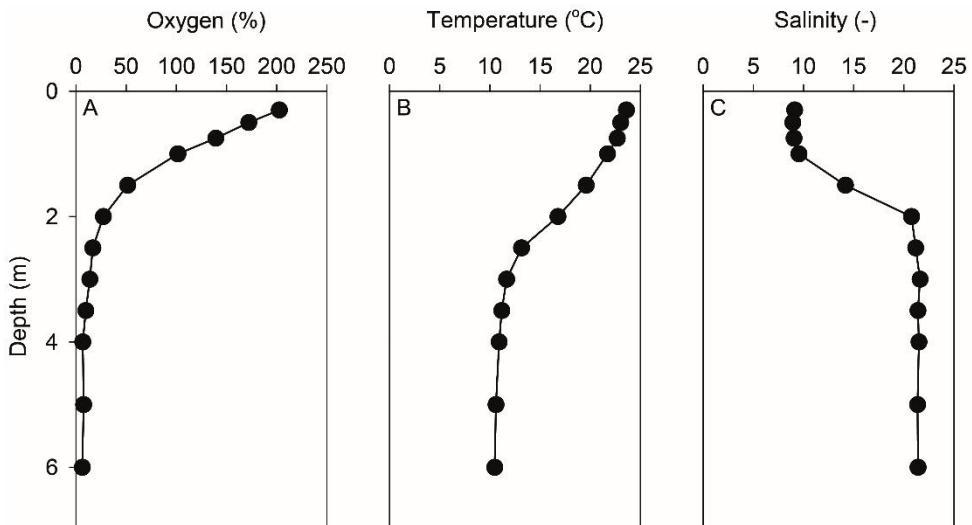


**Figure 6.1** Map of the Ouwerkerkse Kreek. The letters A, B, and C indicate the three areas where the  $\text{H}_2\text{O}_2$  treatment was performed. The creek was separated into a southern part (B) and a northern part (C) by two temporary sand dams. The canal (A) served as pilot study area; it was isolated from the Southern creek (B) by a temporary steel dam. The pumping station at the end of the canal was temporarily switched off to prevent flushing of the *A. ostenfeldii* bloom into the Oosterschelde estuary.

## Materials and methods

### Study area

The Ouwerkerkse Kreek (51°62'N, 3°99'E) is a small brackish lake in the province of Zeeland, The Netherlands (Fig. 6.1). It has a surface area of 0.12 km<sup>2</sup>, an average depth of 5 m, and a maximum depth of 8 m. At its northern side, the Ouwerkerkse Kreek is connected with an extensive network of canals and ditches draining the surrounding agricultural lands. At its south-eastern side, another canal leads from the Ouwerkerkse Kreek toward a pumping station that regularly discharges water into the Oosterschelde estuary. The creek has a vertical salinity stratification with an aerobic brackish top layer of 2 m depth and an oxygen-depleted saline bottom layer (Fig. 6.2). For the purpose of the H<sub>2</sub>O<sub>2</sub> treatment, the creek was divided into three sections via the construction of three temporary dams. The canal (A, in Fig. 6.1) from the creek toward the pumping station became the pilot study area after being isolated from the creek with a temporary steel dam. Subsequently, the creek was partitioned into a southern section (B) and a northern section (C) through construction of a temporary sand-filled dam along the bridge which crosses the creek. A third temporary sand-filled dam isolated the creek from the agricultural canals and ditches at the northern end.



**Figure 6.2** Stratification of the Ouwerkerkse Kreek. Depth profiles of (A) oxygen saturation, (B) temperature, and (C) salinity, measured with a Hydrolab probe (Hach Corporation, Loveland, CO, USA) on 14 August 2012.

## Laboratory study

As a first step, we investigated the H<sub>2</sub>O<sub>2</sub> sensitivity of *Alexandrium ostenfeldii* in laboratory incubations of lake water. Two water samples of 10 L each were collected from the *A. ostenfeldii* bloom in the creek on 11 August 2012 and sent via courier to the Department of Aquatic Microbiology of the University of Amsterdam. Immediately upon arrival, the samples were subdivided over 300 mL incubation flasks and incubated at ambient light and temperature. To determine the optimal H<sub>2</sub>O<sub>2</sub> concentration, we added 3% (w/v) H<sub>2</sub>O<sub>2</sub> concentrate to produce final concentrations of 0, 20, 30, 40, 50, 60, 80, and 120 mg L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>. Each of these 8 treatments was carried out in duplicate, yielding a total of 16 incubations. Changes in the photosynthetic vitality of cells were monitored by pulse amplitude modulation (PAM) fluorescence and the concentration of *Alexandrium* cells was counted by microscope during the first 12 h after H<sub>2</sub>O<sub>2</sub> addition (see details below).

## Field treatments

### *Pilot study in the canal*

Based on the laboratory incubations, 50 mg L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> was deemed the optimal concentration for our field application. To test the effectiveness of this H<sub>2</sub>O<sub>2</sub> dosage, we first ran a pilot study in the 200 m long canal (A, in Fig. 6.1) running from the Ouwerkerkse Kreek toward the pumping station at the Oosterschelde estuary on 17 August 2012. The volume to be treated in the canal was estimated at 5000 m<sup>3</sup>. H<sub>2</sub>O<sub>2</sub> was provided by a 1000-L tank with a 50% (v/v) H<sub>2</sub>O<sub>2</sub> concentration, placed next to the canal. A circulation and mixing scheme was initiated which included two injection points of H<sub>2</sub>O<sub>2</sub>. The first injection point was at the west end of the canal, near the temporary steel dam, where we also placed the outlet of a circulation pipe that ran the length of the canal to create a net circular motion of water throughout the canal. The second injection point was located midway on the canal, where we also placed a bubble screen with compressed air across the width of the canal to enhance mixing of the injected H<sub>2</sub>O<sub>2</sub>. The canal was sampled from a small boat at two stations, at ¼ and ¾ of the length of the canal, before and every hour after the initiation of H<sub>2</sub>O<sub>2</sub> injection. Samples were taken from multiple depths with a 1.2 L Niskin bottle and integrated before analysis. Analysis included the H<sub>2</sub>O<sub>2</sub> concentration, plankton counts, toxin analysis, and photosynthetic yield. Hourly measurements continued until the target concentration of 50 mg L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> was reached

throughout the canal, at which point H<sub>2</sub>O<sub>2</sub> addition was discontinued. Additional samples were collected 24 and 48 h after the initial injection of H<sub>2</sub>O<sub>2</sub>.

### ***Treatment of the entire creek***

After successful completion of the pilot study, the entire creek was treated with 50 mg L<sup>-1</sup> of H<sub>2</sub>O<sub>2</sub> in two stages. First, the southern part of the creek (B, in Fig. 6.1), with an estimated volume of 317,000 m<sup>3</sup>, was treated on 22 August 2012. Subsequently, the northern part of the creek (C), with a volume of 107,000 m<sup>3</sup>, was treated on 24 August 2012. Hydrogen peroxide was administered from a large barge carrying a 15,000 L tank with a 50% (v/v) H<sub>2</sub>O<sub>2</sub> concentration, connected to an intermediary tank for pre-dilution with creek water to 1% (v/v) H<sub>2</sub>O<sub>2</sub>, and onwards to a specially designed injection screen called a “water harrow” injecting 5 mL of the pre-diluted solution per L of creek water (Matthijs et al., 2012). The water harrow consisted of a series of six parallel injection tubes on a metal frame. Each tube contained a series of outlet valves every 20 cm that can be positioned vertically from 50 cm to a maximum of 8 m depth for the dispersal of H<sub>2</sub>O<sub>2</sub>. Additional tubing delivered a stream of compressed air in parallel with the dosing of H<sub>2</sub>O<sub>2</sub> to achieve optimal mixing. Positioning of the tubing and pump rates were computer-controlled using custom-made software. The software integrated 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 H<sub>2</sub>O<sub>2</sub>. The barge was towed by a tug boat traveling in parallel transects throughout the creek until 50 mg L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> was reached. 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 H<sub>2</sub>O<sub>2</sub> from a small boat and mixing of the water by gentle circulation. The creeks were sampled at two stations in the Southern creek and two stations in the Northern creek, in the same manner as the canal, except the northern portion of the creek did not include hourly *Alexandrium ostenfeldii* counts but only cell counts at 0, 24 and 48 h after the onset of H<sub>2</sub>O<sub>2</sub> addition. Data presented are an average of all four stations unless stated otherwise.

