Diurnal changes in the xanthophyll cycle pigments of freshwater algae correlate with the environmental hydrogen peroxide concentration rather than non-photochemical quenching

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- Background and Aims In photosynthetic organisms exposure to high light induces the production of reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂), which in part is prevented by non-photochemical quenching (NPQ). As one of the most stable and longest-lived ROS, H₂O₂ is involved in key signalling pathways in development and stress responses, although in excess it can induce damage. A ubiquitous response to high light is the induction of the xanthophyll cycle, but its role in algae is unclear as it is not always associated with NPQ induction. The aim of this study was to reveal how diurnal changes in the level of H₂O₂ are regulated in a freshwater algal community.

- Methods A natural freshwater community of algae in a temporary rainwater pool was studied, comprising photosynthetic Euglena species, benthic Navicula diatoms, Chlamydomonas and Chlorella species. Diurnal measurements were made of photosynthetic performance, concentrations of photosynthetic pigments and H₂O₂. The frequently studied model organisms Chlamydomonas and Chlorella species were isolated to study photosynthesis-related H₂O₂ responses to high light.

- Key Results NPQ was shown to prevent H₂O₂ release in Chlamydomonas and Chlorella species under high light; in addition, dissolved organic carbon excited by UV-B radiation was probably responsible for a part of the H₂O₂ produced in the water column. Concentrations of H₂O₂ peaked at 2μM at midday and algae rapidly scavenged H₂O₂ rather than releasing it. A vertical H₂O₂ gradient was observed that was lowest next to diatom-rich benthic algal mats. The diurnal changes in photosynthetic pigments included the violaxanthin and diadinoxanthin cycles; the former was induced prior to the latter, but neither was strictly correlated with NPQ.

- Conclusions The diurnal cycling of H₂O₂ was apparently modulated by the organisms in this freshwater algal community. Although the community showed flexibility in its levels of NPQ, the diurnal changes in xanthophylls correlated with H₂O₂ concentrations. Alternative NPQ mechanisms in algae involving proteins of the light-harvesting complex type and antioxidant protection of the thylakoid membrane by de-epoxidized carotenoids are discussed.

Key words: Chlamydomonas, Euglena, algae, diatom, xanthophyll cycle, reactive oxygen species, ROS, NPQ, hydrogen peroxide, diurnal, antioxidant, Chlorella, oxidative stress.

INTRODUCTION

The production of reactive oxygen species (ROS), such as superoxide (O₂⁻) and hydrogen peroxide (H₂O₂), is a well-characterized phenomenon of eukaryotic photosynthetic organisms, particularly under high light (Foyer and Noctor, 2003; Keren and Krieger-Liszkay, 2011). In the presence of transition metals, such as Fe(II) or Cu(II), H₂O₂ forms the highly reactive hydroxyl radical (·OH), which can cause irreversible damage of membranes and proteins (Halliwell, 2006). Conversely, ROS also contribute to redox signalling pathways in development and in stress responses, enabling acclimatization of the photosynthetic organism (Ledford et al., 2007; Urzica et al., 2012; Barth et al., 2014). A variety of antioxidants help maintain a ‘healthy’ cellular redox environment, thereby preventing oxidative stress while allowing local temporary bursts of ROS for signalling purposes (Foyer and Noctor, 2009; Mittler et al., 2011; Miechel et al., 2013). Diurnal rhythms of H₂O₂ and antioxidants occur within organelles of photosynthetic organisms, leading to shifts in localized redox states, which coordinate fundamental processes such as the cell cycle and enable coordination of growth with the environment (Kiyota et al., 2006; Pellny et al., 2009; Lai et al., 2012; Cheng et al., 2013). Diurnal rhythms of H₂O₂ also occur in freshwater lakes and marine waters that peak at midday (Cooper et al., 1988; Herrmann, 1996; Clark et al., 2008). Under high light, photosynthetic organisms are a likely source of this H₂O₂, which can pass through aquaporin membrane channels of the chloroplast and cell (Ishikawa et al., 1993; Mubarakshina et al., 2010), increasing the concentration in the surrounding water or media to low micromolar levels (Collen and Pedersen, 1996; Allorent et al., 2013). In addition, dissolved organic carbon (DOC) also...
produces various oxygen radicals and H$_2$O$_2$ when excited by UV-B radiation in sunlight (Mill et al., 1980), and a positive correlation between DOC concentrations of various water bodies and H$_2$O$_2$ production during daylight has been reported (Scully et al., 1996).

