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Physiological changes in *Chlamydomonas reinhardtii* after 1000 generations of selection of cadmium exposure at environmentally relevant concentrations†

Zhen Yu,‡[a] Huiling Wei,‡[a] Rui Hao,‡[a] Huashuo Chu‡[bc] and Yi Zhu *[a]

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Cadmium (Cd) is a nonessential and toxic trace element widely existing in waters through various anthropogenic activities such as mining and waste disposal. The physiological responses of aquatic organisms to long-term Cd exposure at environmentally relevant concentrations are still not well explored. In the present study, two strains of unicellular green algae *Chlamydomonas reinhardtii*, a walled strain CC125 and a wall-less strain CC406 were selected to investigate the physiological changes of aquatic organisms after long-term Cd exposure at environmentally relevant concentrations (4.92 and 49.2 μg L^{-1}). After about 1000 generations of selection, all of the two strains showed higher intracellular lipid peroxidation and lower photosynthetic activities, and failed to evolve specific adaptation to high levels of Cd (4.92 mg L^{-1}) compared to the control. However, short-term low dose Cd exposure exerted hormetic effects on *C. reinhardtii* and the hormetic stimulation of growth rate, chlorophyll contents and photochemical activities at the lower concentration of Cd (4.92 μg L^{-1}) groups were more pronounced than those at higher ones (49.2 μg L^{-1}). Taken together, this study confirmed that long-term exposure to Cd at environmentally relevant concentrations which were regarded as nontoxic in acute experiments would produce toxic effects on *C. reinhardtii* and should be paid more attention.

Environmental significance

Cadmium enters the environment through various anthropogenic activities and many concerns have been raised about its potential toxicity. In natural settings, long-term exposure regimes at low levels of Cd are most common. Besides, not all calculated "so called" safe concentrations of toxicants turn out to be safe in reality. Therefore, it is necessary to investigate the long-term exposure effects of Cd at environmentally relevant dosages. This study focuses on the physiological changes of green algae after long-term Cd exposure at environmentally relevant concentrations. Our results indicated that, after about 1000 generations, selection cells suffered damage and failed to increase tolerance to high Cd concentrations.

1. Introduction

Cadmium (Cd), a nonessential and toxic trace element, enters the environment through various anthropogenic activities such as lead-zinc mining and disposal of rechargeable nickel–cadmium batteries.[1] In China, the concentration of Cd can reach up to 5.6 mg L^{-1} in fresh waters associated with e-waste recycling activities.[2] In aquatic environments, Cd is easily absorbed by organisms at lower trophic levels and transferred to higher trophic levels in the food chain.[1] Cd is mutagenic and has been classified by the International Agency for Research on Cancer as a group 1 human carcinogen.[3] As for aquatic organisms, Cd inhibits the growth of microalgae, reduces the photosynthetic pigments and destroys physiological activities.[4–6]

Microalgae belong to the first trophic level of the food chain, providing biological energy and oxygen for other trophic levels and playing very important roles in keeping the material balance and energy cycle of aquatic ecosystems. *Chlamydomonas reinhardtii*, a unicellular green microalga regarded as one of the most used model systems, is very sensitive to contaminants and rapidly responds to environmental stresses, such as cadmium.[1,7] In addition, *Chlamydomonas reinhardtii* has short life cycles (approximately 5 hours) and is well suited for performing long-term and life-cycle exposures. Therefore, *Chlamydomonas reinhardtii* is commonly used to assess the toxicity of a hazardous substance in aquatic environments.[8,9]

[a]*College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China. E-mail: zhuyi@cau.edu.cn*

[b]*State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, Guiyang, 550081, China*

[c]*University of the Chinese Academy of Sciences, Beijing, 100049, China*

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‡ These authors contributed equally to this work.

The physiological responses of freshwater algae to short-term Cd exposure have been extensively studied. Toxic effects on growth, metabolism and photosystems were observed upon elevated concentrations of cadmium.[1,5,10] Nevertheless, these studies may not accurately reveal the changes that occur in natural settings, where long-term exposure regimes at lower levels of Cd are most common. For example, the cadmium concentration in agricultural water ranges from 0.011 μg L^{-1} in unpolluted environments[11] to 25 μg L^{-1} in slightly polluted landscape water in China.[12] In addition, not all calculated "so called" safe concentrations of toxicants turn out to be safe in reality.[13] Therefore, it is necessary to investigate the long-term exposure effects of cadmium at environmentally relevant dosages. Previous studies have reported the effect of long-term Cd exposure on algae, but the exposure duration was limited to dozens of generations.[14,15] The physiological changes of algae after a longer Cd exposure duration at environmentally relevant concentrations, such as over a thousand generations, remain unclear.