Total costs for the treatment amounted to €370,000, although regular implementation would most likely reduce the costs for future applications. For the entire operation, permission was obtained from the responsible authorities,

and safety measures were taken in accordance with legislation. Warning signs indicated that the entire creek area and all access roads were closed for the public. Concentrated H<sub>2</sub>O<sub>2</sub> was delivered on site by a certified transport company on the days of usage. For the pilot study in the canal, stocks of H<sub>2</sub>O<sub>2</sub> were stored in a restricted area with entrance on permission only. For the creek treatment, tanker truckspumped the H<sub>2</sub>O<sub>2</sub> directly into the tank on the barge. The application was carried out by professionals experienced in handling H<sub>2</sub>O<sub>2</sub>. Public announcements of the treatment were made on a daily basis in the local newspapers, radio and television.

### **Analysis**

A small on-site laboratory was installed in a workshed adjacent to the Ouwkerkse Kreek, for microscopic cell counts, measurements of photosynthetic yields and sample preparation for further laboratory analysis.

### ***Photosynthetic yield***

The photosynthetic yield of phytoplankton samples was determined with a portable mini-PAM fluorometer (Walz, Effeltrich, Germany). First, a 50 mL water sample was filtered over 25 mm diameter 0.45 µm pore size glass fiber filters (GF/C, Whatman International Ltd., Maidstone, England) using a vacuum manifold (model 1225, Millipore, Eschborn, Germany) with 12 placements. The loaded filters were dark adapted for 10 min before the photosynthetic yield was measured with the sensor of the mini-PAM fluorometer mounted just above the filters on the manifold. The photosynthetic yield ( $\phi_{\text{PSII}}$ , also known as the quantum yield of photosystem II electron transport) was calculated as  $\phi_{\text{PSII}} = (F_m - F_o)/F_m$ , where  $F_o$  is the minimum fluorescence and  $F_m$  is the maximum fluorescence following a saturating light pulse (Genty et al., 1989). The photosynthetic yield of a sample without added H<sub>2</sub>O<sub>2</sub> was used as reference value.

### ***Plankton counts***

During the laboratory incubations and field treatments, *Alexandrium ostenfeldii* cells were counted with an inverted microscope immediately after sampling. For each water sample, a minimum of 200 cells was counted in triplo using a 1 mL volume gridded Sedgewick Rafter counting chamber (Pyser-SGI Ltd, Edenbridge, UK). If less than 200 cells were present, the entire counting

chamber was examined. We distinguished between motile vegetative cells, nonmotile vegetative cells, pellicle cysts and nonviable cells. Nonviable cells were defined as cells with a visibly damaged cell wall and/or without any pigmentation. Viable cells were defined as the sum of the motile vegetative cells, nonmotile vegetative cells and pellicle cysts.

In addition, phytoplankton samples were fixed with Lugol's iodine (1% final concentration) and sent for complete community analysis to plankton experts at Koeman & Bijkerk B.V. (Haren, The Netherlands). Because the different species varied widely in size, the phytoplankton counts were converted to biovolume according to the geometric equations of Hillebrand et al. (1999) for community composition comparisons.

For zooplankton, 20 L of lake water gathered by multiple sampling with a 1.2 L Niskin bottle was filtered over a plankton sieve (mesh size 30  $\mu\text{m}$ ), and the filtered material was pooled, fixed with Lugol's iodine, and sent to Koeman & Bijkerk B.V. for species identification and counting. For each sample, at least 100 individuals per species were counted with an inverted microscope to obtain reliable estimates of their population abundances, or the entire sample volume was counted if it contained less than 100 individuals.

### ***Hydrogen peroxide, PSP toxins, nutrients***

$\text{H}_2\text{O}_2$  concentrations of water samples were measured on site with Quantofix test sticks (Machereye-Merck, Darmstadt, Germany). These ready-to-go test sticks are intended for direct measurement of distinct  $\text{H}_2\text{O}_2$  concentrations of 1, 3, 10, 30 and 100  $\text{mg L}^{-1}$ . We refined the measurements by making photographs of each test stick and subsequent comparison to our own calibration series, diluting the samples when necessary to ensure accuracy of color comparisons.

Toxin concentrations in the whole water samples and in the water phase only (after filtration over 25 mm diameter 0.45  $\mu\text{m}$  pore size glass fiber filters; GF/C, Whatman) were analyzed at the Institute for Food Safety (RIKILT) of Wageningen University. The analysis identified saxitoxin (STX), saxitoxin analogs (mainly gonyautoxin-2 and -3; GTX-2 and -3), and 13-desmethyl spirolide-C (SPX-1). STX and STX analogs were analyzed with hydrophilic interaction liquid chromatography coupled to a Waters Xevo TQ-S tandem mass spectrometer (HILIC-MS/MS; Waters Corporation, Milford, MA, USA)

according to Dell'Aversano et al. (2005). SPX-1 was analyzed with ultra-performance liquid chromatography (UPLC) coupled to a Waters Xevo TQ-S tandem mass spectrometer (LC-MS/MS) according to a modified method of Gerssen et al. (2009). The limits of detection were  $2.3 \mu\text{g L}^{-1}$  for STX,  $2.1 \mu\text{g L}^{-1}$  for GTX-2 and -3, and  $0.7 \mu\text{g L}^{-1}$  for SPX-1. We quantified the saxitoxins as saxitoxin only (STX) and as saxitoxin-equivalents (STXeq; including STX and STX analogs).

Dissolved inorganic nutrients (ammonium, nitrate, nitrite, phosphate) were analyzed spectrophotometrically with a SKALAR spectrophotometer (Skalar Analytical B.V., Breda, The Netherlands) (Murphy and Riley, 1962; Parsons et al., 1984).

### ***Fish, macroinvertebrates, and water plants***

The entire treated area was closely monitored by visual inspection during the first 48 h after  $\text{H}_2\text{O}_2$  addition for mortalities of fish or macroinvertebrates (e.g., shrimp, crab), and for possible damage to the sparse aquatic vegetation. Finally, to assess effects on aquatic macroinvertebrates, we compared the species composition of macroinvertebrates in the treated canal with that in the not yet treated Southern creek on 21 August 2012 (four days after  $\text{H}_2\text{O}_2$  treatment of the canal but one day before treatment of the Southern creek). Macroinvertebrates were collected by drawing 10 times a large macrofauna net (0.5 mm mesh size) over a standardized length of 5 m, making sure to incorporate all microhabitats within a sampling site.

## **Results**

### **Laboratory incubations**

*Alexandrium ostenfeldii* was by far the most dominant phytoplankton species in the water samples of the Ouwkerkse Kreek used for the laboratory incubations, with concentrations of 5.5 million *Alexandrium* cells  $\text{L}^{-1}$ . Within 2 h after  $\text{H}_2\text{O}_2$  addition, the photosynthetic yield was reduced by more than 80% in the treatment with  $30 \text{ mg L}^{-1} \text{H}_2\text{O}_2$  and by more than 95% in all treatments with  $40 \text{ mg L}^{-1} \text{H}_2\text{O}_2$  or higher (Fig. 6.3A). In all treatments, except the control, motility was rapidly reduced, from 21.6% initially to zero within 1 h after  $\text{H}_2\text{O}_2$  addition (data not shown). After 12 h, more than 80% of the *A. ostenfeldii* cells had visibly lost viability (i.e., with disrupted cell walls or

without pigmentation) when exposed to H<sub>2</sub>O<sub>2</sub> concentrations of 40 mg L<sup>-1</sup> or higher (Fig. 6.3B).