Due to the regulatory and potentially destructive nature of H$_2$O$_2$ in biological processes, aquatic microorganisms may have to regulate the level of H$_2$O$_2$ in their environment. As H$_2$O$_2$ can cross membranes, dictated by concentration gradients, environmental H$_2$O$_2$ could be broken down intracellularly by enzymes, such as ascorbate peroxidase and catalase. Hydrogen peroxide could even be an ecological driver affecting species composition as certain cyanobacteria are particularly sensitive to H$_2$O$_2$ (Drábková et al., 2007; Matthijs et al., 2012). The cyanobacterium *Synechocystis* uses flavo-diiron proteins, which allows oxidation of over-reduced electron transport chains and prevents ROS production (Zhang et al., 2009). On the contrary, symbiotic coral dinoflagellates possess a large capacity to photo-reduce O$_2$ (Roberty et al., 2014). In the waters surrounding coral reefs, temporal variation in H$_2$O$_2$ metabolism by the micro-organism community has been reported (Shaked and Armoza-Zvuloni, 2013). To the best of our knowledge it is not known whether H$_2$O$_2$ levels are regulated by photosynthetic organisms in freshwater ecosystems.

It is widely accepted that control of photosynthetic electron transport is paramount in preventing ROS production in the chloroplast in response to the highly variable light intensities experienced in the field (for review see Niyogi and Truong, 2013; Roach and Krieger-Liszkat, 2014). In conjunction with a potent antioxidant system, non-photochemical quenching (NPQ) enables photosynthetic organisms to protect against ROS production in the chloroplast (Roach and Krieger-Liszkat, 2012; Allorent et al., 2013). The reversible attachment of light-harvesting complex II (LHCCI) to photosystem II (PSII), called state transition (qT), is one of the rapidly reversible NPQ mechanisms that lowers excitation pressure and balances excitation energy between photosystems, and is particularly relevant to green algae (Nagy et al., 2014). Another NPQ mechanism, called high energy state quenching, occurs as a result of conformational changes within the thylakoid membrane that lead to the dissipation of excess light energy to heat (Tokutsu and Minagawa, 2013; Niyogi and Truong, 2013). Furthermore, the de-epoxidation/epoxidation cycle of xanthophylls, termed the xanthophyll cycle, is ubiquitously involved in the eukaryotic response to high light (Goss and Lepetit, 2015). The xanthophyll carotenoids are all derived from the $\beta$-carotene branch, but vary phylogenetically; in higher plants and in many green and brown algae the de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin occurs, while in marine dinoflagellates, diatoms and a few other organisms, such as green Euglenophytes (freshwater protists), diadinoxanthin and diatoxanthin are the xanthophyll cycle pigments (Goss and Lepetit, 2015). Whereas NPQ is important in lowering excitation pressure and ROS production under excess light, zeaxanthin is also a potent antioxidant against lipid peroxyl radicals (Lim et al., 1992; Havaux et al., 2007). Moreover, zeaxanthin formation occurs as a high light response of the green alga *Chlamydomonas reinhardtii*, but, unlike in higher plants, it is not associated with qE, which instead is reliant on a key protein of the light-harvesting complex (LHC) type called LHCSR3 (Peers et al., 2009). This indicates that early in eukaryotic evolution the xanthophyll cycle could have had an alternative function, with structural and antioxidant protection of the thylakoid membranes during high light as likely possibilities (Havaux, 1998; Baroli et al., 2003).

We hypothesized that the freshwater community responds to and influences the concentrations of environmental H$_2$O$_2$. We used a natural freshwater community to investigate the mechanisms of H$_2$O$_2$ production and breakdown alongside diurnal changes in high light protective mechanisms. Strikingly, the diurnal changes in the xanthophyll cycle correlated with H$_2$O$_2$ concentrations, while no such relationship was observed with NPQ, strengthening the argument for an antioxidant function of the xanthophyll cycle in protecting the thylakoid membrane from ROS-induced damage.