In the present study, we performed a chronic exposure experiment with *Chlamydomonas reinhardtii* to explore the physiological responses to long-term Cd exposure at environmentally relevant dosages. Since the cell wall is the first structure of algae to come in contact with the external medium and serves as additional protection against Cd,[16] two strains of *C. reinhardtii* with different cell wall characteristics were compared presently: CC125, a walled wild type and CC406, a wall-less mutant. The algae were transferred for approximately 1000 generations under low dosage Cd exposure, and then the cell growth, total chlorophyll contents, intracellular lipid peroxidation and the photosynthetic activity of the photosystem in the algae were examined. In addition, the physiological responses to a high concentration of Cd (4.92 mg L^{-1}) of selection cells after long-term low dose Cd exposure were investigated. Short-term tests with the same low dose Cd exposure were also performed to compare with the long-term Cd exposure.

2. Materials and methods

2.1 *Chlamydomonas reinhardtii* strains and culture conditions

Two strains of unicellular green algae *Chlamydomonas reinhardtii* were obtained from the *Chlamydomonas* Genetic Center (Duke University, Durham, NC, USA): CC125, a walled wild type and CC406, a wall-less mutant. Stock cultures were grown in 50 mL liquid Tris-acetate-phosphate (TAP) medium (ESI Table S1†)[17] in 100 mL Erlenmeyer flasks at 25 ± 1 °C, under a 16 h/8 h light/dark regime with a light density of 100 μmol of photons m^{-2} s^{-1} and were hand-shaken four times a day. The initial pH value of the TAP medium was 7.0. All glassware used was soaked in 10% HNO_3 for at least 48 h and rinsed 7 times with ultrapure water.

2.2 Experimental setup

Before treatment with Cd, the exponentially grown cultures were transferred to fresh, sterile TAP medium. The initial cell density was 2.3–2.75 × 10^5 cells per mL for all experiments. Then, Cd was added into TAP medium as analytical grade cadmium chloride ($CdCl_2 \cdot 2\frac{1}{2}H_2O$; Sinopharm, Shanghai, China) to give final treatment concentrations of Cd (4.92 and 49.2 μg L^{-1}), which were regarded as the environmentally relevant dosages according to the previous reports,[18,19] with three replicate flasks per concentration. The speciation of Cd in the TAP medium was performed using Visual MINTEQ version 3.0 with a fixed pH of 7.0 in the presence of Cd[20] (ESI Table S2†). For short-term cadmium exposure, the whole experiment lasted for 168 h with 7 time points (24, 48, 72, 96, 120, 144 and 168 h) and the specific growth rate of algae was detected at each time point. Besides, the total chlorophyll contents, malondialdehyde (MDA) content, peroxidase (POD) activity and photosynthetic activities of both photosystem I (PSI) and photosystem II (PSII) were measured after 72 h-exposure. For long-term cadmium exposure, algae were transferred every 3 days (exponential growth phase) for approximately 1000 generations for each replicate line. Then, the algae cells were transferred to fresh, sterile TAP medium with Cd and incubated for 168 h with 7 time points (24, 48, 72, 96, 120, 144 and 168 h). The specific growth rate of algae was detected every 24 h. In addition, the algae cells were collected at 72 h and the same indexes as the short-term tests were evaluated to investigate the effects of mechanisms modulating the physiological and biochemical responses of long-term Cd exposure on *Chlamydomonas reinhardtii* at environmentally relevant dosages. Besides, after about 1000 generations, selection cells after long-term low dose Cd exposure were treated with a high concentration of Cd (4.92 mg L^{-1}) for 72 h. The photosynthetic activities of both PSI and PSII and the Cd contents in algae cells were measured to investigate whether they evolved specific adaptation to Cd stress. Each treatment was replicated three times.