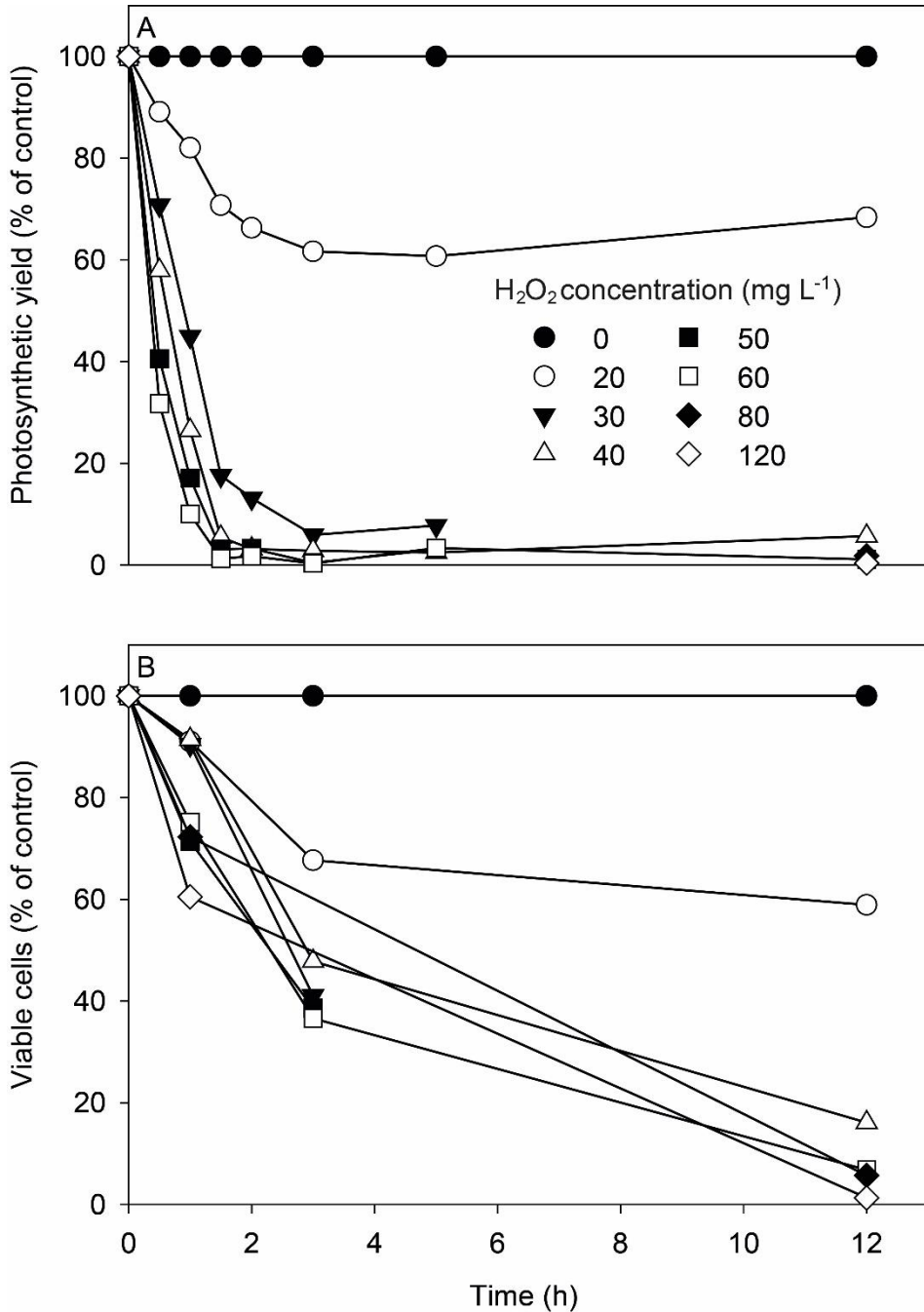
Hence, these lab results indicated that a H<sub>2</sub>O<sub>2</sub> concentration of 40 mg L<sup>-1</sup> should be sufficient to terminate the *Alexandrium* bloom. Treatment of an entire ecosystem is more challenging than a small-scale laboratory experiment, however. To be on the safe side, it was decided in consultation with the management authorities to treat the canal with 50 mg L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>.

### ***Pilot study in the canal***

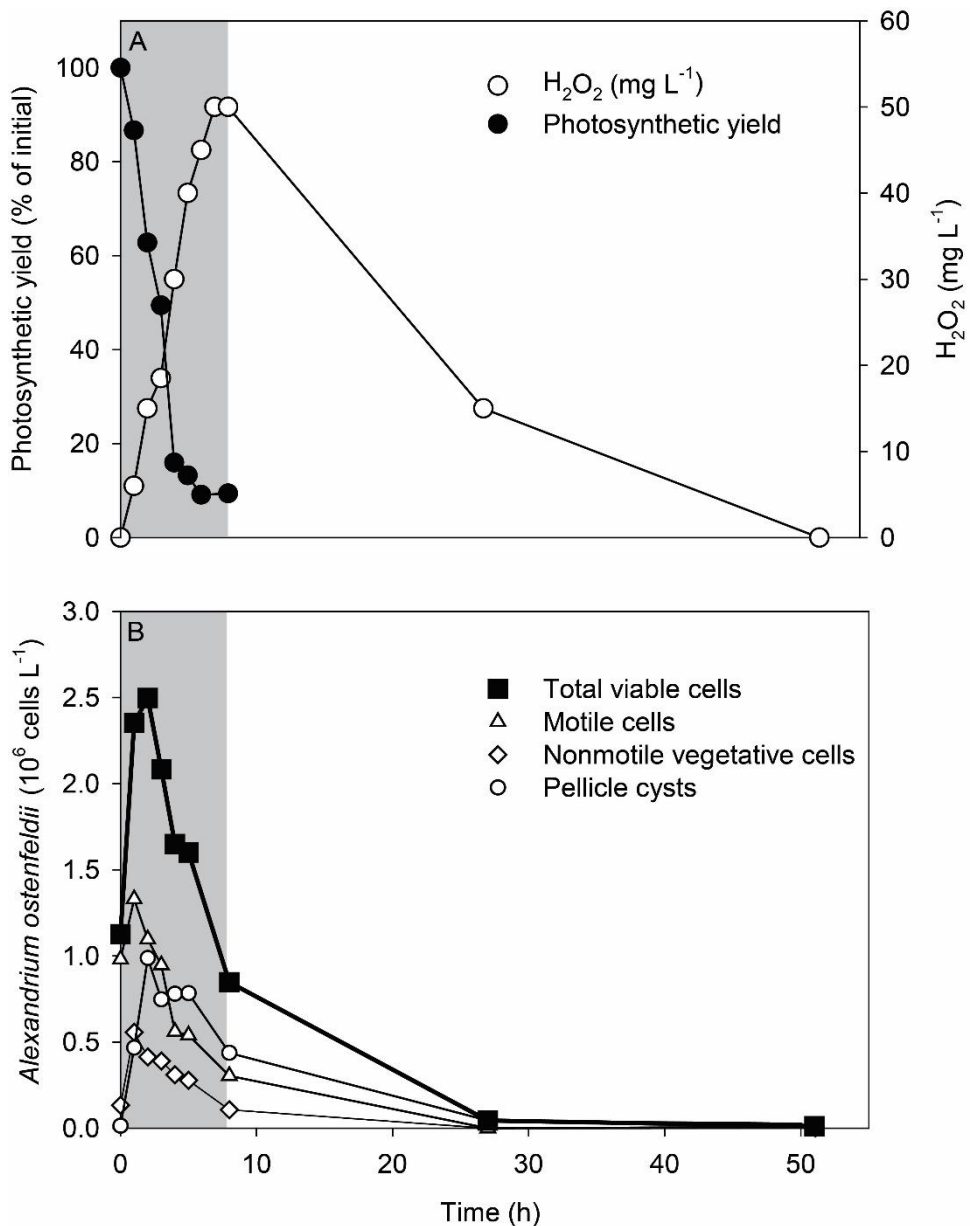
After 7 h of H<sub>2</sub>O<sub>2</sub> injection, the desired concentration of 50 mg L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> was reached throughout the entire canal, and the H<sub>2</sub>O<sub>2</sub> injection was stopped (Fig. 6.4A). Subsequently, the H<sub>2</sub>O<sub>2</sub> concentration declined, and after 51 h it was reduced below the detection limit of the Quantofix test sticks of ~0.1 mg L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>.

