ROS

RESEARCH ARTICLES

Photo-Oxidative Stress in Green Algae and Cyanobacteria

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ABSTRACT | High intensity visible light and ultraviolet radiation (UVR) could trigger oxidative stress (OS) and pigment bleaching in photosynthetic organisms and thus might represent a serious threat for cell survival. In the present study, green alga *Chlamydomonas reinhardtii* and cyanobacterium *Synechocystis* sp. were exposed to solar simulated light with different spectral composition: (i) visible light, (ii) visible light with environmental level of UVR, (iii) visible light with enhanced ultraviolet B (UVB) radiation. Photo-induced increase of the cellular reactive oxygen species (ROS), OS, and damage, as well as the alterations of the cell autofluorescence were thoroughly examined using flow cytometry. A small percentage of the cell population was affected by visible light and light with environmental levels of UVR. Exposure to light with enhanced UVB radiation resulted in severe OS, lipid peroxidation, and membrane damage. Light with enhanced UVB also induced a shift of the photosynthetic pigment fluorescence towards low values. *C. reinhardtii* was more sensitive to UVB as compared with *Synechocystis* sp. The obtained results highlight the considerable difference in the levels of OS induced by visible, UVA, and UVB radiation in both species, as well as the species-specific sensitivity to UVB radiation, which should be taken into account when assessing the impact of enhanced UVB radiation on natural phytoplankton communities.

KEYWORDS | Chlamydomonas; Chlorophyll bleaching; Flow cytometry; Membrane damage; Oxidative stress; Synechocystis

ABBREVIATIONS | FCM, flow cytometry; OS, oxidative stress; PI, propidium iodide; PSI, photosystem I; PSII, photosystem II; ROS, reactive oxygen species; SSR, solar simulated light; SSR_{NAT}, SSR_{VIS} plus UVR radiation; SSR_{UVB}, SSR_{NAT} plus enhanced UVB; SSR_{VIS}, SSR composed of only visible light; UVA, ultraviolet A; UVB, ultraviolet B; UVR, ultraviolet radiation

CONTENTS

1. INTRODUCTION

Oxidative stress (OS) is one of the main consequences in photosynthetic microorganism exposed to excessive levels of visible light and UV radiation (UVR). The effects of UVR on the algal and cyanobacterial growth and photosynthetic activity, production of pigments and UVR absorbing compounds, antioxidant activity, cell ultrastructure alterations, protein and DNA damage are well-documented [1–3]. However, much less attention was paid to UVR-induced reactive oxygen species (ROS) generation and subsequent photo-oxidative stress and damage in microalgae and cyanobacteria. The majority of the available studies focused on the photo-oxidative damages induced by excessive levels of ultraviolet B (UVB) radiation [4–11], while much less is known about the OS caused by light of environmentally relevant intensity and spectral composition [12–14]. The existing studies revealed an increased intracellular ROS concentration in cyanobacterial species exposed to solar simulated light but did not provide information on the possibly associated cellular damages. Moreover, it is recognized that OS occurs when the intracellular ROS overwhelm the antioxidant capacity of the cell [16–18]. The assessment of photo-oxidative stress and damage in addition to intracellular ROS concentration is highly sought for the evaluation of the harmful potential of visible and UV radiation. Recently, it was shown that the exposure to high light generated singlet oxygen in *C. reinhardtii*, reacting with membrane lipids and damaging membranes [15]. Comparison of *pgrl 1*, *npq4*, and *pgrl1npq4* mutants of *Chlamydomonas reinhardtii* during high light acclimation, showed that PGRL1 and/or LHCSR3 regulate electron flow upstream of photosystem I (PSI), and limit the accumulation of electrons on the PSI acceptor side, thus avoiding PSI photo-inhibition [16]. Study of cyanobacterium *Synechococcus elongatus* PCC 7942, overexpressing an iron superoxide dismutase (Fe-SOD) and *Synechocystis* sp. PCC 6803 revealed the overexpression of these ROS-scavenging enzymes might protect the repair of PSII rather photosystem II (PSII) from photo-damage [17]. Together with these important basic knowledge advances, it was recognised that most of the studies were conducted under irradiance conditions that are higher than the natural condition and lack environmental realism.