**MATERIALS AND METHODS**

**Location, environmental parameters and the photosynthetic community under study**

The freshwater community was situated in a temporary rainwater pool at the northern edge of Innsbruck in Tyrol, Austria (47°16'5" N, 11°22'12" E), 690 m above sea level, on clay/humus-based soil with a maximum water depth of 10 cm at the time of investigation. Except for 2.5 h of midday sun, the community was in the shade during the day. The pH of the water was between 7.5 at night and up to 9.5 during the day. All on-site measurements were taken on 22 and 30 March 2014, referred to as Day 1 and Day 9, respectively, and chosen for the similarity of climatic conditions on both days. Air temperature and relative humidity were monitored with WMR88 sensors (Oregon Scientific, Portland, OR, USA) every minute and the O$_2$ concentration of the water at a depth of 2 cm was monitored with a Fibox-3 O$_2$ optode (PreSens, Regensburg, Germany) on Day 1. At regular intervals the photosynthetically active radiation (PAR) and UV-B levels were recorded using a PMA2100 system (Solar Light, Glenaside, PA, USA).

Three algal zones were determined: the top half of the water, the benthic region, which was predominantly composed of algal mats, and the epipelic community on the bare soil, which at the time of measurements was moist but not covered with water (Fig. 1). Organisms were observed with an Olympus BX50 (Olympus Corporation, Tokyo, Japan) differential interference contrast microscope equipped with an Olympus UIS2 objective lens ($\times 100$) using immersion oil (refractive index 1.52). Five samples were collected from each zone and 70 images were taken with an Olympus DP25 camera using Cell D software (Olympus Soft Imaging Solutions, v3.1); organisms were identified using morphological criteria and their representation in the algal communities was calculated as the percentage of total eukaryotic photosynthetic organisms.

**Photosynthetic performance**

Photosynthetic performance of these three zones, as indicated by the effective quantum yield of photosystem II (FPSSII), was measured with a MINI-PAM (Walz, Effeltrich, Germany) attached to a 2-mm diameter optic-fibre probe that could be plunged into the water ($n = 10$ for each zone). We measured NPQ with an AquaPen (PSI, Brno, Czech Republic) using a
blue LED as actinic light (455 nm; 300 μmol quanta m$^{-2}$ s$^{-1}$) and saturating pulses of 1000 μmol quanta m$^{-2}$ s$^{-1}$, after 0.5 h of dark adaptation in a 3-mL cuvette. Equations for calculating ΦPSII and NPQ can be found in Maxwell and Johnson (2000).

Measurements of hydrogen peroxide

To measure H$_2$O$_2$, 1 mL of water was adjusted to pH 7.0 with 50 mM phosphate buffer and then incubated in the dark with 5 μM Ampliflu Red (Sigma-Aldrich, St Louis, MO, USA), and 0.01 U of horseradish peroxidase was added 5 min before centrifugation for 10 min at 26 000 g and 25 °C. The absorbance of the supernatant was measured at 571 nm and measured against a standard that was linear up to 5 μM. To investigate H$_2$O$_2$ production and breakdown in the water, centrifuged (16 000 g for 5 min) and non-centrifuged water samples were placed in 8-cm Petri dishes next to the pool under full sunlight, either unfiltered or placed underneath UV-B-opaque glass for 2 h and then allowed to recover in the shade for 1 h, simulating the light pattern experienced by the freshwater community. The UV-opaque glass reduced PAR by 11.4% and UV-B by 89.7%.

Photosynthetic pigments

To sample the pigments of organisms in the water column, 15-mL samples were taken from the top half of the water column and immediately frozen in liquid nitrogen and lyophilized for 5 d. Pigments in algal mats were also measured after lyophilization (0.13 ± 0.03 g dry weight, n = 10). Photosynthetic
RESULTS