2.3 Specific growth rate and chlorophyll content measurements

During the experiments, the cell density of algae at each time point was determined using a hemocytometer chamber under a light microscope (Shanghai Optical Instrument Factory, Shanghai, China) using the standard procedure. Then, the specific growth rate (μ) was calculated according to the equation described by Manzo:[21]

$$\mu = (\ln N_t - \ln N_0)/(t - t_0)$$

where μ is the growth rate; N_t and N_0 are the algae cell density at times t and t_0, respectively; t is the sample time for counting cell density; and t_0 is the origin time of the treatment.

Total chlorophyll contents were measured as described by Pokora[22] with a little modification. Briefly, photosynthetic pigments were extracted with 80% acetone and the supernatant was collected by centrifugation at 5000 rpm, 4 °C for 5 min. Then, the absorbance at 470, 646 and 663 nm was measured with an ultraviolet spectrophotometer (METASH, Shanghai, China), and chlorophyll contents were calculated according to equations given by Lichtenthaler.[23]

2.4 Malondialdehyde content and peroxidase activity assessment in *Chlamydomonas reinhardtii*

After incubation for 72 h, the algae cells were collected by centrifugation at 5000 rpm, 4 °C for 10 min and disrupted with an ultrasonic cell crusher (Nanjing Immanuel Instrument Equipment Co., Nanjing, China) at 4 °C for 5 min (crushing 5 s and interval 10 s). The activity of POD was determined using guaiacol as a hydrogen donor and the change was measured at 470 nm over 1 min according to Maehly and Chance.[24] The MDA content, a lipid peroxidation product, was analyzed using the thiobarbituric acid (TBA) reacting substance method described by Uchiyama and Mihara.[25]

2.5 Evaluation of the photosynthetic activity

The absorbance changes at 830 nm (ΔA_{830}) reflect the oxidation and reduction of PSI reaction center P700. In order to explore the effect of Cd on the photochemical activity of PSI in *Chlamydomonas reinhardtii*, ΔA_{830} was measured using a dual-wavelength ED-P700DW unit connected to a pulse amplitude modulation (PAM) chlorophyll fluorimeter (model PAM101, Walz, Effeltrich, Germany). Absorbance values were recorded using a PDA-100 PAM data acquisition system. Saturating far-red light (720 nm, 24 μmol m^{-2} s^{-1}) emitted by a far-red diode (102-FR) was applied to oxidize P700 for 45 s. Before every measurement, all the samples were dark adapted for 15 min.

In order to observe the photochemical activity of PSII in microalgae, a double-modulation fluorometer (FL3500, PSI, Inc., Brno, Czech) was employed to measure the polyphasic fast fluorescence induction and Q_A^- reoxidation kinetics. Before measurements, all the samples were dark adapted for 15 min to ensure closure of all PSII reaction centers and estimate the maximum fluorescence yield (F_m). The chlorophyll a fluorescence was measured according to Ran[26] under an actinic light of 3000 μmol photons per m^2 per s and the fluorescence signals were recorded from 10 μs to 1 s. When plotted on a logarithmic time scale, the chlorophyll a fluorescence transient displayed a polyphasic rise including phases O, J, I and P.[27] The O–J–I–P transients were analyzed using the JIP-test (Table 1).[28,29] Q_A^- reoxidation kinetics were measured according to Li[29] with 100% flash intensity of the power and the fluorescence signals were recorded from the 200 μs to 10 s range. Both actinic (30 μs) flashes and measuring (2.5 μs) flashes were provided by red LEDs.

2.6 Cadmium contents in *Chlamydomonas reinhardtii*

In order to investigate the responses of algae after long-term cadmium exposure to a high concentration of Cd, selection cells after long-term cadmium exposure in the exponential growth phase were transferred into new TAP medium with 4.92 mg L^{-1} of Cd and the cadmium contents in algae cells were measured. After 72 h-exposure, algae cells were centrifuged at 5000 rpm, 4 °C for 10 min and digested as described by Ran[30] with minor modification. Briefly, cells were digested with a HNO_3 : $HClO_4$ (9 : 1 v/v) mixture at 110 °C until the yellow color disappeared and "white smoke" appeared. The Cd contents in digestion solution were measured using inductively coupled plasma optical emission spectrometry (ICP-OES) (Agilent 5100, Santa Clara, California, USA). The standard solution was diluted using 6.5% of superior grade nitric acid from the 1 g L^{-1} standard solution obtained from the Chinese National Research Center for Certified Reference Materials.