In this work, the photo-oxidative effects of solar simulated light of different spectral composition on two microorganisms, the cyanobacterium *Synechocystis* sp. and the eukaryotic green alga *Chlamydomonas reinhardtii*, were studied using flow cytometry (FCM). FCM is increasingly adopted in ecotoxicology to examine cellular stress [18], since it allows simultaneous multiparameter analysis at the single cell level. Namely, information on cellular traits, and physiological and structural cell properties can be obtained directly or in combination with specific fluorescent stains. Among the available fluorescent dyes for ROS measurements and oxidative damages [19], only those based on fluorescein diacetate were applied for measurement of ROS generated by UVR [4–6, 8, 9, 12–14, 20]. In the present work, three different probes were used to target specifically the intracellular ROS, lipid peroxidation, and membrane damage in cells exposed to solar simulated light with different spectral composition.

2. MATERIAL AND METHODS

2.1. Test Organisms and Culture Conditions

Chlamydomonas reinhardtii strain CPCC11, obtained from the Canadian Phycological Culture Center (CPCC, Department of Biology, University of Waterloo, Ontario, Canada) was cultivated in 4×-diluted Tris-acetate-phosphate liquid medium [21]. *Synechocystis* sp. PCC 6803, from the Pasteur Culture Collection of Cyanobacteria (PCC, Institute Pasteur, France) was grown in BG11 medium [22]. Axenic cultures were grown in a specialized incuba-

tor (Infors, Bottmingen, Switzerland), under rotary shaking of 100 rpm, temperature of 20°C, and illumination of 100 µmol photons m^{-2} s^{-1}. Cells in their mid-exponential growth were harvested by gentle centrifuged (2,083 *g* for 3 min), rinsed, and suspended with fresh medium to a final cell density of 1×10^6 cells/ml, then exposed to solar simulated light with different spectral composition. Cellular density and characteristics of the inoculum were determined with BD Accuri C6 flow cytometer (BD Biosciences, Switzerland).

2.2. Solar Simulated Light Exposure

Thirty ml of the algal suspensions were exposed to solar simulated light (Sun 2000 solar simulator, Abet Technologies, Milford, CT, USA), with a xenon short arc lamp (type UXL-553, Ushio Inc., Tokyo, Japan) using opened test tubes submersed in a water bath at 20°C to keep the temperature constant during the incubation. Three different combinations of filters (AE filter, AM 1.G filter, UVC blocking filter, and UVABC blocking filter; Abet Technologies) were used to simulate exposure to the light with different spectral composition. SSR_{VIS} (see Abbreviations) is composed of only visible light with an intensity of 234 Wm^{-2}. SSR_{NAT} (see Abbreviations) has visible light intensity comparable to SSR_{VIS} plus UVR (UVA 12.64 Wm^{-2}, UVB 0.63 Wm^{-2}) with values comparable with those measured at midday on a clear sky day on the shore of Lake Geneva. SSR_{UVB} (see Abbreviations) has visible and UVA intensities comparable to $SSR_{NAT,}$ but enhanced UVB radiation (UVB 1.95 Wm^{-2}).

2.3. FCM Measurements and Staining with Fluorescent Probes

To evaluate the time course of the photo-induced OS and damage, aliquots of each test replicate were sampled at 30-min intervals and analyzed by FCM. The flow cytometer was equipped with three detectors for determination of green (530 ± 15 nm), yellow (585 ± 20 nm), and red (670 ± 25 nm) fluorescence upon excitation with a 488 nm argon-ion laser, and with a detector for red (670 ± 12.5 nm) fluorescence upon excitation with a 640 nm. For each sample, 20,000 events were measured using the medium flow rate mode. BD Accuri C6 Software 264.15 was used for data acquisition and analysis. Algal cells were discriminated from other particles by applying the gating strategy as previously described [23]. Intracellular ROS, lipid peroxidation, and membrane damages were examined using the fluorescent probes CellROX® green, C11-$BODIPY^{581/591}$ (Life Technologies Europe B.V., Zug, Switzerland) and propidium iodide (PI) (Sigma–Aldrich, Buchs, Switzerland), respectively. The staining procedures and gating strategies were detailed in our previous study [24]. Gates designed to assess the percentage of cells experiencing OS, lipid peroxidation, and membrane damage, as well as autofluorescence were shown in **Figures 1A** and **2A**.