The initial *Alexandrium ostenfeldii* concentration in the canal was ~1.1 million cells L<sup>-1</sup>, consisting for ~85% of motile cells and 15% of nonmotile cells, while pellicle cysts were rare (Fig. 6.4B). During the first 2 h of H<sub>2</sub>O<sub>2</sub> addition, cell numbers of *A. ostenfeldii* increased, presumably due to resuspension of sedimented cells after the pumps for H<sub>2</sub>O<sub>2</sub> injection and mixing were switched on. During the same time span, the contribution of pellicle cysts increased to 40% of the total *A. ostenfeldii* population, indicating that vegetative cells responded to the oxidative stress by transitioning to this temporary cyst stage. After 5 h, when the H<sub>2</sub>O<sub>2</sub> concentration had increased to ~40 mg L<sup>-1</sup>, the photosynthetic yield of the phytoplankton community was reduced by 90% (Fig. 6.4A).

Microscopic analysis showed that many cells and cysts started to lyse. After 27 h, the *A. ostenfeldii* population was reduced by 99.8%, motile and nonmotile vegetative cells were no longer detected, and the number of potentially viable cysts was reduced to 2500 cysts L<sup>-1</sup>. After 51 h, the total number of viable *A. ostenfeldii* cells was reduced by 99.9%, to below safety regulatory requirements of 1000 cells L<sup>-1</sup>.



**Figure 6.3** Laboratory incubations of creek water with *A. ostenfeldii* at different  $H_2O_2$  concentrations. (A) Photosynthetic yield, expressed as percentage of the control (no  $H_2O_2$  addition). (B) Viable *A. ostenfeldii* cells, expressed as percentage of the control. Data show the mean of two duplicates per treatment



**Figure 6.4** H<sub>2</sub>O<sub>2</sub> treatment of *A. ostenfeldii* during the pilot study in the canal. (A) H<sub>2</sub>O<sub>2</sub> concentration and photosynthetic yield of the phytoplankton community (expressed as percentage of the initial yield). (B) Concentration of *A. ostenfeldii* in the canal; total viable cells is the sum of motile vegetative cells, nonmotile vegetative cells, and pellicle cysts without visible damage. Gray shading indicates the duration of H<sub>2</sub>O<sub>2</sub> injection into the canal. Data show the mean of measurements at two sampling stations.

Total toxin concentrations of SPX-1, STX and STXeq decreased over 27 h, and ultimately both SPX-1 and STXeq were below local regulatory levels of  $15 \mu\text{g L}^{-1}$  (Table 6.1). Prior to the treatment, toxin concentrations in the filtrate comprised  $\sim 60\%$  of the total saxitoxins and as little as 13% of the total spirolides, indicating that most spirolides were intracellular. Only 8 h after initiation of  $\text{H}_2\text{O}_2$  treatment, all saxitoxins and 66% of the spirolides appeared in the filtrate. This shift from intercellular to extracellular presence of the toxins is presumably a result of the release of toxins due to the observed cell lysis.

**Table 6.1** Concentrations [ $\mu\text{g L}^{-1}$ ] of 13-desmethyl spirolide C (SPX-1), saxitoxin (STX) and saxitoxin equivalents (STXeq) during the pilot study in the canal.

Time (hr)	SPX-1 total	SPX-1 filtrate	STX total	STX filtrate	STXeq total	STXeq filtrate
0	22.5	2.9	6.6	3.6	10.5	6.6
5	13.6	5.9	4.7	4.2	6.8	7.0
8	7.6	5.0	4.3	4.6	6.2	6.6
27	8.6	2.8	<LOD	<LOD	<LOD	<LOD

Data show the mean of duplicate samples per timepoint; <LOD, below limit of detection.

### ***Treatment of the entire creek***

The successful termination of the *Alexandrium ostenfeldii* bloom in the canal led to the decision to treat the Southern and Northern creek with  $50 \text{ mg L}^{-1} \text{ H}_2\text{O}_2$ . In contrast to the canal, the creek system was stratified, with a warmer and less saline surface layer of  $\sim 2$  m depth on top of a colder and more saline lower layer devoid of oxygen (Fig. 6.2). The combination of  $\text{H}_2\text{O}_2$  injection and aeration with the water harrow, over the entire depth of the water column, induced a distinct smell of hydrogen sulfide ( $\text{H}_2\text{S}$ ) and during the first hour of  $\text{H}_2\text{O}_2$  injection a milky white precipitate developed in the creek, presumably caused by oxidation of  $\text{H}_2\text{S}$  to elemental sulfur. After the first hour of injection, we therefore decided to limit further  $\text{H}_2\text{O}_2$  injection to the aerobic upper 2 m of the water column. The white precipitate gradually disappeared during the next 24 h.

**Table 6.2** Concentrations [ $\mu\text{g L}^{-1}$ ] of 13-desmethyl spirolide C (SPX-1), saxitoxin (STX) and saxitoxin equivalents (STXeq) during the  $\text{H}_2\text{O}_2$  treatment of the creek.

