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Original Research Article

Repeated release of cerium oxide nanoparticles altered algal responses: Growth, photosynthesis, and photosynthetic gene expression

Saibo Liu [a], Jingheng Han [a], Xiaowu Ma [a], Xiaoshan Zhu [b], Han Qu [c], Guorong Xin [a], Xiaochen Huang [a,*]

[a] State Key Lab of Biocontrol, Guangdong Provincial Key Laboratory of Plant Resources, School of Agriculture and Biotechnology, Shenzhen Campus of Sun Yat-sen University, Shenzhen 518107, China
[b] College of Ecology and Environment, Hainan University, Haikou 570228, China
[c] College of Environment and Ecology, Key Laboratory of the Three Gorges Reservoir Region's Eco-Environment, Ministry of Education, Chongqing University, Chongqing 400045, China

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ABSTRACT

The expanding production of engineered nanomaterials (ENMs) can eventually cause their increased release into and presence in aquatic ecosystems, potentially threatening the health of aquatic organisms and the stability of the ecological environment. Generally, ENMs are repeatedly released into real-world aquatic environments in relatively low concentrations, potentially affecting photosynthesis in primary producers such as algae. However, knowledge regarding the effects of repeated exposure to ENMs on algal photosynthesis is still lacking. Herein, the physiological responses of the freshwater algae *Chlorella vulgaris* following single and repeated exposures to cerium oxide nanoparticles (CeO_2 NPs) were investigated at 10 mg/L, with a focus on photosynthesis. The results showed that repeated exposures triggered increased photosynthetic pigment contents, oxidative stress levels, decreased photosynthetic performance, and lower biomass in *C. vulgaris* compared to a single exposure. Photosynthesis-related genes (i.e., *petA*, *petB*, *psaA*, *atpB*, and *rbcL*) were found to be upregulated following repeated exposures. Particularly for *petB*, repeated rather than single exposure treatment significantly upregulated its expression levels by 2.92–10.24-fold compared to unexposed controls. Furthermore, increased exposure times could aggravate the interaction between CeO_2 NPs and algae, elevating 8.13%, 12.13%, and 20.51% Ce distribution on the algal cell surface or intracellularly, compared to a single exposure. This study is the first to investigate the effects of ENM exposure times on algal photosynthesis, providing new insights into the assessment of the risks these materials pose to real-world aquatic environments.

1. Introduction

Recent advances in nanotechnology have dramatically increased the production of artificially engineered nanomaterials (ENMs), as well as their inevitable release into ecological systems. Indeed, over 10,000 ENMs-based products have been registered in the Nanotechnology Product Database as of 2023 [1]. Among these ENMs, cerium oxide nanoparticles (CeO_2 NPs) can significantly transform between Ce(III) (Ce_2O_3) and Ce(IV) (CeO_2) forms. They have been used as diesel fuel additives [2], mechanical polishers [3], pharmacological agents [4], and catalyzers [5]. However, the usage and unanticipated discharge of ENMs, including CeO_2 NPs, into the environment can mean their eventual migration into bodies of water, where they can pose direct threats to aquatic organisms [6]. The Organization for Economic Cooperation and Development (OECD) has listed CeO_2 NPs as one of 13 priority ENMs for immediate testing and risk assessment since 2008 [7]. To date, several studies have identified the deleterious effects of CeO_2 NPs on aquatic organisms at environmentally relevant concentrations (i.e., 1–100 nmol/L) [8–11].

The final concentrations of ENMs in aquatic environments arise from the combined effects of continuous ENM usage and the repeated discharge of wastewater containing these substances—which, in turn, leads to extensive contact between them and aquatic organisms [12–14]. Previous toxicological studies have generally focused on the effects of single exposure of ENMs on aquatic organisms [15–17], and have thus overlooked the effects of repeated exposures. Considering that the

* Corresponding author.
E-mail address: huangxch55@mail.sysu.edu.cn (X. Huang).

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exposure period of pollutants is deeply connected with the physiological rhythms of the exposed organisms [18], in the long-term biogeochemical cycle, repeated exposure to ENMs may also cause a chronic impact on environmental microorganisms, as well as different biological effects in a single organism, compared to a single exposure [19]. For example, repeated exposure to TiO_2 NPs can induce significant shifts in soil bacterial diversity and community structure, owing to the mobility of these NPs [13]. Regarding aquatic organisms, some studies have found that repeated exposure to ENMs can trigger weaker nanotoxicity responses than single exposure. However, in a recent study, Cao et al. [19] found that although repeated exposure to low concentrations of Ag NPs had little effect on algal growth, it nevertheless accelerated the intracellular accumulation of these NPs. Enhanced NPs internalization into the algal cells suggested that the mode of repeated exposure may aggravate the aquatic ecological risks of ENMs via their migration through food chains [20]. Conflicting results in this field may be attributable to organism-dependent differences in responses to specific ENMs.

Algae represent one of the most important primary producers in many aquatic ecosystems. Their growth and cellular responses to environmental stresses are highly regulated by the light processed in their photosynthetic systems [21]. They produce oxygen and consume CO_2 through photosynthesis, sequestering ~54.9 to 67.7 tons/ha of CO_2 annually via algal photosynthesis, thus representing one of the most critical carbon sequestration pathways in many ecosystems [22]. They are thus generally considered to be important contributors to the goal of achieving global carbon neutrality as soon as possible [23,24]. Zhang et al. [25] reported that several typical indexes (e.g., photochemical quantum yield and non-photochemical quenching) for the degree of photosynthesis in algae were sensitive to ENMs, suggesting photosynthesis as an adjustable parameter through which to adjust the algal response to ENMs. Algal chloroplasts, a source and reservoir for reactive oxygen species (ROS) production and accumulation, may be harmed by exposure to ENMs [26]. Environmental stress-induced ROS overproduction may not only limit CO_2 fixation but also disturb the overall process of photosynthesis [27]. Therefore, photosynthesis can be used as an effective biological indicator to identify the toxicological effects of ENMs [25,28]. Previous studies have found that low-dose or long-term exposure to ENMs not only induced significant variations in growth and/or photosynthesis, but also significantly regulated gene expression [29]. Given the important role of photosynthesis in algae, it is reasonable to hypothesize that changes in exposure to ENMS (e.g., single vs. repeated exposures) may significantly influence algal growth and development by regulating photosynthesis at the genetic level.

Notably, once ENMs are released into aquatic environments, they induce the formation of homo-aggregates or hetero-aggregates with aquatic organisms [30,31]. In contrast to single exposure, repeated releases of CeO_2 NPs may provide more opportunities for interactions with algae, owing to the reduction of homo-aggregates. This may explain the differences in algal growth and photosynthesis observed as a result of exposure to CeO_2 NPs, but this notion merits further investigation. In this study, both single and varying levels of repeated exposures (i.e., 2, 3, and 6 times) to CeO_2 NPs were used to investigate the resultant differences in growth, photosynthesis, and oxidative stress characteristics in the freshwater algae *Chlorella vulgaris*. The expression of photosynthesis-related genes was determined to reveal whether repeated exposures could significantly affect photosynthesis at the genetic level. Finally, the aggregation, the Ce distribution, and the biological effects of direct contact between CeO_2 NPs and algae were investigated to explain the different mechanisms underlying algal responses to single and repeated exposures to CeO_2 NPs. This study will, hopefully, provide valuable insights into the ecological risks ENMs pose to aquatic ecosystems from the perspective of photosynthesis.

2. Materials and methods

The CeO_2 NPs used in this study were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China) and had a purity of >99.95%. The morphology and size of CeO_2 NPs were observed via transmission electron microscopy (TEM; FEI Talos F200x, Thermo Fisher Scientific, USA). The Ce^{3+}/Ce^{4+} ratios on the surface of CeO_2 NPs were determined using X-ray photoelectron spectroscopy (XPS; K-Alpha, Thermo Fisher Scientific, USA), and each spectrum was calibrated using its C1s peak at 284.8 eV. The crystal structure of the nanoparticles was also determined using X-ray diffraction (XRD; Ultima IV, Rigaku Co., Ltd., Japan).

2.1. Algal culture and CeO_2 NPs exposure

C. vulgaris (strain no. FACHB9) was obtained from the freshwater algae culture collection at the Institute of Hydrobiology, Chinese Academy of Science (Wuhan, China). The cultures were pre-cultivated in sterile BG11 medium (pH = 7.0 ± 0.2) in conical flasks at 24 °C, using an illumination shaking incubator at 150 rpm and a light/dark cycle of 14 h/10 h [light intensity: 100 μmol/(m^2•s); red: 23.2%, green: 48.7%, blue: 28.0%]. Once the algae reached the exponential growth phase, CeO_2 NPs were added to the growth substrate in single and repeated additions. The initial concentration of algae was 1×10^6 cells/mL, which were treated with CeO_2 NPs for 72 h. Before the exposure experiment, 1 g/L of CeO_2 NPs stock solution was prepared and sonicated for 20 min. Toxicological assays over a range of concentrations (0, 0.1, 1, 5, 10, 50, and 100 mg/L) of CeO_2 NPs on *C. vulgaris* were conducted to determine the sublethal concentration, which was then used for the repeated exposure experiment. The algal number, biomass, and chlorophyll content of the samples exposed to CeO_2 NPs for 120 h at varying concentrations are shown in Fig. S2. No significant changes in cell numbers were observed at any concentration of CeO_2 NPs from 0 to 120 h. However, when exposed to CeO_2 NPs at 10 mg/L, both algal biomass and the ratio of chlorophyll a to b (Chl a/b) were significantly decreased (Fig. S2). On the other hand, the ROS levels, superoxide dismutase (SOD) activity, and catalase (CAT) activity increased (Fig. S3, $p < 0.05$). Previous studies have shown that the environmentally relevant concentration of CeO_2 NPs, as well as other nanomaterials in the aquatic environment, has reached the range of mg/L [19,32,33]. With the increase in yields of CeO_2 NPs, their amount of repeated release would probably elevate to 10 mg/L and then become a key environmental concern. In addition, current studies have demonstrated the toxicity of CeO_2 NPs to algae when concentrations reached 4.4–29.6 mg/L [16]. Therefore, 10 mg/L was the concentration selected for comparing the algal responses following single and repeated exposures to CeO_2 NPs. The doses and addition times of CeO_2 NPs for repeated exposures varied with the incubation time. As shown in Fig. S4, within a 72-h exposure treatment, the 1/2, 1/3, and 1/6 doses of 10 mg/L CeO_2 NPs were concurrently added to algae-contained media at every 36 h, 24 h, and 12 h, respectively. The control, single exposure, 2× repeated exposure, 3× repeated exposure, and 6× repeated exposure groups were labeled Con, 1-CeO_2 NPs, 2-CeO_2 NPs, 3-CeO_2 NPs, and 6-CeO_2 NPs, respectively. The interactions between the algae and the CeO_2 NPs were determined in the 1-CeO_2 NPs group via scanning electron microscopy (SEM; SU8100, Hitachi Ltd., Japan) equipped with energy dispersive X-ray spectroscopy (EDS; UltimMax65, Oxford Instruments plc., UK). More detailed information regarding the procedure is shown in the supporting information (SI, Text S1).