2.4. Statistical Analysis

Kruscal–Wallis analysis of variance on ranks was applied together with the Student–Newman–Keuls test for multiple comparison ($p < 0.05$) using Sigma Plot 11.0 (Systat Software Inc., San Jose, CA, USA).

3. RESULTS AND DISCUSSION

3.1. SSR-Induced Photo-Oxidative Stress and Damage

Intracellular ROS increase and lipid peroxidation were detectable for both *Synechocystis* sp. and *C. reinhardtii* after 2 h exposure to both SSR_{VIS} and SSR_{NAT}; however, less than 5% of the cells in the populations were affected (**Figure 1B**). The percentage of cells with damaged membranes after 3 h exposure to SSR_{NAT} for *Synechocystis* sp. and after 2 h exposure to SSR_{NAT} for *C. reinhardtii* (**Figure 1B**) was around 5%. These results showed that high saturating light, SSR_{VIS} and environmental levels of UVA and UVB, SSR_{NAT} did not cause short-term severe stress in microorganisms. They were in line with the literature results obtained for cyanobacterium *Anabaena variabilis* exposed to comparable solar simulated light conditions where no increase in cellular ROS was observed within the first 12 h exposure [12, 13]. These observations also suggested that the multiple mechanisms in *C. reinhardtii* to minimize stress caused by excess of light [25] were very efficient under the studied conditions.

By contrast, SSR_{UVB}-induced strong OS and damage in both alga and cyanobacterium (**Figure 1B**). Indeed, more than 90% of *C. reinhardtti* and *Syn-*