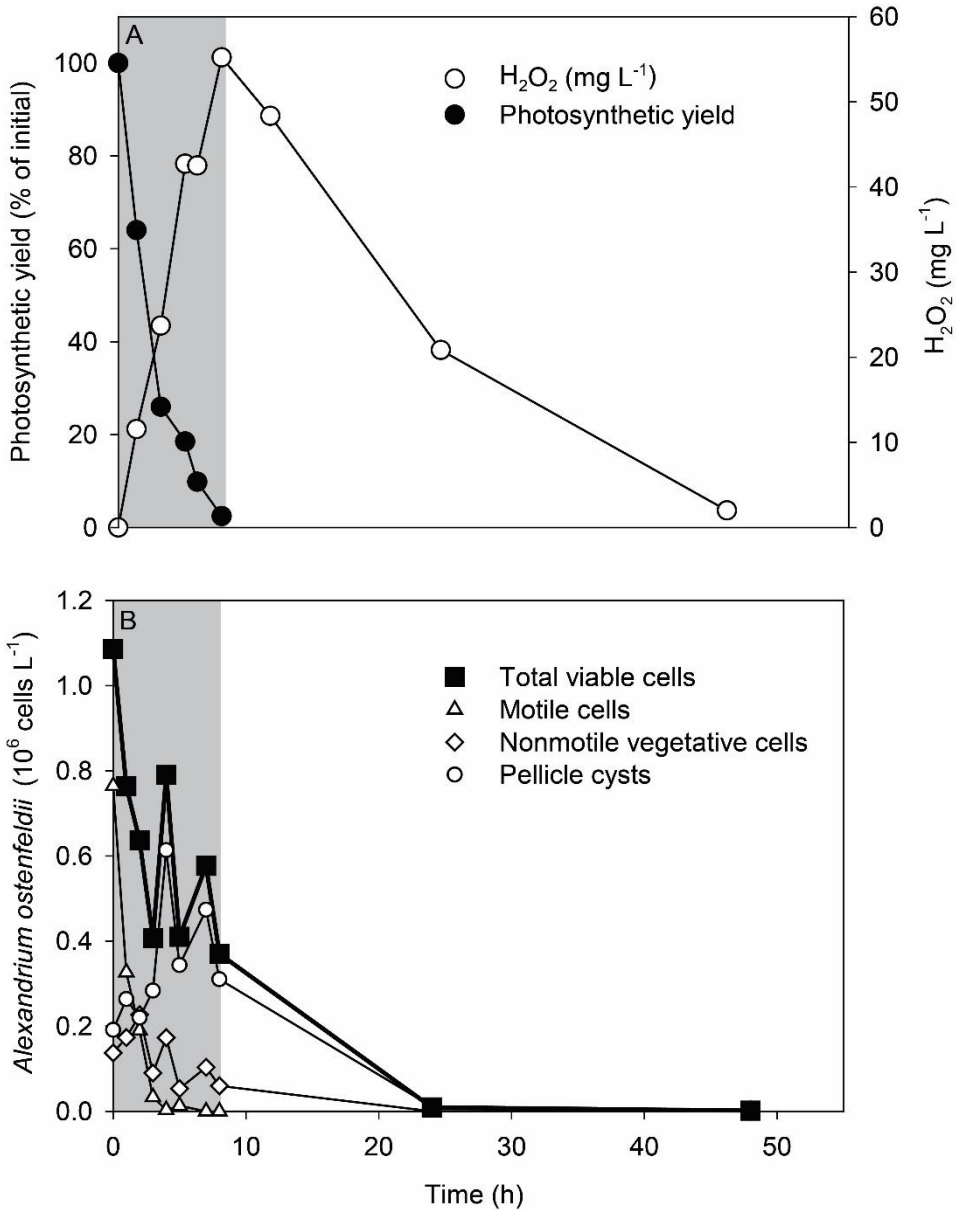
Time (hr)	SPX-1 total	SPX-1 filtrate	STX total	STX filtrate	STXeq total	STXeq filtrate
0	43.4	10.9	10.1	9.2	11.6	10.7
4	28.7	13.3	9.3	7.9	13.0	8.6
7	21.5	9.8	8.1	7.2	8.9	8.5
24	24.6	8.2	7.2	6.8	7.5	7.2
52	ND	12.0	ND	8.4	ND	8.6
72	16.3	6.4	6.4	5.5	7.3	6.4
96	13.1	3.7	5.8	5.2	6.4	6.1

Data show the mean of duplicate samples per timepoint; ND, no data.

The target concentration of  $50 \text{ mg L}^{-1} \text{ H}_2\text{O}_2$  was reached after 8 h of  $\text{H}_2\text{O}_2$  injection (Fig. 6.5A). At this point, the  $\text{H}_2\text{O}_2$  injection was stopped and the  $\text{H}_2\text{O}_2$  concentration gradually declined to natural background levels after 50 h. The *Alexandrium ostenfeldii* population in the creek showed a similar response to the treatment as in the canal. The photosynthetic yield collapsed to less than 5% of its initial value within 8 h after initiation of the  $\text{H}_2\text{O}_2$  treatment (Fig. 6.5A). The concentration of pellicle cysts strongly increased until it comprised  $\sim 75\%$  of the population at 5 h after the onset of the treatment (Fig.6.5B). Microscopic observations confirmed that both vegetative cells and pellicle cysts started to lyse from this point onwards, and the total *A. ostenfeldii* population eventually declined by more than 99.8% from  $\sim 1.1$  million cells  $\text{L}^{-1}$  at the start of the treatment to less than 2000 cells  $\text{L}^{-1}$  after 48 h (Fig. 6.5B). Concentrations of SPX-1 and STXeq were reduced below local regulatory levels of  $15 \mu\text{g L}^{-1}$  after 96 h (Table 6.2).

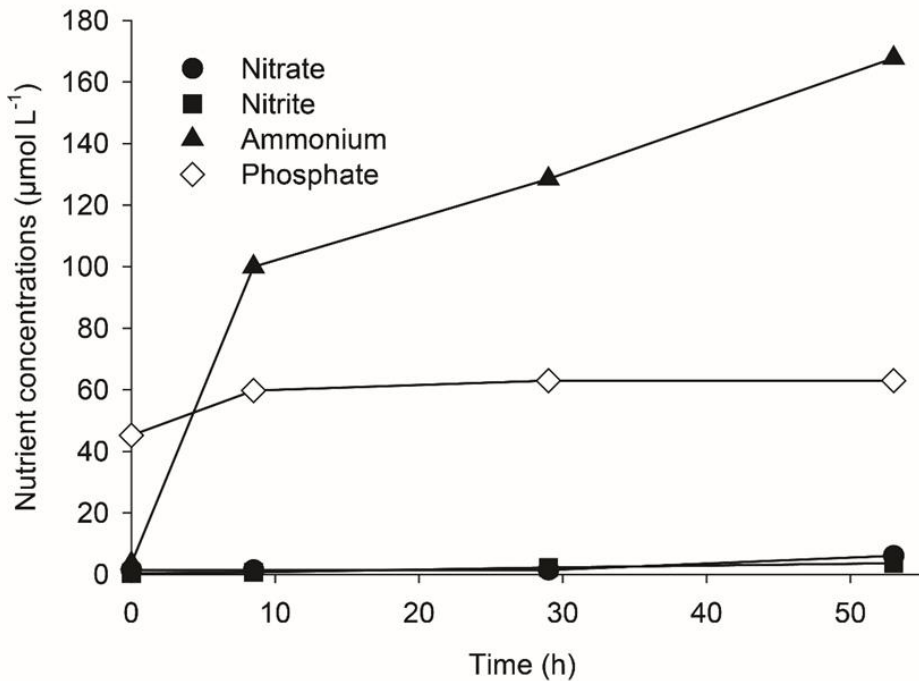
### ***Other effects on the ecosystem***

In addition to the collapse of the *Alexandrium ostenfeldii* bloom,  $\text{H}_2\text{O}_2$  treatment of the creek also affected other components of the ecosystem. For instance, the ammonium concentration increased from  $3.6 \mu\text{mol L}^{-1}$  before  $\text{H}_2\text{O}_2$  injection to  $167 \mu\text{mol L}^{-1}$  after 58 h, probably due to cell lysis and subsequent ammonification of the cellular contents (Fig. 6.6). We also note that the nitrite concentration increased from  $0.36$  to  $3.64 \mu\text{mol L}^{-1}$ . The nitrate and phosphate concentrations were much less affected by the  $\text{H}_2\text{O}_2$  treatment (Fig. 6.6).



**Figure 6.5** H<sub>2</sub>O<sub>2</sub> treatment of *A. ostenfeldii* in the entire creek. (A) H<sub>2</sub>O<sub>2</sub> concentration and photosynthetic yield of the phytoplankton community (expressed as percentage of the initial yield). (B) Concentration of *A. ostenfeldii* in the creek; total viable cells is the sum of motile vegetative cells, nonmotile vegetative cells, and pellicle cysts without visible damage. Gray shading indicates the duration of H<sub>2</sub>O<sub>2</sub> injection into the creek. Data show the mean of measurements in the Southern creek and the Northern creek.

The total phytoplankton (expressed as biovolume) declined by 94%, from  $9.75 \times 10^4 \mu\text{m}^3 \text{L}^{-1}$  at the start of the treatment to  $0.57 \times 10^4 \mu\text{m}^3 \text{L}^{-1}$  after 50 h (Fig. 6.7). During this time span, the phytoplankton species composition changed from strong dominance by *Alexandrium ostenfeldii* (87% of the total phytoplankton biovolume) to co-dominance by small green algae (Chlorophyta), the chain-forming cyanobacterium *Anabaena* sp., our target species *A. ostenfeldii* and euglenophytes (Fig. 6.7). However, the *Anabaena* sp. cells were likely non-viable as they had lost most of their pigmentation. Follow-up samples one week after the  $\text{H}_2\text{O}_2$  treatment showed a complete lack of *Anabaena* sp. and a further decline of *A. ostenfeldii*, whereas the green algae and euglenophytes remained dominant.