Composition of the algal community

Algae from epipelic communities were found above the water level on damp soil (Fig. 1A), in the water column (Fig. 1B) and in algal mats in the benthic region (Fig. 1C). The majority of the water and soil-borne community was composed of a large flagellate protist, *Euglena* sp., morphologically identified as *E. viridis* (Fig. 1D). Green algae were less common (14 % of total organisms) and included *Chlamydomonas* spp. (Fig. 1E) and *Chlorella* spp. (Fig. 1F). Depending on day (see below) and location, pigments were separated by HPLC with a Spherisorb (Waters, Milford, MA, USA) reverse-phase column (5 μm, 250 × 4.6 mm) using solvents and gradients according to Pfeifhofer et al. (2002). To isolate the pigments, lyophilized extracts were suspended in 2 mL of tert-butyl methyl ether containing 0.1 % butylated hydroxytoluene, placed in an ultrasonic bath for 15 min and incubated for 16 h at –20 °C before 1 mL of 20 % methanol was added. The samples were briefly shaken and centrifuged for 5 min at 5000 g and 4 °C. Nine hundred microlitres of the upper phase was collected, evaporated by vacuum centrifugation for 15 min at 30 °C and 50 % methanol before centrifugation for 40 min at 26 000 g and 4 °C and injection of 50 μL. Whole-spectrum absorbance was measured using an Agilent 1100 Series (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode-array detector. For quantification, absorbance at 440 nm was measured against separate standards, except for diatoxanthin, for which zeaxanthin was used after correction for the difference in extinction coefficients. Chlorophyll c was not measured and all pigments were normalized to chlorophyll a. The de-epoxidation ratio (DEP) of the xanthophyll cycle was calculated by dividing de-epoxidized xanthophyll cycle pigments by total xanthophyll cycle pigments (i.e. antheraxanthin + zeaxanthin/antheraxanthin + violaxanthin + zeaxanthin) or (diatixanthin/diatxanthin + diadinoxanthin) or (sum of total de-epoxidized xanthophyll cycle pigments/sum of total xanthophyll cycle pigments).

Isolation and culture of *Chlorophyta* algae

Water samples were diluted in half-strength 3 N Bold's Basal Medium (BBM) at pH 8 and cultured at 50 μmol quanta m⁻² s⁻¹ between 15 and 22 °C. After 3 weeks, samples were diluted and spread on Tris–acetate–phosphate (TAP) 1.5 % agar plates. Colonies derived from individuals were multiplied on TAP agar plates at 20 °C at 70 μmol quanta m⁻² s⁻¹ under a 16h/8h light/dark cycle for 7 d and transferred to BBM liquid culture at pH7 for 14 d. Culture purity was monitored with a microscope (as above) after each multiplication stage. High light at 500 μmol quanta m⁻² s⁻¹ was provided by a compact fluorescent bulb while cultures were gently agitated in the presence or absence of 10 μM nigericin sodium salt dissolved in H₂O (10 mM stock) and the H₂O₂ concentration of the medium was measured with Ampilflu Red after 1.5 h, as described above.

**Fig. 2.** Diurnal changes in light intensity and H₂O₂ concentrations and the H₂O₂ concentration at various depths of the water column. (A) Light intensity (open squares, right y-axis) and concentration of H₂O₂ in the top half of the water on Day 1 (closed circles, left y-axis) or Day 9 (closed squares, left y-axis); n = 5 ± s.d. (B) Concentration of H₂O₂ through the water column at 16:00 h on Day 9; n = 4 ± s.d. Different letters denote significant difference (P < 0.05).

Navicula pennate diatoms (Fig. 1G) made up between 10 % of the water-borne community and 70 % of the algal mats.

**Diurnal O₂ and H₂O₂ levels in the water column**

The H₂O₂ concentration of the water body followed the pattern of light intensity. Before sunrise the H₂O₂ concentration was below 0.25 μM and gradually increased above 0.5 μM during the morning, when still in shade (Fig. 2A). After direct sunlight hit the water the concentration of H₂O₂ increased further before steadily decreasing in accordance with the encroaching shade. Measurements of H₂O₂ at various depths in the water column showed that H₂O₂ concentrations were dependent on depth, being significantly lower in the benthic region next to the algal mats in comparison with the surface (Fig. 2B). Two days were chosen for measurement based on their climatic similarities (Supplementary Data Fig. S1). On Day 1 a peak H₂O₂ concentration of 0.63 μM was recorded, and on the Day 9 the H₂O₂ concentration reached 2.14 μM. However, between these days the chlorophyll a and b concentration of the organisms in the water decreased from 0.67 ± 0.45 to 0.17 ± 0.10 μg mL⁻¹, which was assigned to the stress of the brief cold spell of weather (Supplementary Data Fig. S1), typical of this region. The O₂ concentration increased from 0.18 to 0.65 mm below night and midday. This greatly deviated from the
equilibrium O₂ concentration, by up to 0.13 mm lower at night and 0.4 mm higher in the day (Fig. 3). This deviation clearly showed that photosynthesis increased the O₂ content of the water during daylight, while O₂ was consumed at night, most likely from respiration.