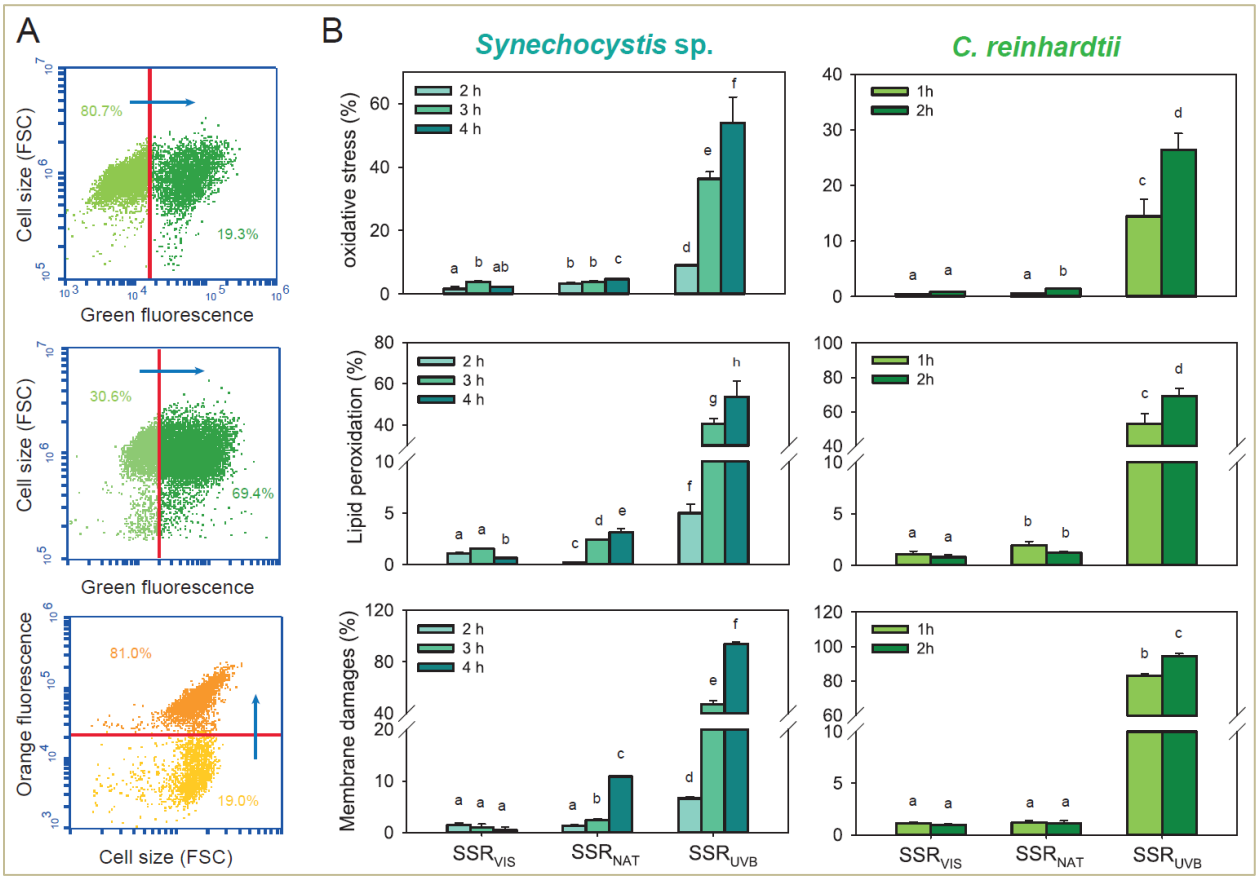

FIGURE 1. Photo-oxidative stress and membrane damage of cells exposed to solar simulated light. (A) Gates applied to determine the percentages of affected cells; from the top to the bottom: *C. reinhardtii* cells exposed for 1 h to SSR_{UVB} and stained with CellROX® green, C11-BODIPY$^{581/591}$, and propidium iodide. (B) Percentages of *Synechocystis* sp. (on the left) and *C. reinhardtii* (on the right) cells affected by oxidative stress, lipid peroxidation, and membrane damage. Values are reported as mean ± standard deviation of three replicates. Different letters indicate statistically significant differences from the values of controls not exposed to solar simulated light ($p < 0.05$).

echocystis sp. cells experienced OS, lipid peroxidation, and membrane damage after 1 h and 3 h exposure, confirming the potential of UVR to cause severe cellular damage even during short-term exposure. The increased number of cells with membrane damage indicated that cell homeostasis was compromised and thus the stress led to cell death. A significant difference was observed between the photo-oxidative effects caused by SSR_{NAT} and SSR_{UVB}. These two exposure conditions correspond to light with comparable spectral composition and differ only in the intensity of UVB radiation, confirming that high intensity of UVB is the main driver of the observed photo-oxidative stress [12, 13]. UVB is known to induce generation of ROS in microalgae by photodynamic action and by damaging the electron transport chain in photosynthesis [6]. Indeed, intracellular ROS [4–6, 8, 9, 12–14, 20] and lipid peroxidation [4, 5, 8, 10, 11, 20] were observed in several microalgae exposed to high levels of UVB radiation. The increase of the percentage of cells with photo-oxidative stress under SSR_{VIS} (no UVR component) and SSR_{NAT} might be associated to an oxidative burst able to activate cellular response to stress. Indeed,

the oxidative burst is one of the earliest response in photosynthetic organisms towards external stressors, and ROS might act as signals for the activation of cell responses and subsequent adaptive processes [26]. On the other hand, the photo-oxidative stress observed in presence of enhanced UVB merely represents a threat for microalgal cells and, given the severe cell damages, an acclimation to these light conditions is implausible.

Different phytoplankton species have different sensitivities to UV radiation [1]. In this work, green alga *C. reinhardtii* exhibited higher sensitivity than cyanobacterium *Synechocystis* sp. when exposed to light with enhanced UVB radiation, as revealed by the extremely high percentage of cells with OS, lipid peroxidation, and membrane damage after 2 h. Interestingly, the photo-oxidative stress response of *Synechocystis* sp. was delayed, as shown by the comparable percentage of the affected cells after 4 h exposure. Such a delay might be related to the presence of a cellular sheath of exopolymeric substances which were reported to reduce UV damages [3]. These results highlight the species-specific differences in the UVB-induced photo-oxidation in microalgae, which are expected to be of primary importance in evaluating the impact of UVB radiation on natural phytoplankton.