2.2. Growth inhibition

After 72 h of exposure to CeO_2 NPs, algae were collected and counted using an automatic cell-counting instrument (Countstar, ALIT Life Science Co. Ltd., China). The biomass of the algae was then calculated via the following equation [34]:

$$\text{Biomass} = \frac{c_1 \times l_1 \times d_1^2}{c_0 \times l_0 \times d_0^2} \times \text{Biomass}_0 \quad (1)$$

where c_1 and c_0 represent the concentration of the algal suspensions in the treatment and control groups, respectively, l_1 and l_0 represent the

average lengths of the algal cells in the treatment and control groups, respectively, and d_1 and d_0 represent the average diameters of the algal cells in the treatment and control groups, respectively. $Biomass_0$ represents the dried algal biomass in the control group, which was prepared via overnight vacuum drying to achieve a constant weight.

The inhibition ratio of algal growth under the different treatments was evaluated using the following equation:

$$\text{Algal growth inhibition ratio} = \frac{N_0 - N_1}{N_0} \times 100\% \quad (2)$$

where N_0 and N_1 represent the algal cell numbers in the control and treatment groups, respectively.

2.3. Photosynthetic pigment contents and chlorophyll fluorescence parameters

The photosynthetic pigments (i.e., Chl a and Chl b) of *C. vulgaris* were extracted and measured using an ultraviolet–visible light (UV–Vis) spectrophotometer (U-3900, Hitachi), following a previously described method with minor modifications [10]. The steps used for pigment extraction and measurement are described in detail in Text S2. The chlorophyll fluorescence of the algae was determined via pulse amplitude modulated (PAM) fluorometry (AP110-C, PSI, Czechia), which was dark-adapted for 15 min before the determination. For each measurement, a 4-mL algal suspension was prepared and added to a four-sided clear cuvette for the measurement of chlorophyll fluorescence. Rapid light curves (RLCs) were tested by exposing the cuvettes to gradients of light intensity [10, 20, 50, 100, 300, and 500 μmol/(m^2·s)] to quantify the respiratory rate of the algae, which were plotted by fitting the relative electron transport rate against the photosynthetically active radiation (PAR) using a double exponential decay equation. The final slope (β) of the RLCs reflected the efficiency of light energy consumption. Several indexes—including non-photochemical quenching (NPQ), photochemical quenching (qL), photochemical quantum yield ($\Phi_{PS\,II}$), and chlorophyll fluorescence transient (OJIP test)—were used to evaluate the algal photosynthesis activity under single and repeated exposures to CeO_2 NPs. Detailed information regarding the formula used to calculate the fluorescence parameters is described in Table S1.

2.4. Oxidative stress

The methods used to measure oxidative stress via the selected physiological indexes have been described in our previous report [35]. Briefly, algal pellets were collected via centrifugation (3,000*g* at 4 °C for 10 min), treated with liquid nitrogen for 20 min, thawed for 30 min at 4 °C, and centrifuged for 10 min (10,000 rpm at 4 °C). The freeze-thaw step was repeated three times to obtain the homogenate for determining the activity levels of the intracellular enzymes. The activity levels of two of the antioxidant enzymes, CAT and SOD, were detected using A007-1-1 and A001-3-2 assay kits (Nanjing Jiancheng Bioengineering Institute, China), respectively. Soluble protein content was also determined using the Coomassie brilliant blue G250 staining method to calculate CAT and SOD activities. To determine the level of intracellular ROS, algal suspensions were treated with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) under dark conditions for 15 min, after which fluorescence was detected at an excitation/emission wavelengths of 485/525 nm using a fluorescent microplate reader (Spark, TECAN, Switzerland). The activity of extracellular lactate dehydrogenase (LDH) was measured using an LDH cytotoxicity detection kit (C0016, Beyotime Institute of Biotechnology, China). Before the determination, the samples were centrifuged at 3,500*g* for 15 min, and the supernatants were collected.

2.5. Quantification of photosynthesis-related gene expression

The expression levels of photosynthesis-related genes in *C. vulgaris* were determined via quantitative real-time polymerase chain reaction (qRT-PCR). They included the *psbA*, *psbD*, *petA*, *petB*, *psaA*, *atpB*, and *rbcL* genes. The amplification of 18S rRNA was used as an internal control reference gene. Prior to the qRT-PCR experiment, algal pellets were obtained from the algal suspension (20 mL) following centrifugation at 8,000 rpm for 15 min. RNA was extracted from the algal pellets using a HiPure Plant RNA Mini Kit (R4151-02, Magen Biotechnology Co. Ltd., Guangzhou, China). The synthesis of cDNA was done using a First Strand cDNA Synthesis Kit (11141, YEASEN Biotechnology Co. Ltd., Shanghai, China). The primers to amplify the target genes were designed using Primer Premier v.6.0 software (Premier Biosoft International, Palo Alto, CA, USA) based on information pulled from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The sequences of the primers are listed in Table S2. The qRT-PCR analysis was conducted with the aid of a 2× Hieff PCR Master Mix (10102ES03, YEASEN) and a real-time PCR thermocycler (ABI 7300, Thermo Fisher Scientific).

2.6. Quantification of cell-associated CeO_2 NPs contents

The 100 mL algae-CeO_2 NPs suspension was concentrated via centrifugation (3,500*g* for 15 min) for the Ce quantification experiments using a density gradient centrifugation. According to the specific method of density gradient centrifugation used [36,37], the suspended CeO_2 NPs were removed, and algae-CeO_2 NPs aggregates were kept, as shown in Fig. S5. The solid samples were then washed with 0.1 M phosphate-buffered saline (PBS), vacuum-dried, and digested using nitric acid in a microwave digestion instrument (Mars 6, CEM Co. Ltd., USA). The concentration of total Ce that included both the NPs attached to the algae and the ones internalized within the algal cells was determined via inductively coupled plasma optical emission spectrometry (ICP-OES; 5110, Agilent Technologies Co. Ltd., USA). The Ce content was calculated according to the biomass of the algal cells.

2.7. Determination of homo- and hetero-aggregation

To compare the differences between single and repeated exposures on the aggregation status of CeO_2 NPs, the hydrodynamic diameters of the CeO_2 NPs in the algal media under different concentrations (10, 10/2, 10/3, and 10/6 mg/L, corresponding to the amount added each time in the single, 2×, 3×, and 6× exposure treatments) were determined using dynamic light scattering (DLS, NanoBrook Omni, Brookhaven Instruments Co., USA) at 0 and 30 min.

Visual image analysis was used to measure the size distribution of algae-CeO_2 NPs' aggregates and the percentage of hetero-aggregation. Briefly, microscopic images of the algal populations were obtained and analyzed using automatic cell counting and analysis instrument (Countstar, ALIT Life Science Co. Ltd., China) along with its accompanying software (Countstar Algae version 1.1, ALIT Life Science Co. Ltd., Shanghai, China). For each treatment, >10,000 cells were collected, and any aggregates composed of >3 cells were counted. The percentage of aggregation (PA) was measured five times for each sample and calculated according to a previously described method, with minor modifications [38,39]. The PA values were calculated using the following equation:

$$\text{PA} = \frac{\text{Aggregates number} \times \text{Average cell number in aggregates}}{\text{Total algal cell number}} \times 100\% \quad (3)$$

2.8. Poly(acrylic acid) stabilized CeO_2 NPs preparation and its effects on the algae

An aqueous dispersion of poly(acrylic acid) stabilized CeO_2 NPs (CeO_2 NPs-PAA) was prepared in a precipitation-redispersion process, according to a previous study [40]. Briefly, both pristine CeO_2 NPs and PAA solutions were prepared at the same concentration (1 wt.%) with a pH of 1.4. The two initial solutions were mixed at the ratio of $V_{CeO_2}/V_{PAA} = 2$, where V_{CeO_2} and V_{PAA} represent the volumes of the CeO_2 NPs and PAA solutions,

respectively. Subsequently, the pH was gradually increased by the addition of ammonium hydroxide (NH_4OH) with continuous stirring. Once the pH reached 9.5, the suspension was neutralized to pH 7.0 with HCl. The effects of CeO_2 NPs-PAA on algal growth and photosynthesis were determined as compared to pristine CeO_2 NPs with the same exposure mode. The hydrodynamic diameters of pristine CeO_2 NPs and CeO_2 NPs-PAA in water with a concentration of 10 mg/L were determined using time-resolved DLS from 1 to 1,800 s.