To investigate H₂O₂ metabolism in the water column, samples with or without algae (removed by centrifugation) were placed for 2 h under sunlight that was either filtered or unfiltered for UV-B radiation. Reducing the UV-B level lowered the production of H₂O₂ during sunlight exposure (Fig. 4). The contribution of UV-B to H₂O₂ production would agree with a role for DOC, observed as a brown tint to the water (Fig. 1C). The presence of algae always lowered the production of H₂O₂ and especially increased H₂O₂ breakdown during recovery after high light (Fig. 4), which explains the rapid decline in H₂O₂ concentrations of the freshwater community in the afternoon shade (Fig. 2A). However, H₂O₂ was never completely broken down (Figs 2A and 4), showing that a basal level of H₂O₂ was always present.

**Photosynthetic performance and non-photochemical quenching of the communities**

The ΦPSII decreased between 12:00 and 14:00 h, when direct sunlight hit the water, and more so in the epipelic community (Fig. 5A). The air humidity above the epipelic community fell to 25% when temperatures rose above 20 °C (Supplementary Data Fig. S2). The photosynthetic performance of the organisms at the top of the water column was slightly higher than that of those in the benthic region (Fig. 5A). On Day 1 the NPQ increased up to 0.43 during the period of direct sunlight.

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*Fig. 3. Water temperature and O₂ concentration of the water on Day 1. Also shown is the hypothetical O₂ content of the water at equilibrium with the atmospheric O₂ content at that temperature.*

*Fig. 4. Sources of H₂O₂ production and breakdown in the water. Concentration of H₂O₂ in removed water samples before and after 2 h of sun exposure and after 1 h of recovery in shade, with (A) and without (B) the sunlight being filtered, and with and without microorganisms removed, as indicated by +/− algae under the x-axis; n = 4 ± s.d., different letters denote significant difference when comparing all data (P < 0.05).*

*Fig. 5. Diurnal changes in light use efficiency, NPQ and xanthophyll de-epoxidation (DEP) ratios of the freshwater communities, and the correlation of DEP with NPQ or H₂O₂ concentration of the water. (A) Relative quantum yield of photosystem II (ΦPSII) of the organisms in the top half (surface) of the water, in the benthic zone and on the soil (epipelic) on Day 1; n = 10 ± s.d. (B) NPQ of the organisms in the top half of the water, comparing violaxanthin and diadinoxanthin cycles on Day 1 and Day 9. Asterisks show significant differences between the DEP ratio of the violaxanthin and diadinoxanthin cycles (P < 0.05). (D) Correlation of the average combined DEP ratio of both xanthophyll cycles with either NPQ (R² = −0.34, line not shown) or H₂O₂ concentration (R² = 0.83).*
sunlight and decreased to 0.18 in the shade (Fig. 5B). Only the epipelagic community had a high NPQ of 1.1 ± 0.3 after 1.5 h of full sun (Fig. S2). The NPQ in samples taken at midday on Day 9 was twice as high as on Day 1, and kept a value near 1 well into the afternoon shade, only decreasing when it became dark again. State transitions were observed during the day and night, whereas only during the day could algae induce major qE (Supplementary Data Fig. S3). Furthermore, although the location of the algae during full sunlight influenced the level of qE (e.g. epipellic > benthic), the chlorophyll fluorescence response of qT and a transition to state I during 2 min of actinic illumination was a consistent NPQ characteristic (Supplementary Data Fig. S3).