3.2. SSR-Induced Alteration in Cell Autofluorescence

Photosynthetic cells are naturally fluorescent due to the presence of pigments such as chlorophylls and phycobilins. Bleaching of these pigments is a recognized photo-oxidative damage caused by UVR [27]. In this study, no changes of chlorophyll and phycocyanin fluorescence were observed after short-term exposure to SSR_{VIS} and to SSR_{NAT} (**Figure 2B**) despite the saturating levels of the visible light for both green alga and cyanobacterium [28]. By contrast, cell autofluorescence was strongly altered under SSR_{UVB} (**Figure 2B**). Significant shift of chlorophyll fluorescence toward lower values was found in both *C. reinhardtii* and *Synechocystis* sp. populations after 2 h and 3 h exposure, respectively. For *Synechocystis* sp. a decrease of phycocyanin fluorescence was also observed after 3 h. These results were consistent with the significant OS experienced by the cells under the studied conditions. Furthermore, the UVB-induced bleaching of phycocyanin fluorescence was considerably higher than the decrease of chlorophyll fluorescence in *Synechocystis* sp. This finding was in agreement with previous observation for cyanobacterium *Anabaena* sp. [8]. The UVB-induced decrease of the algal fluorescence might be associated with two phenomena: direct chlorophyll degradation mediated by intracellular ROS [7] and downregulation of the genes involved in the synthesis of photosynthetic pigments [29]. As phycocyanin exhibits considerable antioxidant and radical-scavenging activity [30], the decrease of the phycocyanin fluorescence was in agreement with the enhanced oxidative stress (**Figures 1** and **2**) in *Synechococcus* sp.

In parallel to the decrease of the fluorescence of photosynthetic pigment, a significant increase in the green and orange autofluorescence of both *C. reinhardtii* and *Synechocystis* sp. was found for cells exposed to SSR_{UVB} (**Figure 2**). A shift towards higher values of green and orange autofluorescence was also observed in *Synechocystis* sp. exposed to SSR_{NAT} after 1 to 2 h. It is difficult to link the changes of green and orange autofluorescence of microalgal cells with specific pigments. Nonetheless, microorganisms are known to produce a vast array of substances and antioxidant molecules under light stress conditions which might be responsible for the observed increase in autofluorescence [1]. For example, *Synechocystis* sp. PCC6803 is known to produce mycosporine-like amino acids under photo-oxidative stress [31] with fluorescence emission spectrum maximum in the blue light close to the absorbance band of chlorophyll *a* [32]. Carotenoids were shown to confer green fluorescence to the green alga *Dunaliella salina* [33], while increase in orange fluorescence due to phychoerythrin was reported upon exposure of two cyanobacterium *Nostoc* species to UVB [34].

4. CONCLUSION

Short-term exposure to SSR_{NAT} and SSR_{VIS} had no significant effects on the membrane permeability and pigment bleaching in *C. reinhardtii* and *Synechocystis* sp. A small percentage of cells in the population experienced oxidative stress and lipid peroxidation, showing that these phytoplankton species were able to face short-term variations in natural light intensities even if they were at the saturation levels. The