2.7 Statistical analysis

The data were shown as mean ± standard deviation (SD). All statistical analyses were carried out using SPSS 16.0 software (SPSS, Chicago, USA). The significance levels of differences were determined using ANOVA one-way analysis of variance followed by a Duncan post hoc test for multiple comparisons between the control and treatment groups, and significant differences were reported for $p \leq 0.05$. The figures were completed using origin 9.1 (OriginLab, Northampton, MA, USA).

Table 1 The parameters calculated from the fluorescence transient

Formulae and terms	Illustrations
F_o	Minimal recorded fluorescence intensity (O step). At this time all reaction centers (RCs) are open
F_m	Maximal recorded fluorescence intensity (P step). At this time all RCs were closed after illumination
$F_{300\ \mu s}$	The fluorescence at 300 μs
$F_{2\ ms}$	The fluorescence intensity at the J step (at 2 ms)
$M_o = 4(F_{300\ \mu s} - F_o)/(F_m - F_o)$	Approximated initial slope of the fluorescence transient
$V_j = (F_{2\ ms} - F_o)/(F_m - F_o)$	Relative variable fluorescence intensity at the J-step
$\varphi_{Po} = TRo/ABS = [1 - (F_o/F_m)] = F_v/F_m$	Maximum quantum yield for primary photochemistry (at $t = 0$)
$\Psi_o = ETo/TRo = (1 - V_j)$	Probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^- (at $t = 0$)
$\varphi_{Eo} = ETo/ABS = [1 - (F_o/F_m)]\Psi_o$	Quantum yield for electron transport (at $t = 0$)
$ABS/RC = Mo(1/V_j)(1/\varphi_{Po})$	Absorption flux per RC
$TRo/RC = Mo(1/V_j)$	Trapped energy flux per RC (at $t = 0$)
$ETo/RC = Mo(1/V_j)\Psi o$	Electron transport flux per RC (at $t = 0$)
$DIo/RC = ABS/RC - TRo/RC$	Dissipated energy flux per RC (at $t = 0$)
$PI_{ABS} = (RC/ABS)\varphi_{Po}/(1 - \varphi_{Po})\Psi_o/(1 - \Psi_o)$	Performance index on absorption basis

3. Results and discussion

3.1 Effects of Cd on the cell proliferation and chlorophyll contents of *Chlamydomonas reinhardtii*

In short-term exposure tests (Fig. 1a: CC125-S-4.92 and CC125-S-49.2; Fig. 1b: CC406-S-4.92 and CC406-S-49.2, where the letter "S" represents short-term), the specific growth rate of the two algae increased significantly after exposure to both 4.92 and 49.2 μg L^{-1} Cd compared to the control in the exponential phase of growth (within 3d), but did not exhibit further promotion as the Cd concentration increased from 4.92 to 49.2 μg L^{-1}. However, in long-term exposure tests (Fig. 1a: CC125-L-4.92 and CC125-L-49.2; Fig. 1b: CC406-L-4.92 and CC406-L-49.2, where the letter "L" represents long-term), there were no significant changes in the growth rate of the two algae in the logarithmic growth phase. Besides, when the exposure time extended, algae cells shifted to the stationary phase and the growth of CC125-L-49.2 indicated a downward trend compared to the control (Fig. 1a). These data above indicated that 4.92 and 49.2 μg L^{-1} of Cd presented hormetic effects towards the growth of *C. reinhardtii* in short-term exposure tests. This was in accordance with an earlier report that low concentrations of Cd exposure caused hormesis in the growth of another algal species.[18] Hormesis is a dose–response relationship characterized by a biphasic dose response to stressors with low-dose stimulation and high-dose inhibition.[31] Hormetic stimulation has been observed that initially there would be a disruption in homeostasis, followed by an overcompensatory response that would be seen as stimulation.[32] Many metal ions presented hormetic stimulation effects at low doses, and Cd (about 10^{-7} to 10^{-3} mmol L^{-1}) had a higher stimulatory effect towards *Vibrio fischeri* than other metal ions after 24 h exposure.[19] In addition, Mohammadi-Bardbori and Rannug reported that NADPH oxidase is central to the mechanism of Cd-mediated stimulation of cell proliferation.[33]