2.9. Statistical analysis

All treatments were done in at least three independent replicates ($n \geq 3$). The experimental data are presented as the mean value and standard deviation (mean ± SD). One-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) multiple comparison was performed to analyze the significant differences for each physiological index across the different treatments, with the aid of SPSS version 22.0 (IBM Corp., Armonk, NY, USA). A p-value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Characterization of CeO_2 NPs

As shown in Fig. 1A–D, most of the CeO_2 NPs were spherical, with diameters of ~10 to 20 nm and had lattice fringe spacings of ~0.312 nm, which was attributed to (111) reflection. XPS results showed that the percentage of Ce^{3+} content on their surface was ~15.06%. CeO_2 NPs entered the algal medium and interacted with the algae to form algae-CeO_2 NPs hetero-aggregates (Figs. 1 and S5). After being treated with ethylenediaminetetraacetic acid (EDTA), the surface-bound CeO_2 NPs could not be removed from the algal cell surface (Fig. S5), suggesting the presence of an interaction between CeO_2 NPs and the algal cells. The presence of the CeO_2 NPs structurally disrupted the algal cells, as was revealed through SEM (Fig. 1). It was thus hypothesized that the varying exposure times of CeO_2 NPs to algae led to different responses induced by varying levels of surface-bound CeO_2 NPs on the algal cells.

3.2. Repeated exposure to CeO_2 NPs reduced algal biomass

After both single and repeated exposures to CeO_2 NPs for 72 h, no significant effects on the number of algal cells were observed in the 1-CeO_2 NPs and 2-CeO_2 NPs groups (Fig. 2A). The 3-CeO_2 NPs group showed growth inhibition of 10.97% but the 6-CeO_2 NPs group had an increased growth of 110.3% (Fig. S6). This agreed with the results of previous studies, which also found that low concentrations of ENMS stimulated algal growth [41,42]. This is attributable to the hormesis response of algae to ENMs, regardless of the exposure pattern [43]. Notably, repeated exposure to CeO_2 NPs in six doses induced higher algal cell numbers but also resulted in a significant reduction to 76.12% of the total biomass when compared to the single exposure group (Fig. 2A and B). This inconsistency was mainly attributed to differences in cell sizes (Fig. S7), which likely resulted from inhibited photosynthesis. Notably, the algal biomass in the 6-CeO_2 NPs group was even lower than that of the group exposed to one addition of 100 mg/L CeO_2 NPs. A similar trend was also found during photosynthesis was assessed, as discussed in the next section. These results indicated that: 1) the negative effects of repeated exposure to CeO_2 NPs cannot be overlooked and may trigger severe cellular toxicity compared to single-exposure treatments, even when the latter are at high concentrations, such as 100 mg/L; 2) the evaluation of algal growth inhibition under ENM-induced stress should focus on both cell number and biomass; and 3) repeated exposures may cause significant differences in intercellular photosynthetic efficiency in algae, compared to single ones.

3.3. Repeated exposure disturbed the photosynthetic performance of algae

Photosynthesis, an essential energy-producing physiological process, is a sensitive biomarker that adjusts to environmental stresses [44]. In addition to photosynthesis, pigment content, changes in chlorophyll fluorescence measured using a pulse amplitude-modulated fluorometer can also reflect photosynthetic performance [45]. As demonstrated by the RLC results, the relative electron transport rate (rETR) first increased with the elevation of PAR, which then decreased in all treatment groups (Fig. 3A–E). The β values for RLC were higher in

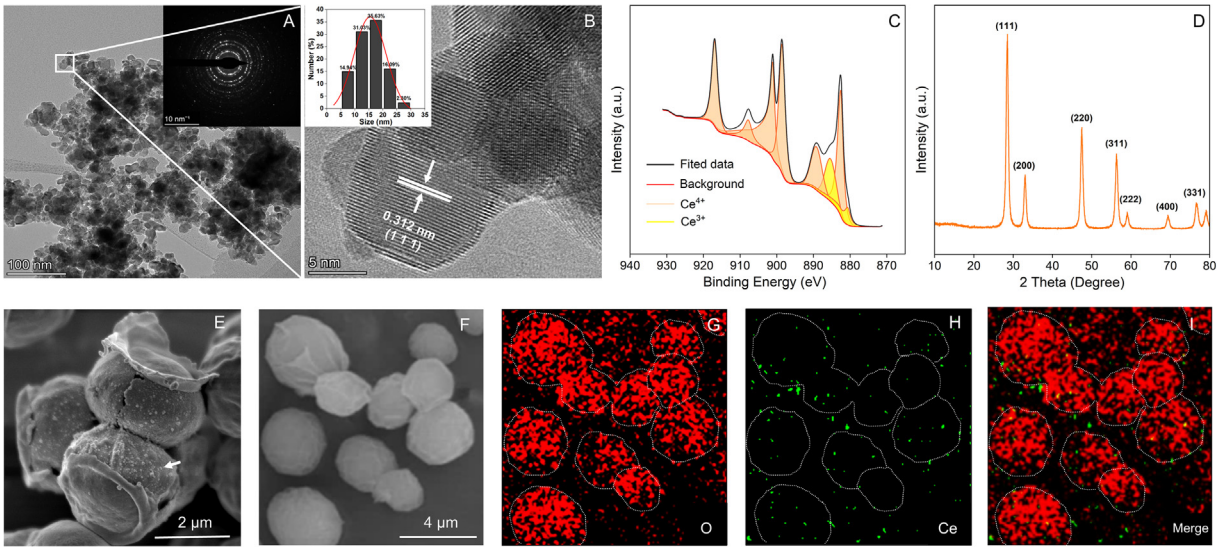

Fig. 1. Characterization of CeO_2 NPs and algae-CeO_2 NPs hetero-aggregates. (A) Representative TEM image of CeO_2 NPs and (B) their size distribution by image. (C) XPS analysis of CeO_2 NPs. (D) XDS analysis of CeO_2 NPs. (E) Structural damage induced by CeO_2 NPs (arrow). (F) Representative SEM image of algae-CeO_2 NPs hetero-aggregates and elemental disturbance of O (G) and Ce (H) mapping by EDS in the 1-CeO_2 NPs group (10 mg/L). The boundaries of the algal cells are represented by dots. (I) Merged image of G and H. CeO_2 NPs, cerium oxide nanoparticles; TEM, transmission electron microscopy; XPS, X-ray photoelectron spectroscopy; SEM, scanning electron microscopy; EDS, energy dispersive X-ray spectroscopy.

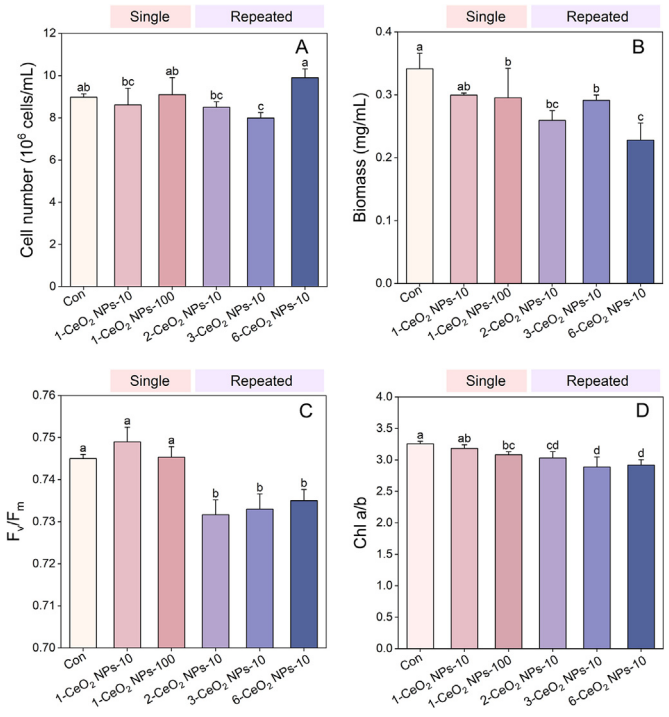

Fig. 2. Effects of CeO_2 NPs on (A) cell number, (B) biomass, (C) F_v/F_m, and (D) Chl a/b. The 1-CeO_2 NPs-10 and 1-CeO_2 NPs-100 labels denote the groups exposed to one addition of 10 mg/L and 100 mg/L CeO_2 NPs, respectively. The 2-CeO_2 NPs-10, 3-CeO_2 NPs-10, and 6-CeO_2 NPs-10 ones denote exposures to two, three, and six additions of 10 mg/L, respectively.

the 2-CeO_2 NPs and 3-CeO_2 NPs groups compared to the control and 1-CeO_2 NPs groups, suggesting that repeated exposures could induce a higher consumption efficiency of light energy than single exposure (Fig. 3F). In addition, both single and repeated exposures were able to elevate NPQ values relative to the control group. In particular, the 3-CeO_2 NPs and 6-CeO_2 NPs treatments induced higher NPQ but lower qL and $\Phi_{PS\ II}$ than the 1-CeO_2 NPs and 2-CeO_2 NPs ones (Fig. 3G–I). The $\Phi_{PS\ II}$ value represents the global photochemical effective quantum yield [46], and the lower $\Phi_{PS\ II}$ values observed following repeated exposures suggest that overall photosynthesis was inhibited by CeO_2 NPs. Compared to the control and 1-CeO_2 NPs groups, increased NPQ and decreased qL values were observed following the 2-CeO_2 NPs, 3-CeO_2 NPs, and 6-CeO_2 NPs treatments. This indicated that repeated exposure to CeO_2 NPs elevated algal heat dissipation and inhibited photosynthesis [47,48].