**Diurnal changes in xanthophyll cycles**

Both the violaxanthin and diadinoxanthin cycles were detected in the algal population by HPLC (Supplementary Data Fig. S4), with the diadinoxanthin cycle pigments of diatoms and the *Euglena* sp. being much more prevalent (69–80 % of total xanthophylls) than the violaxanthin cycle pigments of the green algae (5–6 % of total xanthophylls; Supplementary Data Fig. S4). Early in the day the DEP ratio of both cycles increased in the morning shade, with the violaxanthin cycle significantly more advanced than the diadinoxanthin cycle (Fig. 5C). The DEP ratio of both cycles was higher on Day 9 than on Day 1, in accordance with higher NPQ on this day. However, on both days the DEP ratio of the xanthophyll cycle decreased rapidly in the afternoon shade, more so for the violaxanthin cycle pigments, so that by evening antheraxanthin and zeaxanthin were not detectable, whereas diatoxanthin remained present into the night (Fig. 5C). Relating the DEP ratio of the xanthophyll cycle pigments to qE showed that the DEP ratio matched the NPQ pattern on Day 1. On Day 9 the DEP ratio of both xanthophyll cycles fell in the afternoon shade, as it did on Day 1, but the qE response of NPQ remained high. Moreover, the combined DEP ratio of both xanthophyll cycles showed no positive correlation to NPQ, but a positive correlation was observed between the DEP ratio and the concentration of H2O2 (Fig. 5D).

Photosynthetic pigments were also assessed in algal mats that grew on the bed of the pool and could be clearly distinguished by their release of gas bubbles during the day (Fig. 1B). The pigments of the organisms found in these mats included the diadinoxanthin cycle pigments, but strikingly more fucoxanthin (Supplementary Data Fig. S4), both of which are found in diatoms (Table 1). As diatoms have no chlorophyll *b* and instead use chlorophyll *c*, the high chlorophyll *a:* *b* ratio of 18 ± 0 agrees with the identification of 70 % of the photosynthetic organisms in the mats as *Navicula* diatoms. Considering the specific photosynthetic pigments found in the various organisms of this freshwater community (Table 1), two interesting correlations of the algal mats could be observed. After data had been normalized to chlorophyll *a*, which is present in all photosynthetic organisms, a strong positive linear correlation was found between chlorophyll *b* and diadinoxanthin ($R^2 = 0.85$, Supplementary Data Fig. S4) and to a lesser extent chlorophyll *b* and neoxanthin ($R^2 = 0.41$, data not shown) and a strong negative linear correlation was found between chlorophyll *b* and fucoxanthin ($R^2 = 0.90$; Supplementary Data Fig. S4) and to a lesser extent between chlorophyll *b* and lutein ($R^2 = 0.39$, data not shown). Neoxanthin is specific to the green lineage of photosynthetic organisms, whose presence is associated with the appearance of chlorophyll *b* and is therefore absent in diatoms (Takaichi and Mirauro, 1998). Of all the algae identified here, only *Euglena* sp. possesses diadinoxanthin and chlorophyll *b* (Table 1; Takaichi, 2011), and only diatoms possess fucoxanthin, less lutein, but no neoxanthin, so that these pigments indicate the presence of diatoms in the water samples.

In this respect, the changes in the pigments between Day 1 and Day 9 related a reduction in fucoxanthin and lutein, but an increase in diadinoxanthin/diatoxanthin and neoxanthin (Supplementary Data Fig. S4), which agrees with the less frequent observation of diatoms in the water samples on Day 9 and the chlorophyll *a:* *b* ratio dropping from 6.4 ± 1.3 ($n = 25$) to 1.6 ± 0.3 ($n = 18$).

**Non-photochemical quenching effect on high-light-induced H2O2 production**

Finally, to test whether qE of NPQ prevents the production of H2O2 during exposure to high light, two isolated green algae species from the freshwater community (*Chlamydomonas* sp. and *Chlorella* sp.) were treated with high light in the presence or absence of the qE inhibitor nigericin. As an uncoupler of proton gradients, nigericin significantly reduced the NPQ, which was accompanied by increased release of H2O2 in both species (Fig. 6), indicating that qE in the Chlorophyta algae was able to prevent the over-excitation of photosynthetic pigments that otherwise leads to H$_2$O$_2$ release from the cells.