The total chlorophyll contents of CC125 and CC406 exposed to Cd for short-term and long-term are shown in Fig. 1c and d. In short-term Cd stress tests, the total chlorophyll contents increased by 28.4% and 41.5% for CC125, and by 35.1% and 26.9% for CC406 after 4.92 and 49.2 μg L^{-1} Cd exposure respectively. However, there were no significant differences between the control and long-term Cd exposure groups. Our data

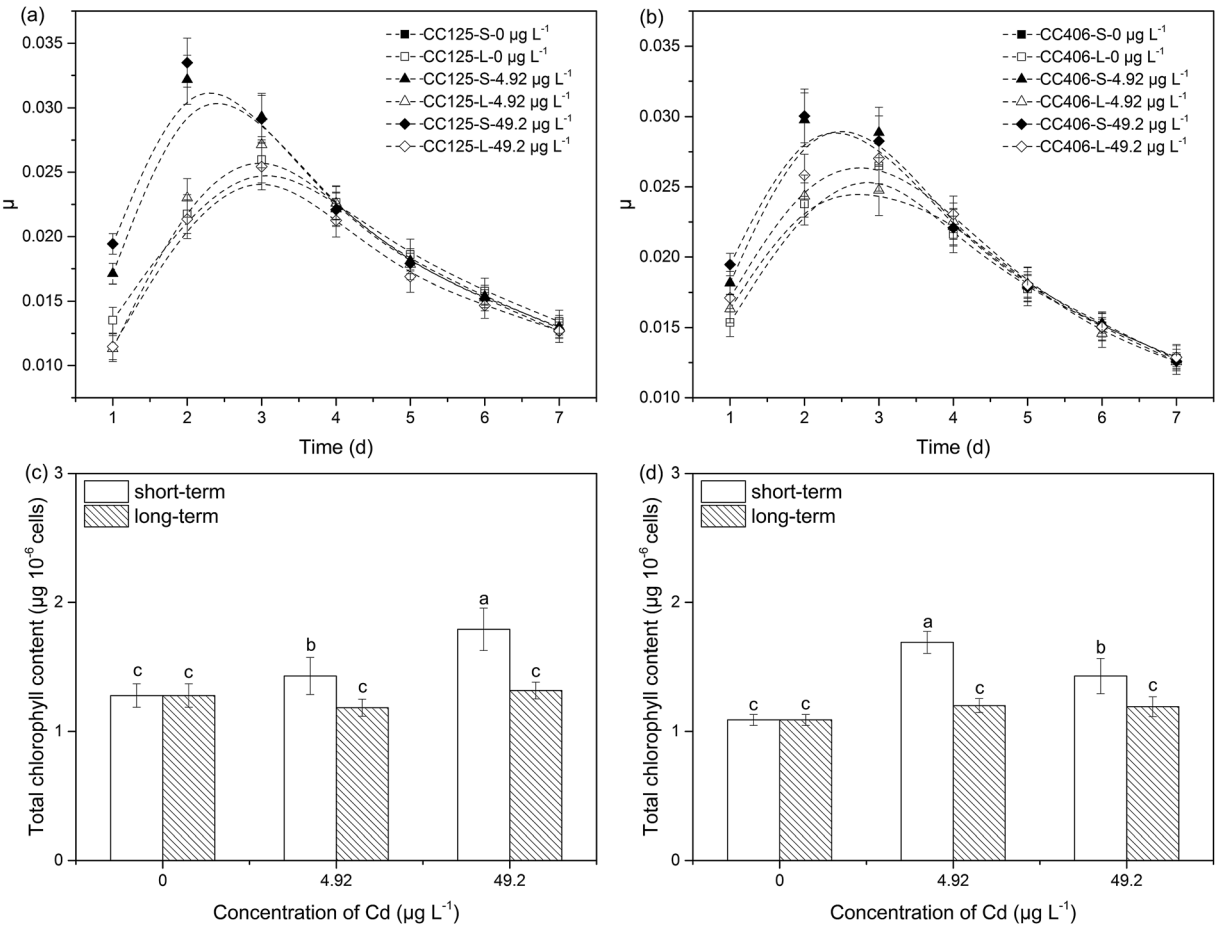

Fig. 1 Cytotoxicity of short-term and long-term exposure at 4.92 and 49.2 μg L^{-1} Cd on the specific growth rate (a and b, where the letters "s" and "l" represent short-term and long-term, respectively) and total chlorophyll contents (c and d) of walled (CC125, a and c) and wall-less (CC406, b and d) *Chlamydomonas reinhardtii* strains. Values represent mean ± s.d (n = 3). Those with different letters on error bars are significantly different (p < 0.05), where the letter "a" is assigned to the groups with the highest values.