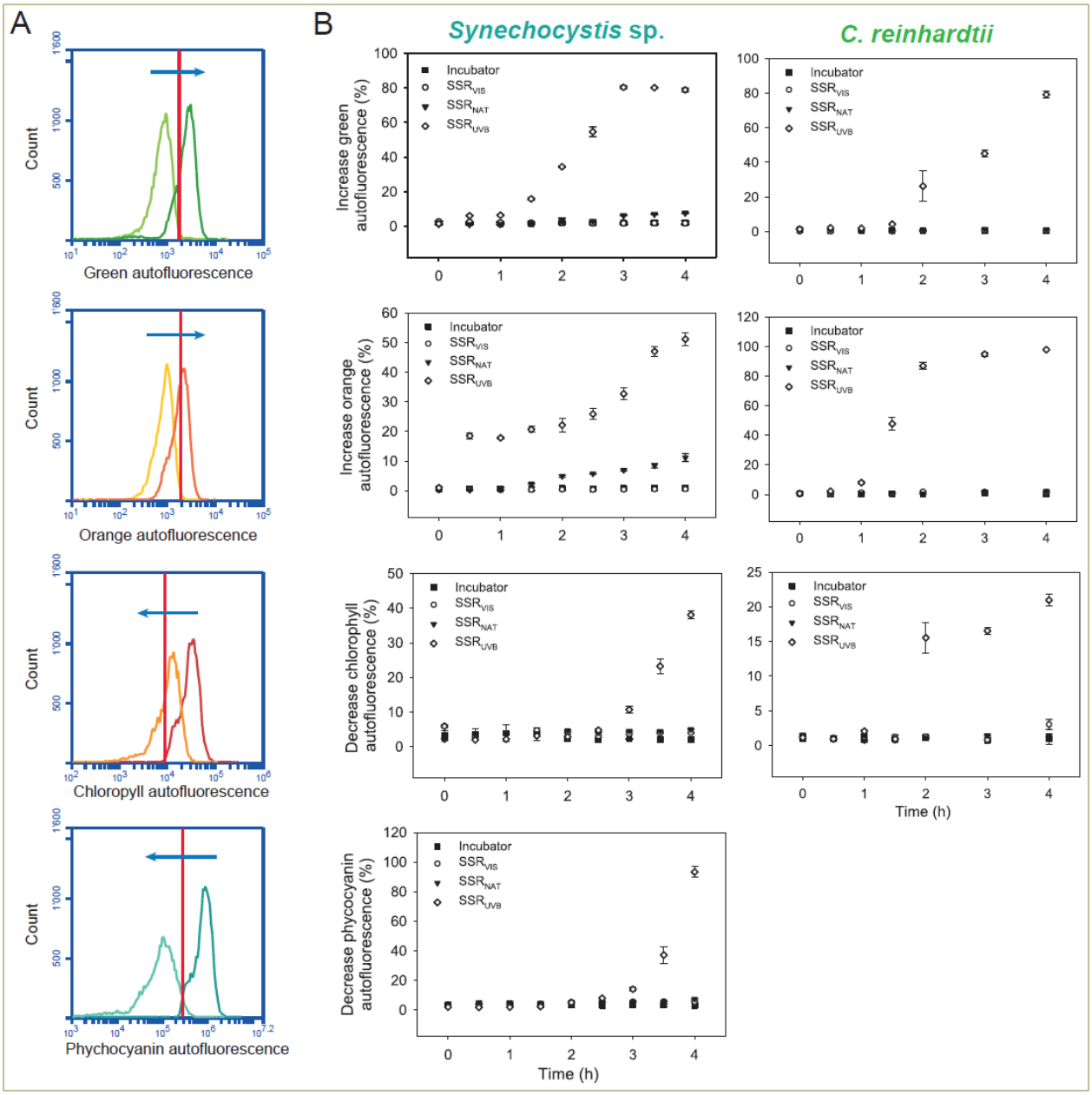

FIGURE 2. Cell autofluorescence versus exposure time. (A) Gates applied to evaluate autofluorescence alterations; form the top to the bottom: overlay of green, orange, chlorophyll, and phycocyanin cytograms of *Synechocystis* sp. prior and after 4 h exposure. (B) Percentages of cells with altered autofluorescence. Values are reported as mean ± standard deviation of three replicates.

enhanced UVB radiation caused severe short-term effects on microalga and cyanobacterium: excessive cellular ROS generation, lipid peroxidation, compromised cell membrane, and bleaching of photosynthetic pigments. The present results confirmed that enhanced UVB radiations might have lethal effects

on photosynthetic aquatic microorganism even when they were exposed for short time. Photo-oxidative damages occurred earlier in *C. reinhardtii* than in *Synechocystis* sp. suggesting that species-specific sensitivity should be taken into account when assessing the impact of enhanced UVB radiation on natural phytoplankton communities.

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