OJIP transient diagrams were constructed to reflect the performance of the photosynthetic system (Figs. 2C, 3J, and S9). As shown in Fig. 2C, a significant reduction in the maximum photochemical quantum yield of photosystem II (F_v/F_m) was observed in all repeated exposure treatments (in contrast to any single exposure treatment), regardless of the exposure concentration. Specifically, the F_v/F_m value was significantly reduced from 0.745 in the control group to 0.732 in the 2-CeO_2 NPs one, 0.733 in the 3-CeO_2 NPs one, and 0.739 in the 6-CeO_2 NPs one (Fig. 2C), suggesting that the mode of repeated exposure weakened the photosynthetic activity [29]. The increases observed in minimal fluorescence (F_0) and maximal fluorescence (F_m) and the decreased F_m/F_0 and F_v/F_0 ratios in the 2-CeO_2 NPs, 3-CeO_2 NPs, and 6-CeO_2 NPs groups indicated that repeated exposure treatments could trigger irreversible damage to photosystem II (Fig. 3J). In addition, the fluorescence relative variable values in the J (V_j) and I phases (V_i) were also obtained from the OJIP transient value, representing the electron flow resistance from Q_A to Q_B in photosystem II and from plastoquinone (PQ) to photosystem I, respectively [49,50]. As shown in Fig. 3J, both single and repeated exposure treatments decreased V_j and elevated V_i, indicating that the electron flow resistance from Q_A to Q_B was mitigated, but it was increased from PQ to photosystem I. Compared to single exposure, repeated exposures to CeO_2 NPs were able to increase the resistance of electron flow. It is reasonable to believe that the blocked electron flow from PQ to photosystem I during photosynthesis may lead to electron accumulation and ROS generation [51].

In terms of photosynthetic pigments, CeO_2 NPs exposure lowered the Chl a/b ratio mainly through an increased chlorophyll b (Chl b) content (Figs. 2D and S8). Under unfavorable conditions, Chl b can be converted into chlorophyll a (Chl a) as a self-defensive mechanism [52]. The elevated Chl b content in the CeO_2 NPs-treated groups indicated that the photosynthetic system of the algae was perturbed, and its defense mechanism was activated. In line with these results, previous studies have also reported the inhibition of photosynthetic pigment synthesis by CuO NPs and nanodiamonds, likely owing to the overgeneration of ROS in chloroplasts [25,53].

3.4. Repeated exposure triggered oxidative stress

Indeed, repeated exposure treatments stimulated higher levels of ROS than single exposure, with the increases ranging from 154.95% to 166.29% (Fig. 4A). The overgenerated ROS indicated that CeO_2 NPs at a concentration of 10 mg/L could trigger oxidative stress in algal cells, which might result from electron transfer in the bio-nano interface [54]. SOD and CAT, as two important antioxidative enzymes in response to oxidative stress, varied significantly with the change in exposure times. SOD can scavenge intracellular superoxide anion ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2), and CAT is mainly responsible for the elimination of excessive H_2O_2 to water and O_2 [55]. Single-exposure treatment of 10 mg/L CeO_2 NPs induced higher SOD activity than any repeated exposure treatment or control treatment ($p < 0.05$, Fig. 4B). The elevated SOD activity in CeO_2-exposed treatments played a role in the balance of oxidative stress levels to protect algae from extrinsic pollutants, which was inconsistent with previous work [56]. Similarly, the repeated exposure treatments also induced lower CAT activities than the single exposure treatment. However, compared to the control, the activity of CAT was lowered after exposure to CeO_2 NPs, both in single and repeated treatments (Fig. 4C), which is in line with the change under 10 mg/L biochar nanoparticle exposure [57]. This is probably due to the low levels of Ce^{3+}/Ce^{4+} redox-state ratios on the surface of CeO_2 NPs, as shown in the results of XPS (Fig. 1C). A previous study found that the relatively low and high Ce^{3+}/Ce^{4+} redox-state ratios could exhibit CAT and SOD mimetic activity, respectively [58,59]. This explains why the lower CAT activities were determined in CeO_2 NP-exposure treatments than the control, attributing to the involvement of CeO_2 NPs themselves for H_2O_2 scavenging. Therefore, it is reasonable to believe that the CeO_2 NPs used in our study may act as an H_2O_2 but not an $O_2^{\bullet -}$ scavenger (Fig. 4D). Repeated exposures may not exceed the antioxidant defense capacity of algae; however, according to the decreased biomass, this may still be at the expense of the overall algal energy budget. In addition, both membrane lipid peroxidation induced by excessively accumulated ROS and the physical characteristics of CeO_2 NPs could damage the algal cell membrane [60], which can be revealed by the activity of extracellular LDH. In our work, no significant change was observed in repeated treatments, but an elevation to 116.89% was observed in a single treatment (Fig. S10A). Consistent with our study, Cao et al. [19] also determined more injured membrane cells in a single exposure to AgNPs than in repeated exposures. In general, ENMs could damage cell membranes via physical contact and/or chemical oxidation [35]. Indeed, considering the correlation between intracellular ROS levels and extracellular LDH activity was not significant (Fig. S10B), the membrane damage was triggered by both physical destruction and oxidative stress.

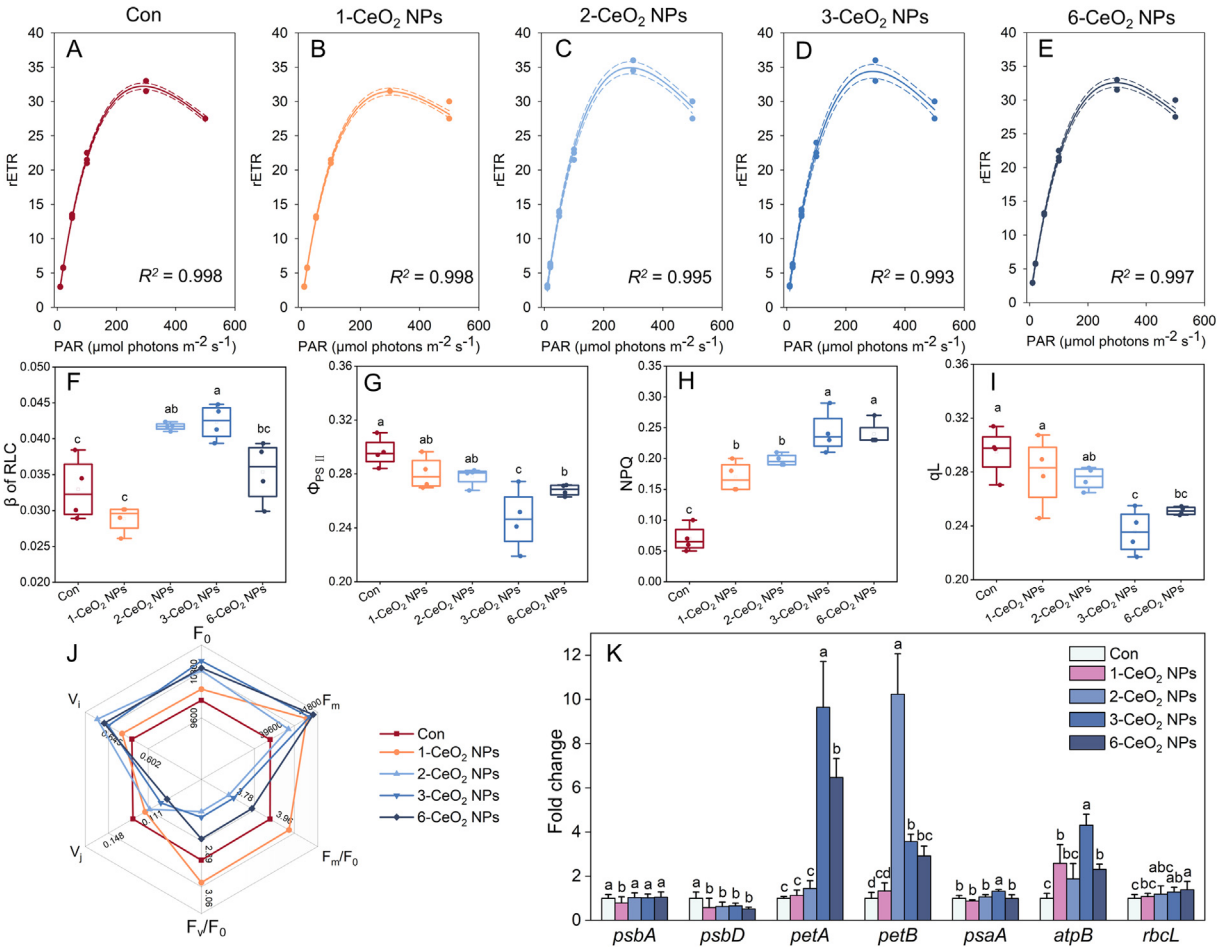

Fig. 3. Effects of CeO_2 NPs on photosynthetic performance and gene expression levels of photosynthesis-related genes in *C. vulgaris* after exposure to CeO_2 NPs in single and repeated doses. (A–E) RLC. (F) β of RLC. (G) $\Phi_{PS\ II}$. (H) NPQ. (I) qL. (J) Critical OJIP parameters. (K) The expression levels of seven genes related to photosynthesis in *C. vulgaris*. RLC, rapid light curves; NPQ, nonphotochemical quenching.