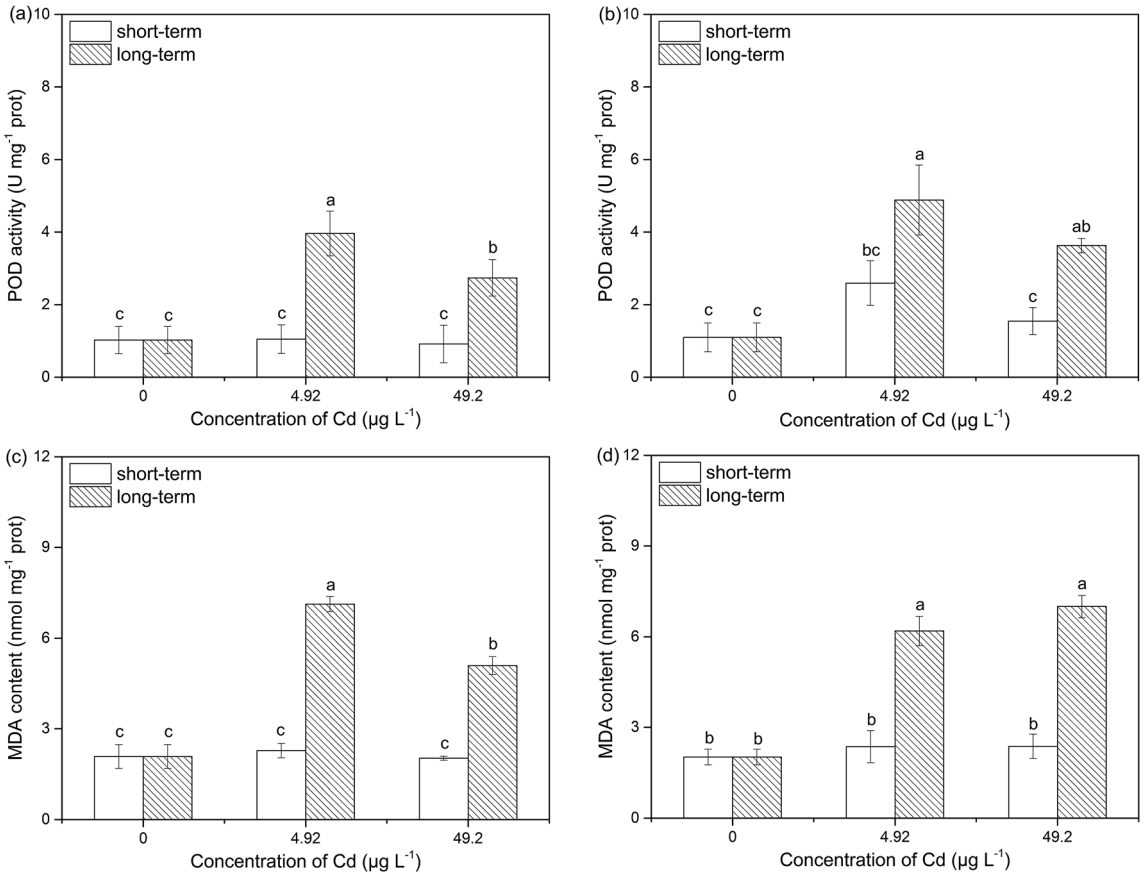

Fig. 2 Peroxidase (POD, a and b) activities and malondialdehyde (MDA, c and d) contents of walled (CC125, a and c) and wall-less (CC406, b and d) *Chlamydomonas reinhardtii* strains exposed to 4.92 and 49.2 μg L^{-1} Cd for short-term and long-term. Values represent mean ± s.d ($n = 3$). Those with different letters on error bars are significantly different ($p < 0.05$), where the letter "a" is assigned to the groups with the highest values.

revealed that the synthesis of chlorophyll was affected by exposure to Cd for short term, which is in accordance with an earlier study where Cd exposure at higher concentrations decreased the content of chlorophyll of *Chlorella vulgaris* significantly.[5] Besides, in short-term Cd stress tests, the addition of Cd stimulated the synthesis of algae chlorophyll, which might be due to the Cd-induced hormetic effects on algae. Accordingly, a report on *Lonicera japonica* indicated that low Cd concentrations resulted in the increase of chlorophyll, which might be related to the Cd-induced increase of Fe uptake.[34]