**DISCUSSION**

Hydrogen peroxide contributes to an array of signalling pathways, although in excess it can imbalance the cellular redox state, split to form ‘OH radicals and lead to oxidative stress (Halliwell, 2006). In this study, UV-B irradiation was active in producing H2O2 (Fig. 4), most likely through photo-excitation of DOC, which releases a variety of ROS (Mill *et al.*, 1980; Scully *et al.*, 1996), of which H2O2 is the most stable and can accumulate. Concentrations of H2O2 rose to >2 μM (Fig. 2A) and then rapidly decreased, likely broken down by the organisms present. As shown in Fig. 4, organisms contributed to

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<th>Pigment</th>
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H$_2$O$_2$ breakdown at the concentrations measured here. In this study, ascorbate peroxidase is a likely candidate for H$_2$O$_2$ breakdown, suggesting that the surrounding H$_2$O$_2$ concentration of the water column was higher than that in the algae, which would enable passive diffusion into the cells, where it could be broken down by antioxidant enzymes. Ascorbate peroxidase, ubiquitously found in eukaryotic photosynthetic organisms (Shigeoka et al., 2002), efficiently breaks down H$_2$O$_2$ at these concentrations. A rapid increase in the transcription of the ascorbate peroxidase gene is a response of *C. reinhardtii* to high light (Michelet et al., 2013; Barth et al., 2014), which agrees with increased ascorbate-dependent H$_2$O$_2$ breakdown following 1 h of high light (Roach et al., 2015). In the study by Roach et al. (2015), breakdown of 10 $\mu$M H$_2$O$_2$ by total cell extracts of *C. reinhardtii* was ascorbate-dependent and was abolished by azide, which inhibits ascorbate peroxidase. Altogether, this suggests that ascorbate peroxidase is a likely candidate for H$_2$O$_2$ breakdown at the concentrations measured here. In the current investigation some H$_2$O$_2$ breakdown in the afternoon shade also occurred in the absence of algae (Fig. 4), indicating that dissolved minerals, such as certain metal ions that catalyse the Fenton reaction, were also breaking down some H$_2$O$_2$. It is not possible to conclude that certain alga species broke down more H$_2$O$_2$ than others; however, lower H$_2$O$_2$ was measured next to the diatom-rich algal mats (Fig. 2B). Moreover, fewer diatoms were present on Day 9 (perhaps due to the cold spell), when H$_2$O$_2$ concentrations reached higher levels (Fig. 2B), but the general algal population was also reduced, so that high H$_2$O$_2$ breakdown activity cannot be assigned to diatoms. The lowered algal population of the water column on Day 9 would have reduced the shading and increased UV-B penetration into the water, which may also have contributed to increased H$_2$O$_2$ production on Day 9. It is relevant to mention that diatoms collect organic material to form benthic mats (Vos et al., 1988), also observed here under the microscope (R. Miller, pers. observ.). The UV-B-induced production of H$_2$O$_2$ may be higher in the proximity of organic material when exposed to the sun, indicating that diatoms may need elevated H$_2$O$_2$ breakdown defences. In summary, water-borne organisms in this freshwater community apparently scavenged the H$_2$O$_2$ produced from DOC in the presence of UV-B.

The production of ROS in the chloroplast is an unavoidable consequence of photosynthesis as excited pigments and charged electron transport chains operate in the O$_2$-rich environment of the chloroplast. High photosynthetic rates leading to net accumulation of O$_2$ in the water column (Fig. 3) would have exacerbated this process. The isolated green algae *Chlamydomonas* sp. and *Chlorella* sp. released more H$_2$O$_2$ under elevated light in the absence of qE, which was achieved with nigericin (Fig. 6). The result of using nigericin as an uncoupler of the pH gradient also rules out the mitochondria as a source of ROS, a process that requires an inner mitochondrial membrane pH gradient (Lambert and Brand, 2004). This is in agreement with a role for qE in protecting against ROS production inside the chloroplast under excess light (Roach and Krieger-Liszka, 2012; Allorent et al., 2013; Roach et al., 2015).