3.5. Repeated exposure regulated the expression of photosynthesis-related genes

The expression levels of seven photosynthesis-related genes were further determined to reveal the different photosynthetic responses of algae to single and repeated CeO_2 NPs exposures at the transcriptional level. Single and repeated exposures induced the same variation trends in the gene expression levels of algal cells, with five of seven analyzed genes being upregulated following exposure to CeO_2 NPs compared to the control treatment. However, significant differences in these gene expression levels were also determined between single and repeated exposures. For example, the relative expression levels of the *petA*, *petB*, *psaA*, and *atpB* genes were upregulated to 9.64, 3.57, 1.32, and 4.31-fold higher levels, respectively, in the 3-CeO_2 NPs group. The upregulation of photosynthesis-related genes indicated that *C. vulgaris* took compensatory measures to maintain essential activities in response to CeO_2 NPs-induced stress at the experimental concentration [61]. The Cyt b6/f-complex was responsible for the rate-limiting step of the photosynthetic electron transport chain [62,63]. The relative expression levels of the *petA* gene were upregulated to 1.44, 9.64, and 6.47-fold increases in the 2-CeO_2, 3-CeO_2, and 6-CeO_2 NPs groups, respectively. Similarly, the relative expression levels of the *petB* gene were upregulated to 10.24, 3.57, and 2.92-fold higher levels in the 2-CeO_2, 3-CeO_2, and 6-CeO_2 NPs groups, respectively. The repeated exposure to CeO_2 NPs may have significantly upregulated the expression levels of *petA* and *petB*, suggesting that the thylakoid Cyt b6/f-complex suffered more severe effects from multiple exposures compared to a single one [29]. This is consistent with the result of an elevated electron flow resistance from PQ to photosystem I that was observed in the repeated treatment groups, as indicated by higher V_i values (Fig. 3J). Additionally, the *atpB* gene, involved in producing adenosine triphosphate synthetase (ATPase) and thus determining adenosine triphosphate (ATP) synthesis levels, was upregulated in both the single and repeated exposure groups. Damaging the light reaction may lower the levels of ATP and nicotinamide adenine dinucleotide phosphate (NADPH) are subsequently provided to the Calvin cycle and hinder the efficiency of carbon fixation [64]. This may explain the observed upregulation of the *rbcL* gene, which encodes the large subunit of Rubisco and catalyzes carbon fixation in the 3-CeO_2 and 6-CeO_2 NP groups. However, compared to the control, the mRNA level of *psbD* in all of the CeO_2 NP-exposed treatment groups was significantly downregulated, regardless of the exposure mode. The reduced expression of *psbD* may be related to weakened electron resistance from Q_A to Q_B in photosystem II, as indicated by a lower V_j [65] (Fig. 3J).

All of the examined *C. vulgaris* genes were altered, indicating that the whole electron transport chain was damaged after being exposed to CeO_2 NPs, as illustrated in Fig. 5. The upregulation of photosynthesis-related genes was generally explained as resulting from stimulation by low doses of the ENMs [29,66]. However, the decreased photosynthetic efficiency was observed concurrently with the upregulation of photosynthesis-related genes. Therefore, it was hypothesized that the upregulation of photosynthesis-related genes was a compensatory measure to maintain the essential physiological processes in response to the stresses induced by the ENMs.

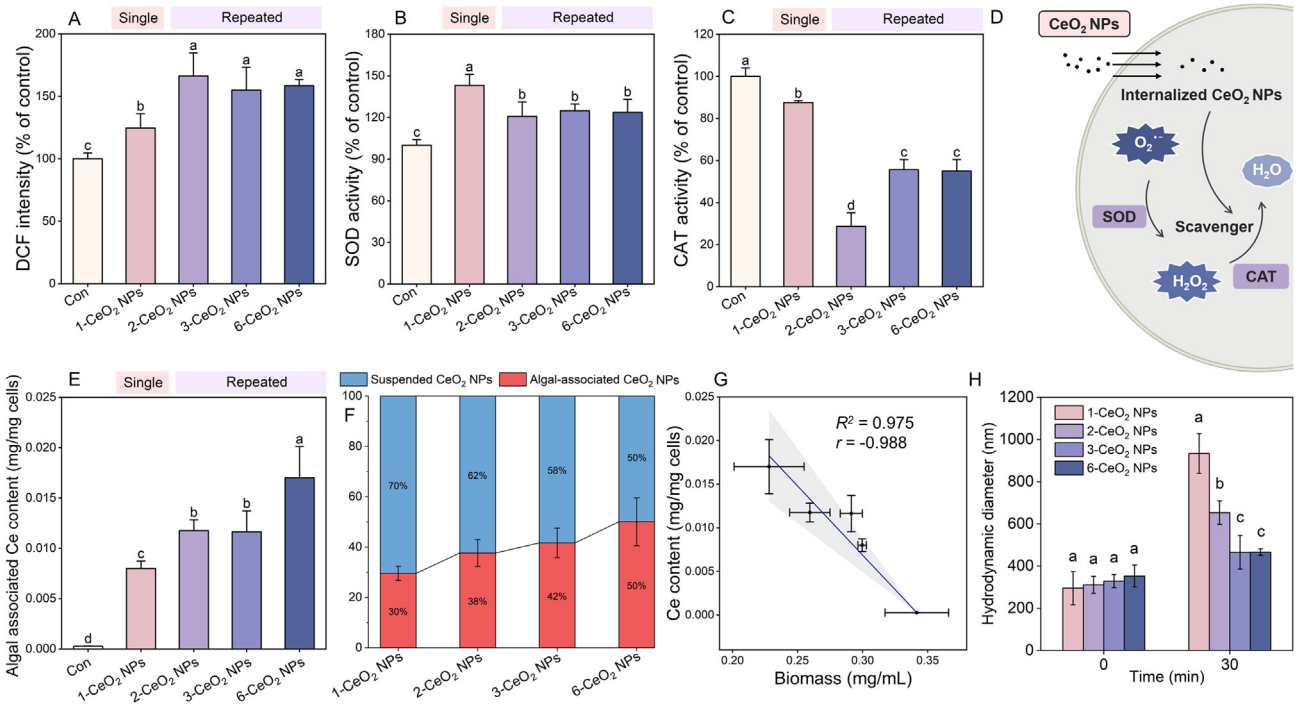

Fig. 4. (A) The DCF intensity, (B) SOD activity, and (C) CAT activity of *C. vulgaris* exposed to CeO_2 NPs in a single and repeated pattern. (D) Schematic diagram illustrating the role of CeO_2 NPs in reducing peroxide. (E) The content of algal-associated Ce. (F) The ratio of algal-associated CeO_2 NPs and suspended CeO_2 NPs. (G) Correlation analysis between Ce content and algal biomass. (H) Hydrodynamic diameters of CeO_2 NPs with 10 mg/L, 10/2 mg/L, 10/3 mg/L, and 10/6 mg/L additions at 0 and 30 min, respectively. SOD, superoxide dismutase; CAT, catalase.

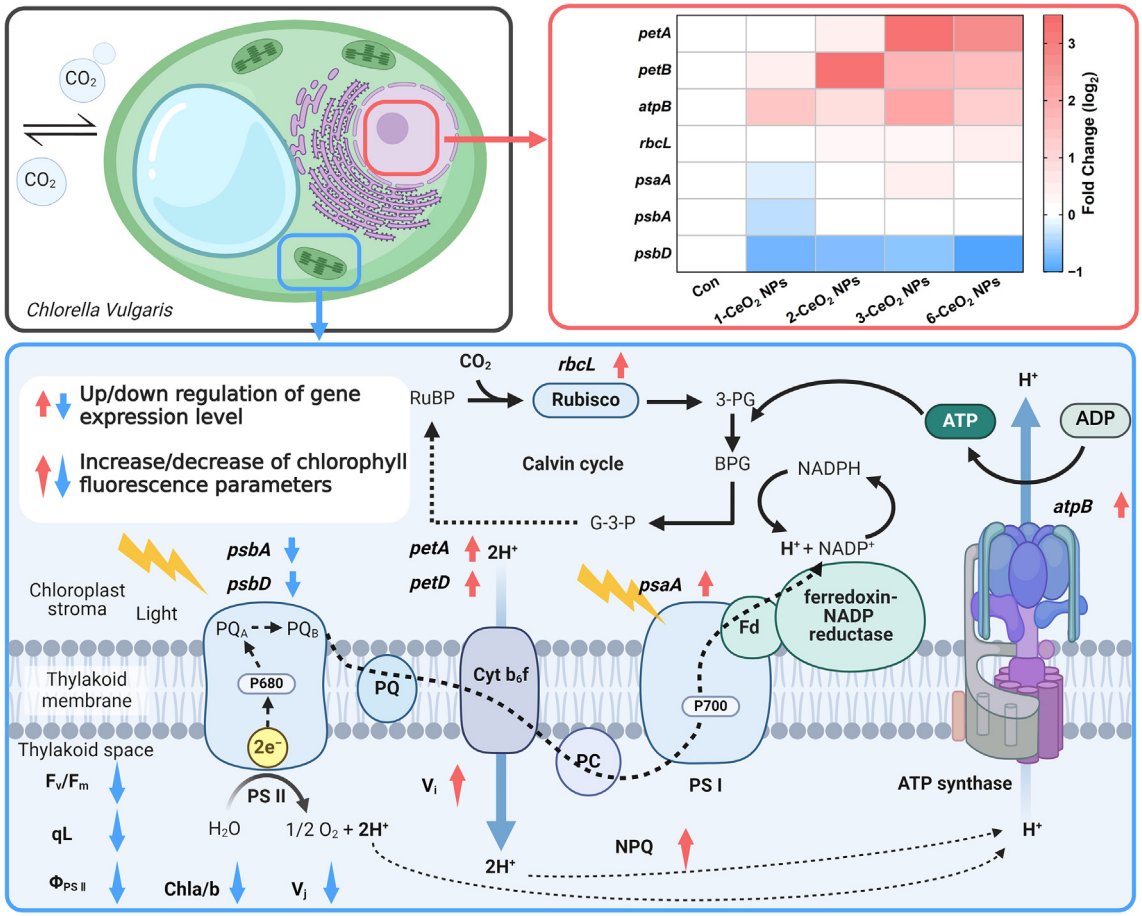

Fig. 5. Schematic diagram of the effect of CeO_2 NPs on the photosynthetic electron transport chain, ATP synthesis, and subsequent carbon assimilation in *C. vulgaris*.