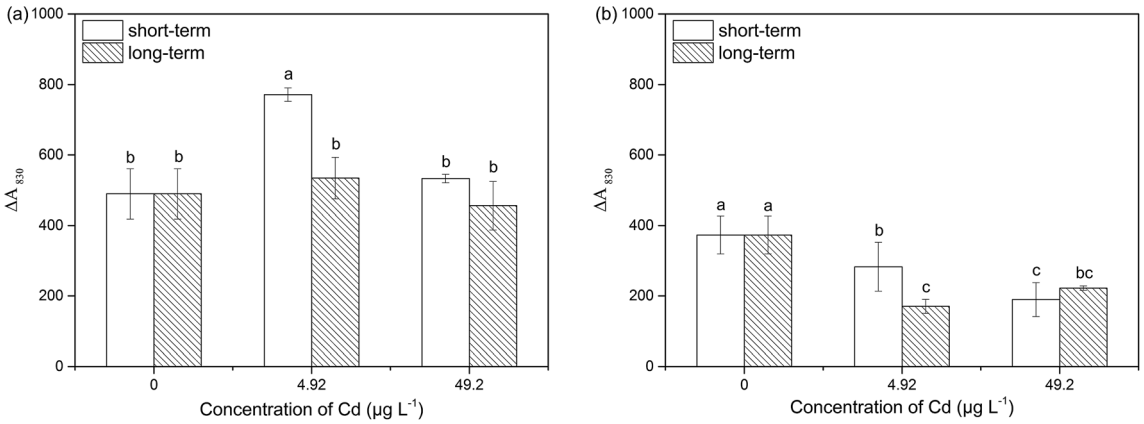

Fig. 3 Amplitudes of far-red-light-induced P700 absorbance changes at approximately 830 nm (ΔA_{830}) of walled (CC125, a) and wall-less (CC406, b) *Chlamydomonas reinhardtii* strains exposed to 4.92 and 49.2 μg L^{-1} Cd for short-term and long-term. Values represent mean ± s.d ($n = 3$). Those with different letters on error bars are significantly different ($p < 0.05$), where the letter "a" is assigned to the groups with the highest values.

3.2 Effects of Cd on lipid peroxidation and antioxidant enzyme activities of *Chlamydomonas reinhardtii*

The activities of POD and MDA contents of CC125 and CC406 exposed to Cd for short-term and long-term are depicted in Fig. 2. In long-term tests, after 4.92 and 49.2 μg L^{-1} Cd exposure, the POD activities showed a 2.8 and 1.7-fold increase for CC125 and a 3.4 and 2.3-fold increase for CC406 compared to the control. Similar trends were observed in MDA contents. The