Concerning the other organisms found in the freshwater community, very little is known of the NPQ mechanisms in photosynthetic Euglenophytes, except that NPQ is independent of the xanthophyll cycle despite the fact that diaoxanthin accumulation occurs in response to high light in *E. gracilis* (Doege et al., 2000). This indicates that qE induction is more likely regulated by an LHCx protein, similar to LHCSR3 of *C. reinhardtii* (Peers et al., 2009). The chloroplasts of photosynthetic Euglenophytes were derived from a secondary endosymbiosis event with a prasinophyte (Green, 2011); the prasinophytes are a group of green algae that includes *Osterococcus tauri*, which also possesses LHCSR3-related NPQ induction (Peers et al., 2009). As the *Euglena* species were the most prevalent and largest organisms (Fig. 1) they likely contributed the most to the chlorophyll fluorescence. Increased synthesis of an LHCx protein, possibly in response to the cold spell, could be an explanation for the sustained NPQ (Fig. 5B). In agreement with Doege et al. (2000) the lack of correlation between NPQ and the DEP ratio of the diadinoxanthin cycle on Day 9 (Fig. 5) indicates that in *Euglena* sp. the diadinoxanthin cycle was not a major contributor to NPQ. This is in contrast to the qE response of diatoms, which possess a qE-type response using diatoxanthin (Chukhutsina et al., 2014). The LHCs of diatoms bind fucoxanthin, which can be partially replaced by diatoxanthin under high light (Gundermann and Büchel, 2008). When diatoxanthin de-epoxidase was knocked out in the diatom *Phaeodactylum tricornutum* the NPQ response was reduced (Lavaud et al., 2012). However, the LHCx1 protein of *P. tricornutum* is also an essential component of the NPQ response, and the level of qE in LHCx1-deficient mutants is only half that in wild-type cells (Baillieul et al., 2010), confirming that the xanthophyll cycle is not the only factor affecting NPQ in diatoms.

Chlorophyll fluorescence, as used to measure NPQ, is complex to interpret in green algae because it is difficult to clearly...
distinguish the overlapping NPQ mechanisms (Allorent et al., 2013). Chlorophyll fluorescence quenching from qE induction is a typical hallmark of NPQ. However, the transition to state II in qT also decreases chlorophyll fluorescence because LHClII de-attaches from PSII, which happens during dark adaptation, as shown in C. reinhardtii (Roach et al., 2015). Conversely, a rise in chlorophyll fluorescence occurs during transition to state I in qT, because LHClII re-attaches to PSII (Allorent et al., 2013; Nagy et al., 2014). It has been shown that transition to state I during actinic illumination functions to build up sufficient absorption capacity to facilitate qE induction (Allorent et al., 2013; Roach et al., 2015). All chlorophyll fluorescence traces of the water column showed characteristics of qT (Supplementary Data Fig. S3), indicating that the Euglena species, which contributed most to chlorophyll fluorescence, possessed high levels of qT, similar to C. reinhardtii.

Considering that the NPQ responses of some of the organisms identified here are independent of the xanthophyll cycle raises the question of the function of de-epoxidized xanthophylls. The lack of correlation between the DEP ratio and NPQ responses identified here are independent of the xanthophyll cycle possessed high levels of qT, similar to species, which contributed most to chlorophyll fluorescence, level. The correlation between the xanthophyll cycle and H2O2 production to organisms and concentrations must be kept below a critical value. It is unlikely that de-epoxidized xanthophylls break down H2O2 directly, but they can react with peroxyl radicals or structurally alter the membrane architecture (Lim et al., 1992; Havaux, 1998), thereby protecting the membrane against lipid peroxidation when H2O2 concentrations and light intensities are high.

In conclusion, the diurnal cycling of H2O2 was apparently modulated by the organisms in this freshwater community. Hydrogen peroxide is a reactive molecule that can cause stress to organisms and concentrations must be kept below a critical level. The correlation between the xanthophyll cycle and H2O2 concentration rather than NPQ agrees with a role for de-epoxidized xanthophylls in protecting the thylakoid membrane from ROS-induced damage. Furthermore, fundamental processes of photosynthetic organisms, such as the cell cycle, are redox-controlled, which may require regulation of environmental H2O2.

**SUPPLEMENTARY DATA**

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. **Figure S1:** air temperatures between Day 1 and Day 9, as indicated in the colour key. **Figure S2:** air humidity 0.5 m from the freshwater community during Day 1. **Figure S3:** chlorophyll fluorescence traces of the algae on Day 1 as used for measuring NPQ. **Figure S4:** HPLC measurements of photosynthetic pigments on Day 1 and Day 9 and in algal mats.

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**LITERATURE CITED**