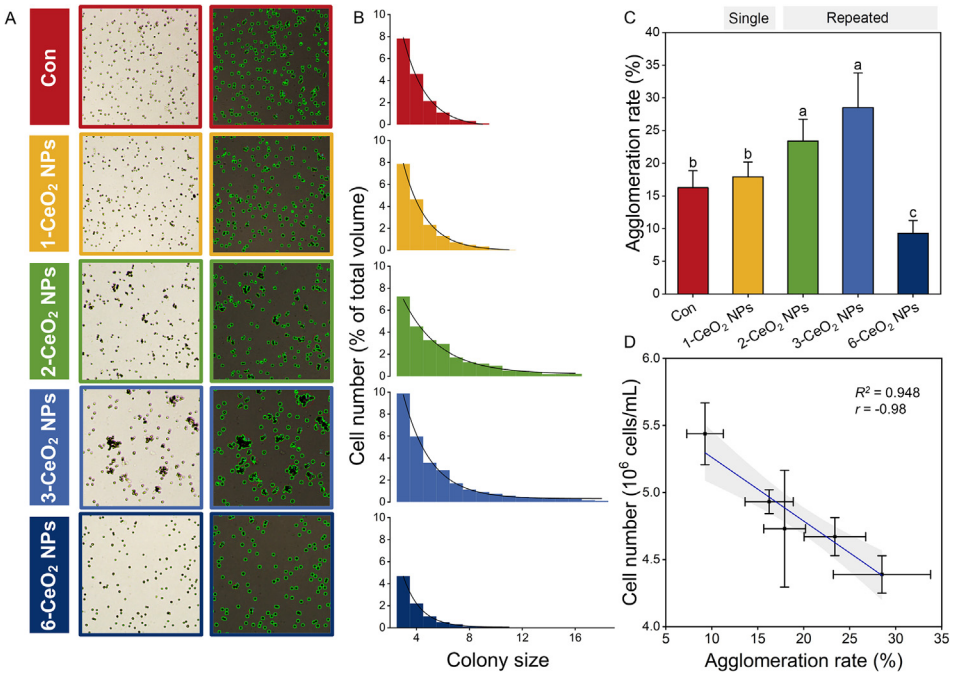

Fig. 6. (A) Representative image of algae-CeO_2 NPs aggregates in each group. The boundaries of the aggregates are represented by green lines. (B) The distribution of colony size. (C) The agglomeration rate in each group. (D) Correlation analysis between cell number and the algal agglomeration.

3.6. *Different mechanisms of single and repeated exposures to CeO_2 NPs*

The Ce contents in both the algae-associated and suspended CeO_2 NPs were determined. Increases in the repeated exposure times of CeO_2 NPs elevated the algae-associated Ce contents from 0.008 to 0.017 mg/mg cells and ratios from 29.55% to 50.06% compared to suspended Ce ions (Fig. 4E and F). It was, therefore, thought that repeated low-dose exposures could provide more opportunities for CeO_2 NPs to interact with the algae, ultimately attaching to the cellular surfaces or entering inside. In addition, the algae-associated CeO_2 NP content correlated negatively with the algal biomass ($r = -0.988$; Fig. 4G), indicating that the algal energy utilization was mainly inhibited by the surface-attached or internalized CeO_2 NPs.

Various exposure modes at each time interval provided different concentrations of CeO_2 NPs in the culture medium. This induced differences in homo-aggregation, further influencing physical contact with the algae. The time-resolved DLS results showed that 10 mg/L of CeO_2 NPs could rapidly homo-aggregate, while 5 mg/L, 3.33 mg/L, and 1.67 mg/L of CeO_2 NPs were relatively stable (Fig. 4H). This indicated that repeated exposures could elevate the interaction between CeO_2 NPs and algae. In addition, an aqueous dispersion of CeO_2 NPs-PAA was prepared to verify the role that aggregation played in the differences in toxicity observed among the various exposure groups. The DLS results suggested that aggregation was not observed in the CeO_2 NPs-PAA group (Fig. S11). The 72-h algal growth (i.e., cell number, Chl a, and biomass) and photosynthesis-related indexes (i.e., OJIP, NPQ, qL, and $\Phi_{PS\ II}$) of *C. vulgaris* were also investigated under exposure to CeO_2 NPs-PAA. As shown in Figs. S13 and S14, no significant differences in algal biomass and photosynthetic physiological indexes were observed among the different treatments. The Chl a content decreased to 1.92, 2.09, 2.06, and 2.21 mg/L in the 1-CeO_2 NPs-PAA, 2-CeO_2 NPs-PAA, 3-CeO_2 NPs-PAA, and 6-CeO_2 NPs-PAA, respectively (Fig. S13). In addition, the value of F_v/F_m was decreased to 0.71 in all of the treatment groups (Fig. S14). These results suggested that increased repeated exposure times of CeO_2 NPs in the algal medium could enhance their direct contact with algae via mitigating homo-aggregation, which was responsible for the significant differences observed in growth and physiological indexes when compared to a single exposure.

Finally, the effects of CeO_2 NP exposure times on hetero-aggregation were investigated, finding that 2-CeO_2 NPs and 3-CeO_2 NP treatments were highly prone to forming larger aggregates, as shown in Fig. 6A–C. The percentage of aggregated algal cell numbers rose to 23.39% and 28.51% in the 2-CeO_2 NPs and 3-CeO_2 NPs treatment groups, respectively, compared to the 16.24% and 17.92% levels observed in the control and 1-CeO_2 NPs groups. However, given the lower aggregated cell number percentage in the 6-CeO_2 NPs group compared to all others, it was thought that repeated exposures of >3 times would not be beneficial for the formation of algae-CeO_2 NPs aggregates (Fig. 6A). The algae-CeO_2 NPs hetero-aggregates traveled vertically and settled at the bottom of the medium [67]. Furthermore, these formed hetero-aggregates could reduce the light available to the algae, eventually altering their growth conditions [9,39]. Analogously, algae balance light harvesting with available light energy for cellular growth when their living space is limited [68]. Similar to the results of a previous experiment [39], a strong negative correlation between the percentage of aggregates and cell number was also observed in this study ($r = -0.98$, $p < 0.05$; Fig. 6D), but not with other biomarkers (Fig. S15), suggesting a potential relationship between aggregation and cell proliferation in the presence of CeO_2 NPs.

4. Conclusion

The repeated discharge of CeO_2 NPs into aquatic environments is widespread, and their effects on freshwater algae can no longer be overlooked. This study found that while single exposures to CeO_2 NPs at experimental concentrations (10 mg/L) did not significantly affect algal growth, repeated exposures could either inhibit or stimulate the proliferation of *C. vulgaris*, depending on the number of repeated exposures. The differences in oxidative stress suggested that repeated exposures could increase the contributions of CeO_2 NPs acting as CAT scavengers but not SOD ones. In addition to photosynthetic performance, the upregulation of photosynthesis-related genes (i.e., *petA*, *petB*, *psaA*, *atpB*, and *rbcL*) played a compensatory rather than a positive stimulatory role in algal responses to repeated CeO_2 NPs exposure. Compared to a single exposure, repeated exposure can exhibit different regulatory effects on algae, as revealed by mitigating homo-aggregation. This study

emphasized the importance of taking exposure time into account when assessing the nanotoxicity of ENMs in real-world environments. Notably, photosynthesis-related indexes, particularly at the transcriptional level, can be used to evaluate algal responses to ENMs, providing novel information to understand the safety of ENMs in aquatic ecosystems.

CRediT authorship contribution statement

S.B.L.: conceptualization, investigation, experiment, data curation, software, writing–original draft. J.H.H.: software, data curation. X.W.M.: experiment. X.S.Z. and H.Q.: writing–review & editing. G.R.X.: supervision. X.C.H.: conceptualization, supervision, funding acquisition, writing–original draft, writing–review & editing.

Declaration of competing interests

The authors have declared no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eehl.2024.04.002.