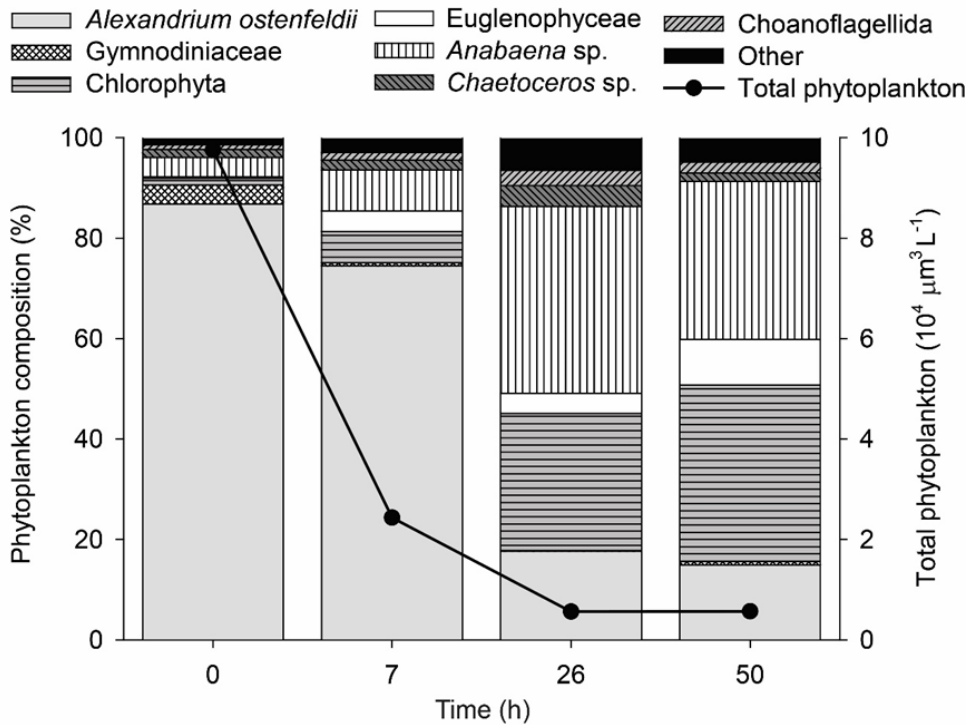


**Figure 6.6** Changes in nutrient concentrations during the  $\text{H}_2\text{O}_2$  treatment of the entire creek.

The zooplankton community was very sensitive to the  $\text{H}_2\text{O}_2$  treatment, and declined from over 40,000 individuals per liter at the start to less than 15 individuals per liter after 50 h (Fig. 6.8). Yet, the  $\text{H}_2\text{O}_2$  treatment did not result in strong changes in species composition. Of the seven most dominant zooplankton groups before addition of  $\text{H}_2\text{O}_2$ , only the rotifers *Hexarthra mira*

and *Pompholyx sulcata* were reduced below detection levels after 50 h, while rotifers of the genera *Rotatoria* and *Brachionus* remained numerically dominant (Fig. 6.8).

About 40 three-spined sticklebacks (*Gasterosteus aculeatus*) and 4 ragworms (*Nereis diversicolor*) were found dead near the H<sub>2</sub>O<sub>2</sub> injection point at the west end of the canal, where we had also placed the outlet of a water circulation pipe. These deaths might be due to locally higher H<sub>2</sub>O<sub>2</sub> concentrations near the injection point, or to the strong turbulence generated by the water circulation system. We did not find any further dead sticklebacks or ragworms, neither in other parts of the canal nor in the Southern or Northern creek. One dead European eel (*Anguilla anguilla*) was found in the Southern creek three days after the H<sub>2</sub>O<sub>2</sub> treatment.



**Figure 6.7** Changes in phytoplankton community structure during the H<sub>2</sub>O<sub>2</sub> treatment of the entire creek. Closed circles indicate the total phytoplankton biovolume. The bar graph represents the relative contribution of different phytoplankton groups to the total biovolume.

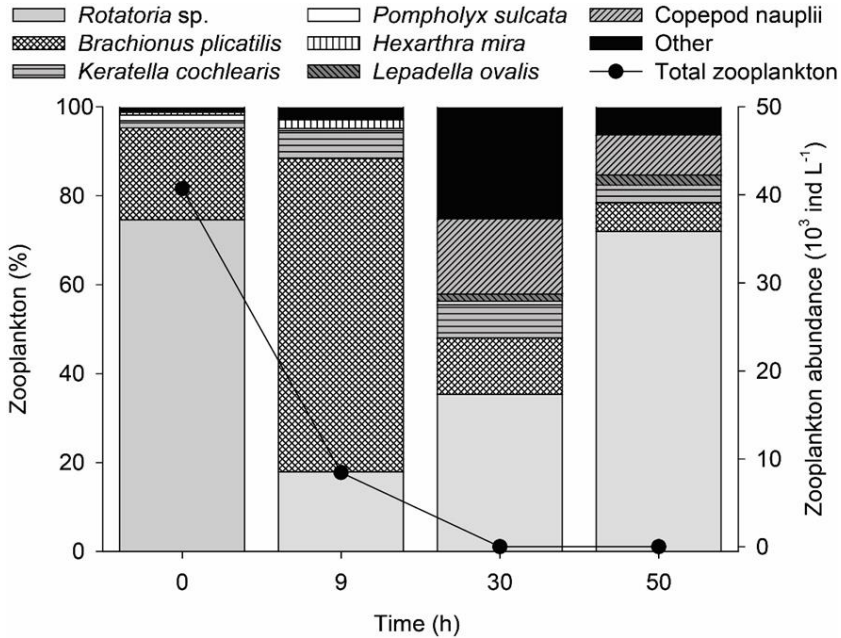
Comparison of macroinvertebrates in the H<sub>2</sub>O<sub>2</sub> treated canal and not yet treated Southern creek did not show a clear response of macroinvertebrates to the H<sub>2</sub>O<sub>2</sub> treatment (Table 6.3). Gammarid shrimps (*Gammarus zaddachi* and *G. duebeni*) and the Atlantic ditch shrimp (*Palaemonetes varians*) were common at both sites, mud snails (*Hydrobiidae* sp.) and the isopod *Lekanosphaera hookeri* were common in the treated canal, whereas midge larvae (*Chironomus* spp.) were abundant in the Southern creek.

Microscopic observations showed localized damage to the gill covers (operculum) of Atlantic ditch shrimps and the legs of a few gammarid shrimps, but did not reveal visual damage to any of the other species. All macroinvertebrate species remained abundant and active during and after the H<sub>2</sub>O<sub>2</sub> treatment. Aquatic vegetation was dominated by stands of common reed (*Phragmites australis*) and sea club-rush (*Scirpus maritimus*) along the shores, and did not show signs of damage due to the H<sub>2</sub>O<sub>2</sub> treatment.

**Table 6.3** Abundances of the most dominant macroinvertebrate species in the untreated Southern creek versus the treated canal.

Species	Untreated creek	Treated canal
<i>Chironomus</i> sp.	34	0
<i>Chironomus aprilinus</i>	26	0
<i>Hydrobiidae</i> sp.	0	156
<i>Gammarus duebeni</i>	82	25
<i>Gammarus zaddachi</i>	532	119
<i>Lekanesphaera hookeri</i>	0	111
<i>Palaemonetes varians</i>	22	33
Other	19	35

Abundances represent the number of individuals caught by a macrofauna net drawn over 10 m length on 21 August 2012 (four days after H<sub>2</sub>O<sub>2</sub> treatment of the canal but one day before H<sub>2</sub>O<sub>2</sub> treatment of the Southern creek).