References

[1] StatNano, Nanotechnology products database. https://product.statnano.com/, 2023.
[2] A.T. Hoang, Combustion behavior, performance and emission characteristics of diesel engine fuelled with biodiesel containing cerium oxide nanoparticles: a review, Fuel Process. Technol. 218 (2021) 106840, https://doi.org/10.1016/j.fuproc.2021.106840.
[3] X. Bi, C. Zeng, P. Westerhoff, Adsorption of arsenic ions transforms surface reactivity of engineered cerium oxide nanoparticles, Environ. Sci. Technol. 54 (2020) 9437–9444, https://doi.org/10.1021/acs.est.0c02781.
[4] L. Zeng, H. Cheng, Y. Dai, Z. Su, C. Wang, L. Lei, et al., In vivo regenerable cerium oxide nanozyme-loaded pH/H_2O_2-responsive nanovesicle for tumor-targeted photothermal and photodynamic therapies, ACS Appl. Mater. Inter. 13 (2020) 233–244, https://doi.org/10.1021/acsami.0c19074.
[5] A. Beck, D. Kazazis, Y. Ekinci, X. Li, M.E.A. Gubler, A. Kleibert, et al., The extent of platinum-induced hydrogen spillover on cerium dioxide, ACS Nano 17 (2022) 1091–1099, https://doi.org/10.1021/acsnano.2c08152.
[6] N. Joudeh, D. Linke, Nanoparticle classification, physicochemical properties, characterization, and applications: a comprehensive review for biologists, J. Nanobiotechnol. 20 (2022) 262, https://doi.org/10.1186/s12951-022-01477-8.
[7] S. Majumdar, J. Trujillo-Reyes, J.A. Hernandez-Viezcas, J.C. White, J.R. Peralta-Videa, J.L. Gardea-Torresdey, Cerium biomagnification in a terrestrial food chain: influence of particle size and growth stage, Environ. Sci. Technol. 50 (2016) 6782–6792, https://doi.org/10.1021/acs.est.5b04784.
[8] H. Zhang, X. He, Z. Zhang, P. Zhang, Y. Li, Y. Ma, et al., Nano-CeO_2 exhibits adverse effects at environmental relevant concentrations, Environ. Sci. Technol. 45 (2011) 3725–3730, https://doi.org/10.1021/es103309n.
[9] K. Van Hoecke, J.T.K. Quik, J. Mankiewicz-Boczek, K.A.C. De Schamphelaere, A. Elsaesser, P. Van De Meeren, et al., Fate and effects of CeO_2 nanoparticles in aquatic ecotoxicity tests, Environ. Sci. Technol. 43 (2009) 4537–4546, https://doi.org/10.1021/es9002444.
[10] W. Zou, Q. Zhou, X. Zhang, X. Hu, Environmental transformations and algal toxicity of single-layer molybdenum disulfide regulated by humic acid, Environ. Sci. Technol. 52 (2018) 2638–2648, https://doi.org/10.1021/acs.est.7b04397.
[11] Y. Dai, C. Sun, R. Hou, R. Lan, W. Su, J. Zhao, et al., Transfer of CeO_2 nanoparticles between freshwater omnivorous organisms: effect of feces and necrophagy, J. Hazard. Mater. 451 (2023) 131137, https://doi.org/10.1016/j.jhazmat.2023.131137.
[12] A. Panacek, L. Kvitek, M. Smekalova, R. Vecerova, M. Kolar, M. Roderova, et al., Bacterial resistance to silver nanoparticles and how to overcome it, Nat. Nanotechnol. 13 (2018) 65–71, https://doi.org/10.1038/s41565-017-0013-y.
[13] Y. Zhai, G. Liu, T. Bosker, E. Baas, W.J.G.M. Peijnenburg, M.G. Vijver, Compositional and predicted functional dynamics of soil bacterial community in response to single pulse and repeated dosing of titanium dioxide nanoparticles, NanoImpact 16 (2019) 100187, https://doi.org/10.1016/j.impact.2019.100187.
[14] M. Simonin, J.M. Martins, G. Uzu, E. Vince, A. Richaume, Combined study of titanium dioxide nanoparticle transport and toxicity on microbial nitrifying communities under single and repeated exposures in soil columns, Environ. Sci. Technol. 50 (2016) 10693–10699, https://doi.org/10.1021/acs.est.6b02415.
[15] D. Wu, J. Zhang, W. Du, Y. Yin, H. Guo, Toxicity mechanism of cerium oxide nanoparticles on cyanobacteria *Microcystis aeruginosa and* their ecological risks, Environ. Sci. Pollut. Red. 29 (2022) 34010–34018, https://doi.org/10.1007/s11356-021-18090-1.
[16] C. Xie, Y. Ma, P. Zhang, J. Zhang, X. Li, K. Zheng, et al., Elucidating the origin of the toxicity of nano-CeO_2 to *Chlorella pyrenoidosa*: the role of specific surface area and chemical composition, Environ. Sci. Nano 8 (2021) 1701–1712, https://doi.org/10.1039/d0en01177k.
[17] J.Q. Xiong, S. Ru, Q. Zhang, M. Jang, M.B. Kurade, S.H. Kim, et al., Insights into the effect of cerium oxide nanoparticle on microalgal degradation of sulfonamides, Bioresour. Technol. 309 (2020) 123452, https://doi.org/10.1016/j.biortech.2020.123452.
[18] L. Xiao, P. Antczak, J. Ruegg, L. Behrendt, Zeitotox: toxicology and the rhythms of life, Environ. Sci. Technol. 56 (2022) 11100–11102, https://doi.org/10.1021/acs.est.2c02961.
[19] M. Cao, X. Huang, F. Wang, Y. Zhang, B. Zhou, H. Chen, et al., Transcriptomics and metabolomics revealed the biological response of *Chlorella pyrenoidesa* to single and repeated exposures of AgNPs at different concentrations, Environ. Sci. Technol. 55 (2021) 15776–15787, https://doi.org/10.1021/acs.est.1c04059.
[20] S. Mariano, E. Panzarini, M.D. Inverno, N. Voulvoulis, L. Dini, Toxicity, bioaccumulation and biotransformation of glucose-capped silver nanoparticles in green microalgae *Chlorella vulgaris*, Nanomaterials 10 (2020) 1377, https://doi.org/10.3390/nano10071377.
[21] Y. Wu, P. Guo, X. Zhang, Y. Zhang, S. Xie, J. Deng, Effect of microplastics exposure on the photosynthesis system of freshwater algae, J. Hazard. Mater. 374 (2019) 219–227, https://doi.org/10.1016/j.jhazmat.2019.04.039.
[22] K. Kumar, C.N. Dasgupta, B. Nayak, P. Lindblad, D. Das, Development of suitable photobioreactors for CO_2 sequestration addressing global warming using green algae and cyanobacteria, Bioresour. Technol. 102 (2011) 4945–4953, https://doi.org/10.1016/j.biortech.2011.01.054.
[23] J. Zeng, Z. Wang, G. Chen, Biological characteristics of energy conversion in carbon fixation by microalgae, Renew. Sustain. Energy Rev. 152 (2021) 111661, https://doi.org/10.1016/j.rser.2021.111661.
[24] D. Krause-Jensen, C.M. Duarte, Substantial role of macroalgae in marine carbon sequestration, Nat. Geosci. 9 (2016) 737–742, https://doi.org/10.1038/NGEO2790.
[25] C. Zhang, X. Chen, W. Chou, S. Ho, Phytotoxic effect and molecular mechanism induced by nanodiamonds towards aquatic *Chlorella pyrenoidosa* by integrating regular and transcriptomic analyses, Chemosphere 270 (2021) 129473, https://doi.org/10.1016/j.chemosphere.2020.129473.
[26] F. Li, Z. Liang, X. Zheng, W. Zhao, M. Wu, Z. Wang, Toxicity of nano-TiO_2 on algae and the site of reactive oxygen species production, Aquat. Toxicol. 158 (2015) 1–13, https://doi.org/10.1016/j.aquatox.2014.10.014.
[27] J. Chen, S. Chen, N. He, Q. Wang, Y. Zhao, W. Gao, et al., Nuclear-encoded synthesis of the D1 subunit of photosystem II increases photosynthetic efficiency and crop yield, Nat. Plants 6 (2020) 570–580, https://doi.org/10.1038/s41477-020-0629-z.
[28] G. Liu, R. Jiang, J. You, D.C.G. Muir, E.Y. Zeng, Microplastic impacts on microalgae growth: effects of size and humic acid, Environ. Sci. Technol. 54 (2019) 1782–1789, https://doi.org/10.1021/acs.est.9b06187.
[29] H. Zhang, Z. Chen, Q. Huang, Study of the toxicity of ZnO nanoparticles to *Chlorella sorokiniana* under the influence of phosphate: spectroscopic quantification, photosynthetic efficiency and gene expression analysis, Environ. Sci. Nano 7 (2020) 1431–1443, https://doi.org/10.1039/c9en01464k.
[30] A.A. Keller, H. Wang, D. Zhou, H.S. Lenihan, G. Cherr, B.J. Cardinale, et al., Stability and aggregation of metal oxide nanoparticles in natural aqueous matrices, Environ. Sci. Technol. 44 (2010) 1962–1967, https://doi.org/10.1021/es902987d.
[31] J. Zhao, Y. Dai, Z. Wang, W. Ren, Y. Wei, X. Cao, et al., Toxicity of GO to freshwater algae in the presence of Al_2O_3 particles with different morphologies: importance of heteroaggregation, Environ. Sci. Technol. 52 (2018) 13448–13456, https://doi.org/10.1021/acs.est.8b00815.
[32] S. Shen, K. Yang, D. Lin, Biomacromolecular and toxicity responses of bacteria upon the nano-bio interfacial interactions with $Ti_3C_2T_x$ nanosheets, Environ. Sci. Technol. 57 (2023) 12991–13003, https://doi.org/10.1021/acs.est.3c02397.
[33] G. You, J. Hou, Y. Xu, C. Wang, P. Wang, L. Miao, et al., Effects of CeO_2 nanoparticles on production and physicochemical characteristics of extracellular polymeric substances in biofilms in sequencing batch biofilm reactor, Bioresour. Technol. 194 (2015) 91–98, https://doi.org/10.1016/j.biortech.2015.07.006.
[34] H. Hillebrand, C.D. Dürselen, D. Kirschtel, U. Pollingher, T. Zohary, Biovolume calculation for pelagic and benthic microalgae, J. Phycol. 35 (1999) 403–424, https://doi.org/10.1046/j.1529-8817.1999.3520403.x.
[35] X. Huang, Y. Huang, D. Wang, M. Liu, J. Li, D. Chen, Cellular response of freshwater algae to halloysite nanotubes: alteration of oxidative stress and membrane function, Environ. Sci. Nano 8 (2021) 3262–3272, https://doi.org/10.1039/d1en00531f.
[36] X. Hou, X. Hu, Self-assembled nanoscale manganese oxides enhance carbon capture by diatoms, Environ. Sci. Technol. 56 (2022) 17215–17226, https://doi.org/10.1021/acs.est.2c04500.
[37] Y. Yang, J. Hou, P. Wang, C. Wang, X. Wang, G. You, Influence of extracellular polymeric substances on cell-NPs heteroaggregation process and toxicity of cerium