**Figure 6.8** Changes in zooplankton community structure during the  $\text{H}_2\text{O}_2$  treatment of the entire creek. Closed circles indicate the total zooplankton abundance. The bar graph represents the relative contribution of different zooplankton groups to the total zooplankton abundance.

## Discussion

This research was the result of an emergency situation, where an intense toxic bloom of *Alexandrium ostenfeldii* in a small brackish lake was held responsible for the death of a dog, led to closure of the lake for recreation, and threatened commercial shellfisheries in the adjacent Oosterschelde estuary. Our results show that injection of  $50 \text{ mg L}^{-1} \text{ H}_2\text{O}_2$  into the lake led to a rapid decline of the photosynthetic yield of the *A. ostenfeldii* bloom, induced the formation of pellicle cysts, and resulted in cell lysis of both vegetative cells and pellicle cysts within just 8 h after the onset of the treatment. The *A. ostenfeldii* population declined by 98% within 24 h, and by 99.8% within two days. Substantial reductions of the saxitoxin and spirolides concentrations were measured after 24 h, and both toxins were reduced to below local regulatory limits by 96 h after initial treatment. Finally, after cessation of the  $\text{H}_2\text{O}_2$  injection,  $\text{H}_2\text{O}_2$  concentrations returned to background levels within 48 h. In total, these results demonstrate that a confined bloom of *A. ostenfeldii* can be terminated by

H<sub>2</sub>O<sub>2</sub> within a timespan of only two days. It is the first example, that we know of, where a dinoflagellate bloom has been so rapidly and effectively remedied.

The Ouwerkerkse Kreek bloom was a unique occurrence for several reasons. First, although one of the first scientific descriptions of an *Alexandrium ostenfeldii* bloom comes from this region (Woloszynska and Conrad, 1939), PSP-producing blooms are very rare in The Netherlands. Less than 1% of all European PSP and diarrhetic shellfish poisoning (DSP) events occurred within The Netherlands between 1989 and 1998 (Scatasta et al., 2003). A monitoring study of marine toxins in shellfish harvested in The Netherlands in 2007 and 2008 revealed very low concentrations of spirolides (SPX-1) in a few samples from the northern part of The Netherlands (Gerssen et al., 2010). Second, *A. ostenfeldii* blooms typically produce either saxitoxins or spirolides (Suikkanen et al., 2013). *A. ostenfeldii* blooms producing saxitoxins, but not spirolides, have occurred regularly in the Baltic Sea since at least 2001 (Kremp et al., 2009; Hakanen et al., 2012). Blooms of *A. ostenfeldii* producing only spirolides have been reported on both sides of the North Atlantic, in coastal waters of Nova Scotia, the Gulf of Maine and Ireland (Cembella et al., 2000; Gribble et al., 2005; Maclean et al., 2003; Otero et al., 2010; Touzet et al., 2011). The bloom in the Ouwerkerkse Kreek is one of the few European cases, together with earlier reports from Denmark and Scotland (MacKinnon et al., 2006; Brown et al., 2010), where *A. ostenfeldii* produces both saxitoxins and spirolides. This sketches the coarse contours of a biogeographical pattern where co-production of saxitoxins and spirolides can be found as a transition zone, in northwestern Europe, between the saxitoxin-producing strains in the Baltic Sea and the spirolides-producing strains in the North Atlantic. Further studies may shed more light on this possible biogeographical pattern. Third, *A. ostenfeldii* seldom develops blooms; usually it occurs in relatively low numbers as background species (Moestrup and Hansen, 1988; Gribble et al., 2005; Touzet et al., 2011). Thus far, dense *A. ostenfeldii* blooms with more than 1 million cells L<sup>-1</sup> have been reported only for coastal areas along the Baltic Sea (Kremp et al., 2009; Hakanen et al., 2012). Fourth, to our knowledge, this is the first time that the death of a dog has been linked to *A. ostenfeldii*, which might be due to the exceptionally high density of the bloom.

Although blooms described for the Baltic Sea produced only saxitoxins, they share several environmental factors with the Ouwerkerkse Kreek. In both

the Baltic Sea and Ouwerkerkse Kreek, dense *Alexandrium ostenfeldii* blooms developed in enclosed and semi-enclosed shallow brackish waters of 8–10 m depth (Kremp et al., 2009; Hakanen et al., 2012) when water temperatures were between 20 and 25 °C in late summer (August–September). Beyond these physical consistencies, the exact explanation of why the Ouwerkerkse bloom occurred is still in need of further investigation. Similar to several other harmful dinoflagellates, laboratory studies have shown that *A. ostenfeldii* can utilize small organic nitrogen compounds as well as inorganic nitrogen (Maclean et al., 2003; Gobler et al., 2012). Also, *A. ostenfeldii* is known to be phagotrophic (Jacobson and Anderson, 1996) and allelopathic (Arzul et al., 1999; Tillmann et al., 2007), allowing it to utilize a broad mix of resources. We do not know if and to what extent these characteristics may have influenced the development of the Ouwerkerkse bloom.

Our choice for H<sub>2</sub>O<sub>2</sub> treatment of the *Alexandrium* bloom was motivated by recent H<sub>2</sub>O<sub>2</sub> applications that successfully ended cyanobacterial blooms in Lake Koetshuis (Matthijs et al., 2012), three other freshwater lakes (H.C.P. Matthijs and R. Talens, unpublished), and wastewater treatment ponds (Barrington et al., 2011; 2013). A key issue for the treatment of harmful algal blooms with H<sub>2</sub>O<sub>2</sub> is the selection of a suitable dosage. Several studies have indicated that cyanobacteria appear to be more sensitive to H<sub>2</sub>O<sub>2</sub> than most eukaryotic phytoplankton species (Barroin and Feuillade, 1986; Drábková et al., 2007a, b; Barrington and Ghadouani, 2008). Due to their sensitivity, Matthijs et al. (2012) could selectively remove a bloom of the freshwater cyanobacterium *Planktothrix agardhii* with a low dosage of only 2 mg L<sup>-1</sup> of H<sub>2</sub>O<sub>2</sub>, without any major effects on the remaining eukaryotic phytoplankton community. Recent laboratory incubations indicate that the eukaryotic “brown tide” species *Aureococcus anophagefferens* can also be suppressed by similarly low H<sub>2</sub>O<sub>2</sub> levels (Randhawa et al., 2012, 2013). In contrast, studies on the removal of dinoflagellate cysts from ballast water indicated that eradication of dinoflagellate species requires considerably higher H<sub>2</sub>O<sub>2</sub> concentrations, ranging from 30 mg L<sup>-1</sup> for *Alexandrium catenella* (Ichikawa et al., 1992) to more than 5000 mg L<sup>-1</sup> for *Gymnodinium catenatum* (Bolch and Hallegraeff, 1993). Our laboratory incubations showed that vegetative cells and pellicle cysts of *Alexandrium ostenfeldii* were effectively removed at a H<sub>2</sub>O<sub>2</sub> dosage of 40 mg L<sup>-1</sup>. We do not know whether this dosage was also effective against sexual resting cysts of *A.*

*ostenfeldii*, as we did not observe sexual resting cysts during our study. It might be suggested that the higher H<sub>2</sub>O<sub>2</sub> tolerance of dinoflagellates in comparison to cyanobacteria is due to their relatively large and armored cells. However, this is contradicted by our observations that small green algae (~5 µm diameter) and unarmored euglenophytes survived the H<sub>2</sub>O<sub>2</sub> dosage that we applied to the Ouwerkerkse Kreek. Hence, our results re-emphasize that the effective H<sub>2</sub>O<sub>2</sub> dosage is very species-specific. Also for future H<sub>2</sub>O<sub>2</sub> applications, laboratory verification of the suitable H<sub>2</sub>O<sub>2</sub> dosage (similar to our lab incubations) is essential before H<sub>2</sub>O<sub>2</sub> is applied to the field.

Field applications are inherently less predictable than small-scale laboratory incubations. For instance, some of the added H<sub>2</sub>O<sub>2</sub> might be rapidly consumed by chemical oxidation–reduction reactions in the water (Matthijs et al., 2012) and the degradation rate of H<sub>2</sub>O<sub>2</sub> might increase to some extent with algal densities (Randhawa et al., 2013), thereby reducing the contact time during which the algae are exposed to H<sub>2</sub>O<sub>2</sub>. Hence, although the laboratory results suggested that a dosage of 40 mg L<sup>-1</sup> might suffice, we chose a slightly higher dosage of 50 mg L<sup>-1</sup> of H<sub>2</sub>O<sub>2</sub> to ensure an effective field treatment. Moreover, we first tested the field application in a pilot study in the canal, before it was successfully applied to the entire creek system.

A key question is whether the *Alexandrium ostenfeldii* population will return after the H<sub>2</sub>O<sub>2</sub> treatment. Due to the short-term nature of the H<sub>2</sub>O<sub>2</sub> treatment, where the added H<sub>2</sub>O<sub>2</sub> decays within a few days, a permanent eradication of *A. ostenfeldii* is not guaranteed. In the Ouwerkerkse Kreek, the population remained very low throughout the remainder of the year in which it was treated. It is conceivable, however, that *A. ostenfeldii* might reoccur in subsequent years. For instance, sexual resting cysts in the sediment may have survived the H<sub>2</sub>O<sub>2</sub> treatment, as they have thicker cell walls and hence are likely to be more resistant to H<sub>2</sub>O<sub>2</sub> than vegetative cells and pellicle cysts. Rapid degradation of H<sub>2</sub>O<sub>2</sub> in the sediment will also reduce the contact time during which sedimented cysts are exposed to oxidative stress. However, no cyst survey was performed on the creek after the treatment, and hence we have not been able to assess their potential survival in the sediment. Furthermore, it might also be that the H<sub>2</sub>O<sub>2</sub> treatment was less effective in some parts of the creek system that were difficult to reach with the water harrow (e.g., dense reed beds along the shoreline).

One year after the H<sub>2</sub>O<sub>2</sub> treatment (summer 2013), cell counts revealed a mix of *Alexandrium ostenfeldii* and a smaller *Alexandrium* species (most likely *A. tamarense*) at concentrations of  $8.0 \times 10^3$  cells L<sup>-1</sup> in the treated creek area, which is more than two orders of magnitude lower than during the *A. ostenfeldii* bloom of August 2012. In a small pond and canal upstream of the treated area *Alexandrium* reached local concentrations of  $1.8 \times 10^6$  cells L<sup>-1</sup> in 2013, which might provide a source area for the development of a new *A. ostenfeldii* population. Hence, *A. ostenfeldii* has not been completely removed from the Ouwerkerkse Kreek, and it might potentially develop dense blooms again in the future. The H<sub>2</sub>O<sub>2</sub> treatment may therefore require repeated use over multiple years, especially when shellfishery protections or public health risks demand action. In this respect, it compares to other emergency treatments of dinoflagellate blooms, such as yearly clay flocculation treatments of recurrent *Cochlodinium* blooms in Korea (Kim, 1998; Lee et al., 2008). There are several strategies that could help to suppress the return of *A. ostenfeldii* after an H<sub>2</sub>O<sub>2</sub> treatment. For instance, regular flushing might prevent the build-up of a new population. Moreover, a consequence of the differential sensitivity of phytoplankton species to H<sub>2</sub>O<sub>2</sub> is that treatments will not only lead to a collapse of the phytoplankton community, but also to shifts in the relative species composition. The remaining phytoplankton species provide the new starting material for the establishment of a new phytoplankton community (Dakos et al., 2009). Hence, once the target species has been eradicated, it might be of interest to actively introduce a new phytoplankton community (e.g., from nearby waters) that may serve to counter re-invasion of the harmful target species. Reduction of the total nutrient influx to the system could ultimately prevent future bloom developments. Unfortunately, the Ouwerkerkse Kreek is located in a region dominated by intense agriculture and a nearby village, where nutrient reduction is very challenging. A long-term management plan which includes routine monitoring and early response efforts is now required to ensure the threat of an *A. ostenfeldii* bloom does not re-emerge.

One of the primary concerns with respect to the H<sub>2</sub>O<sub>2</sub> treatment in Ouwerkerkse Kreek was the potential impact on other organisms. The 94% decrease of total phytoplankton was anticipated as the applied dosage of 50 mg L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> concentration was higher than any other study of this kind. Likewise, the total zooplankton abundance dramatically declined due to the

treatment. This was also anticipated as previous research has shown high zooplankton mortalities at H<sub>2</sub>O<sub>2</sub> concentrations from 1 to 10 mg L<sup>-1</sup> (Kuzirian et al., 2001; Meinertz et al., 2008; Matthijs et al., 2012). Larger organisms showed less negative responses to hydrogen peroxide. Macroinvertebrate species composition did differ between the treated canal and the non-treated open waters of the creek (Table 6.3). However, macroinvertebrates were still abundant after the H<sub>2</sub>O<sub>2</sub> treatment, and the differences between the two areas seem more likely to be associated with different habitat preferences of the species than with a response to the H<sub>2</sub>O<sub>2</sub> treatment.

Although the H<sub>2</sub>O<sub>2</sub> treatment led to local mortality of three-spined sticklebacks in the canal, we did not detect any fish mortality within the creek apart from a single eel three days after treatment. Hence, the impact of the H<sub>2</sub>O<sub>2</sub> treatment on the fish population was only minor. This result aligns with several studies on the use of H<sub>2</sub>O<sub>2</sub> as a disinfectant in aquaculture at concentrations ranging from 10 to 1000 mg L<sup>-1</sup> depending on the fish species, life-stage, and temperature of the water (Rach et al., 1997; Gaikowski et al., 1999; Small, 2004). Some fish species are relatively sensitive to H<sub>2</sub>O<sub>2</sub>. For instance, Clayton and Summerfelt (1996) report 1.3% mortality of walleye juveniles when exposed to 50 mg L<sup>-1</sup> of H<sub>2</sub>O<sub>2</sub> for 60 min. The lethal concentration (LC<sub>50</sub>) for juveniles of two trout species is 189–280 mg L<sup>-1</sup> of H<sub>2</sub>O<sub>2</sub> after exposure for 120 min (Arndt and Wagner, 1997). Most fish species that have been investigated appear sensitive only at higher H<sub>2</sub>O<sub>2</sub> concentrations of 200–1000 mg L<sup>-1</sup> (Rach et al., 1997; Gaikowski et al., 1999; Avendaño-Herrera et al., 2006).

In conclusion, our results show that an *Alexandrium* bloom can be terminated with H<sub>2</sub>O<sub>2</sub> within a few days. Key advantages of the treatment are that it is rapid and very effective, with minimal environmental damage as effects on fish and macroinvertebrates are minor and the applied H<sub>2</sub>O<sub>2</sub> concentration quickly decays. The treatment does have some disadvantages, however. Depending on the H<sub>2</sub>O<sub>2</sub> concentration required, the impact on co-occurring phytoplankton and zooplankton species can be severe. Also, especially with cysting HAB species, complete decimation for multiple years is probably difficult to achieve with a single treatment. Thus, we recommend the use of H<sub>2</sub>O<sub>2</sub> as an effective short-term emergency treatment when the rapid and

complete termination of a harmful algal bloom from a relatively enclosed area is required.

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