dioxide NPs to *Microcystis aeruginosa*, Environ. Pollut. 242 (2018) 1206–1216, https://doi.org/10.1016/j.envpol.2018.08.005.
[38] X. Luo, H. Zhang, Q. Li, J. Zhang, Effects of static magnetic field on *Chlorella vulgaris*: growth and extracellular polysaccharide (EPS) production, J. Appl. Phycol. 32 (2020) 2819–2828, https://doi.org/10.1007/s10811-020-02164-7.
[39] F. Schwab, T.D. Bucheli, L.P. Lukhele, A. Magrez, B. Nowack, L. Sigg, et al., Are carbon nanotube effects on green algae caused by shading and agglomeration? Environ. Sci. Technol. 45 (2011) 6136–6144, https://doi.org/10.1021/es200506b.
[40] A. Sehgal, Y. Lalatonne, J.-F. Berret, M. Morvan, Precipitation–redispersion of cerium oxide nanoparticles with poly (acrylic acid): toward stable dispersions, Langmuir 21 (2005) 9359–9364, https://doi.org/10.1021/la0513757.
[41] X.Y. Deng, J. Cheng, X.L. Hu, L. Wang, D. Li, K. Gao, Biological effects of TiO_2 and CeO_2 nanoparticles on the growth, photosynthetic activity, and cellular components of a marine diatom *Phaeodactylum tricornutum*, Sci. Total Environ. 575 (2017) 87–96, https://doi.org/10.1016/j.scitotenv.2016.10.003.
[42] D. Wu, S. Yang, W. Du, Y. Yin, J. Zhang, H. Guo, Effects of titanium dioxide nanoparticles on *Microcystis aeruginosa* and microcystins production and release, J. Hazard. Mater. 377 (2019) 1–7, https://doi.org/10.1016/j.jhazmat.2019.05.013.
[43] E. Morelli, P. Cioni, M. Posarelli, E. Gabellieri, Chemical stability of CdSe quantum dots in seawater and their effects on a marine microalga, Aquat. Toxicol. 122 (2012) 153–162, https://doi.org/10.1016/j.aquatox.2012.06.012.
[44] C. Zhang, X. Huang, Y. Chu, N. Ren, S.-H. Ho, An overlooked effect induced by surface modification: different molecular response of *Chlorella pyrenoidosa* to graphitized and oxidized nanodiamonds, Environ. Sci. Nano 7 (2020) 2302–2312, https://doi.org/10.1039/d0en00444h.
[45] R.C. Gebara, L.O.G. Alho, A.D.S. Mansano, G.S. Rocha, M.D.G.G. Melão, Single and combined effects of Zn and Al on photosystem II of the green microalgae *Raphidocelis subcapitata* assessed by pulse-amplitude modulated (PAM) fluorometry, Aquat. Toxicol. 254 (2023) 106369, https://doi.org/10.1016/j.aquatox.2022.106369.
[46] Z. Liu, W. Chen, X. He, Influence of Cd^{2+} on growth and chlorophyll fluorescence in a hyperaccumulator: Lonicera japonica Thunb, J. Plant Growth Regul. 34 (2015) 672–676, https://doi.org/10.1007/s00344-015-9483-z.
[47] G. Markou, O. Depraetere, K. Muylaert, Effect of ammonia on the photosynthetic activity of Arthrospira and Chlorella: a study on chlorophyll fluorescence and electron transport, Algal Res. 16 (2016) 449–457, https://doi.org/10.1016/j.algal.2016.03.039.
[48] D.M. Kramer, G. Johnson, O. Kiirats, G.E. Edwards, New fluorescence parameters for the determination of QA redox state and excitation energy fluxes, Photosynth. Res. 79 (2004) 209–218, https://doi.org/10.1023/B:PRES.0000015391.99477.0d.
[49] Z. Wang, Y. Wang, J. Zhao, L. Ma, Y. Wang, X. Zhang, et al., Effects of GeO_2 on chlorophyll fluorescence and antioxidant enzymes in apple leaves under strong light, Photosynthetica 56 (2018) 1081–1092, https://doi.org/10.1007/s11099-018-0807-7.
[50] H. Jiang, L. Chen, J. Zheng, S. Han, N. Tang, B.R. Smith, Aluminum-induced effects on Photosystem II photochemistry in Citrus leaves assessed by the chlorophyll a fluorescence transient, Tree Physiol. 28 (2008) 1863–1871, https://doi.org/10.1093/treephys/28.12.1863.
[51] Y. Liu, L. Yue, C. Wang, X. Zhu, Z. Wang, B. Xing, Photosynthetic response mechanisms in typical C_3 and C_4 plants upon La_2O_3 nanoparticle exposure, Environ. Sci. Nano 7 (2020) 81–92, https://doi.org/10.1039/c9en00992b.
[52] P. Xiong, X. Yan, Q. Zhu, G. Qu, J. Shi, C. Liao, et al., A review of environmental occurrence, fate, and toxicity of novel brominated flame retardants, Environ. Sci. Technol. 53 (2019) 13551–13569, https://doi.org/10.1021/acs.est.9b03159.
[53] Z. Wang, L. Xu, J. Zhao, X. Wang, J.C. White, B. Xing, CuO nanoparticle interaction with *Arabidopsis thaliana*: toxicity, parent-progeny transfer, and gene expression, Environ. Sci. Technol. 50 (2016) 6008–6016, https://doi.org/10.1021/acs.est.6b01017.
[54] Y. Wang, R. Cai, C. Chen, The nano–bio interactions of nanomedicines: understanding the biochemical driving forces and redox reactions, Acc. Chem. Res. 52 (2019) 1507–1518, https://doi.org/10.1021/acs.accounts.9b00126.
[55] R. Mittler, Oxidative stress, antioxidants and stress tolerance, Trends Plant Sci. 7 (2002) 405–410, https://doi.org/10.1016/S1360-1385(02)02312-9.
[56] Y. Li, S. Liu, Z. Ji, J. Sun, X. Liu, Distinct responses of *Chlorella vulgaris* upon combined exposure to microplastics and bivalent zinc, J. Hazard. Mater. 442 (2023) 130137, https://doi.org/10.1016/j.jhazmat.2022.130137.
[57] Y. Huang, J. Lv, S. Liu, S. Zhu, W. Yao, J. Sun, et al., Physicochemical properties of nanosized biochar regulated by heat treatment temperature dictates algal responses: from the perspective of fatty acid metabolism, J. Hazard. Mater. 444 (2023) 130342, https://doi.org/10.1016/j.jhazmat.2022.130342.
[58] T. Pirmohamed, J.M. Dowding, S. Singh, B. Wasserman, E. Heckert, A.S. Karakoti, et al., Nanoceria exhibit redox state-dependent catalase mimetic activity, Chem. Commun. 46 (2010) 2736–2738, https://doi.org/10.1039/b922024k.
[59] E.G. Heckert, A.S. Karakoti, S. Seal, W.T. Self, The role of cerium redox state in the SOD mimetic activity of nanoceria, Biomaterials 29 (2008) 2705–2709, https://doi.org/10.1016/j.biomaterials.2008.03.014.
[60] X. Huang, S. Zhu, H. Zhang, Y. Huang, X. Wang, Y. Wang, et al., Biochar nanoparticles induced distinct biological effects on freshwater algae via oxidative stress, membrane damage, and nutrient depletion, ACS Sustain. Chem. Eng. 9 (2021) 10761–10770, https://doi.org/10.1021/acssuschemeng.1c01814.
[61] A. Middepogu, J. Hou, X. Gao, D. Lin, Effect and mechanism of TiO_2 nanoparticles on the photosynthesis of *Chlorella pyrenoidosa*, Ecotoxicol. Environ. Saf. 161 (2018) 497–506, https://doi.org/10.1016/j.ecoenv.2018.06.027.
[62] W. Yamori, S. Takahashi, A. Makino, G.D. Price, M.R. Badger, S. von Caemmerer, The roles of ATP synthase and the cytochrome b 6/f complexes in limiting chloroplast electron transport and determining photosynthetic capacity, Plant Physiol 155 (2011) 956–962, https://doi.org/10.1104/pp.110.168435.
[63] A.N. Tikhonov, The cytochrome b6f complex at the crossroad of photosynthetic electron transport pathways, Plant Physiol. Biochem. 81 (2014) 163–183, https://doi.org/10.1016/j.plaphy.2013.12.011.
[64] X. You, M. You, Y. Lyu, G. Peng, W. Sun, Single and combined exposure to micro(nano)plastics and azithromycin disturbing the photosynthetic carbon fixation of *Synechocystis* sp. Environ. Sci. Nano 9 (2022) 4354–4366, https://doi.org/10.1039/d2en00204c.
[65] J. Liu, H. Zhang, L. Yan, P.G. Kerr, S. Zhang, Y. Wu, Electron transport, light energy conversion and proteomic responses of periphyton in photosynthesis under exposure to AgNPs, J. Hazard. Mater. 401 (2021) 123809, https://doi.org/10.1016/j.jhazmat.2020.123809.
[66] X. Xu, R. Shen, L. Mo, X. Yang, X. Chen, H. Wang, et al., Improving plant photosynthesis through light-harvesting upconversion nanoparticles, ACS Nano 16 (2022) 18027–18037, https://doi.org/10.1021/acsnano.2c02162.
[67] Q. Chen, Y. Li, B. Li, Is color a matter of concern during microplastic exposure to *Scenedesmus obliquus* and *Daphnia magna*? J. Hazard. Mater. 383 (2020) 121224 https://doi.org/10.1016/j.jhazmat.2019.121224.
[68] K.A. Moore, S. Altus, J.W. Tay, J.B. Meehl, E.B. Johnson, D.M. Bortz, et al., Mechanical regulation of photosynthesis in cyanobacteria, Nat. Microbiol. 5 (2020) 757–767, https://doi.org/10.1038/s41564-020-0684-2.