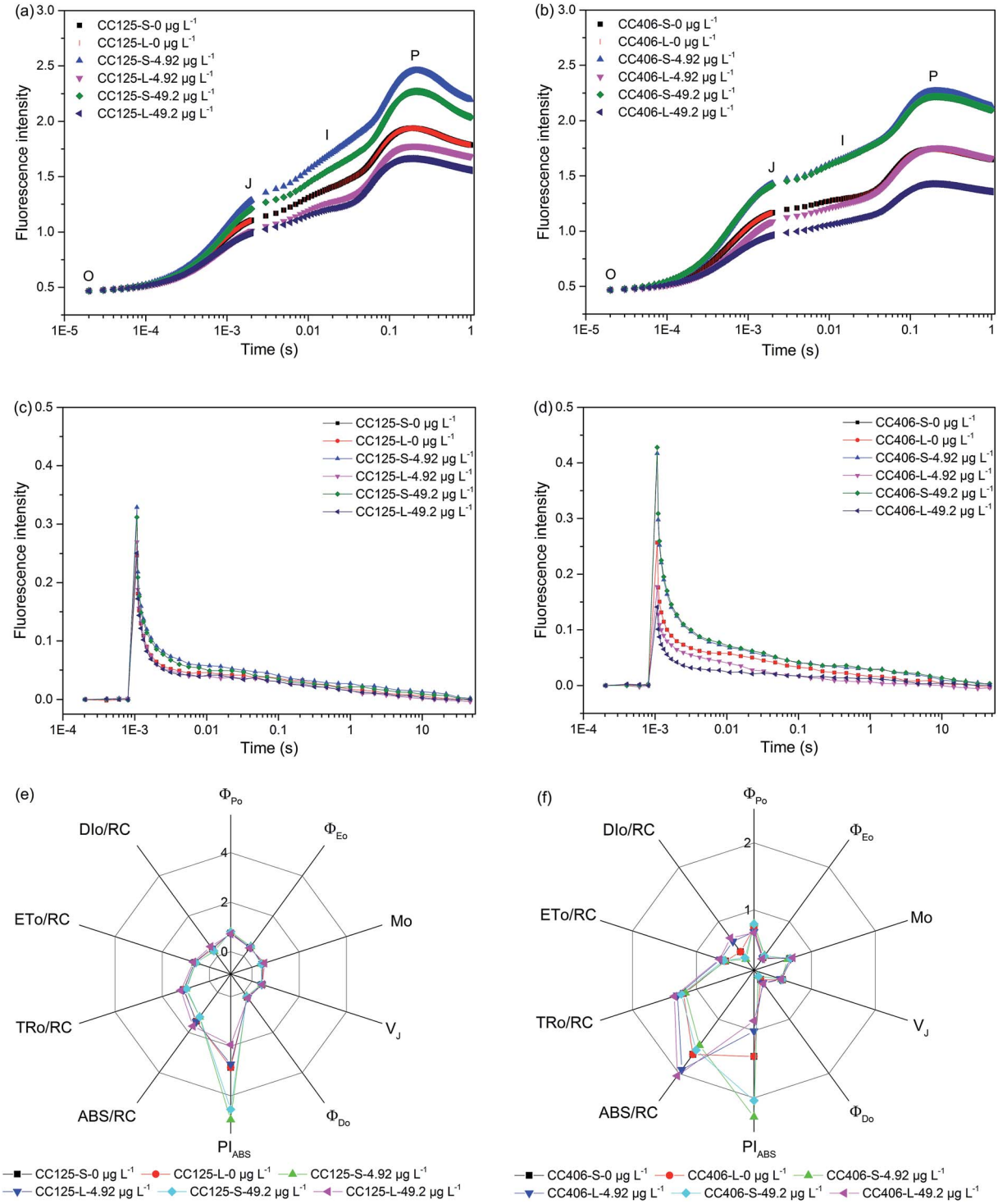

Fig. 4 Effects of short-term and long-term exposure at 4.92 and 49.2 μg L^{-1} Cd on the photosystem II (PSII) activity of walled (CC125, a, c, and e) and wall-less (CC406, b, d, and f) *Chlamydomonas reinhardtii* strains. (a and b) Chlorophyll a fluorescence (OJIP) transients; (c and d) the Q_A^- reoxidation kinetics; (e and f) photosynthetic parameters deduced with the JIP test analysis of OJIP transients. The letters "s" and "l" represent short-term and long-term, respectively. Values represent mean ± s.d ($n = 3$).

MDA contents increased 2.4 and 1.5 times for CC125, and 2.1 and 2.5 times for CC406 respectively. Nevertheless, short-term Cd stress tests had no significant effect on POD activities and MDA contents of the two algae. Oxidative stress is considered as the main mechanism of Cd-induced ecotoxicity and intracellular antioxidant defence mechanisms prevent it from exceeding toxic thresholds.[35] Ran showed that the activities of antioxidant enzymes increased to alleviate the Cd-induced damage of other algae.[26] The present data suggested that long-term exposure to low dose Cd caused oxidative stress in *Chlamydomonas reinhardtii* and induced lipid peroxidation. It might be because of the fact that intracellular Cd accumulation increases with exposure duration and can reach toxic levels after long-term exposure to nontoxic Cd concentrations. Chandurvelan reported that with increased exposure duration, effects on energy metabolism induced by high Cd in *Paratya curvirostris* became apparent at lower exposure concentrations.[36]

3.3 Effects of Cd on the photosystem I (PSI) activity of *Chlamydomonas reinhardtii*

In photosynthetic organisms, photosynthesis is one of the main targets of the toxic effects of Cd[37,38] and PSI is considered to be sensitive to heavy metal stresses.[39,40] In order to explore the effect of short-term and long-term Cd exposure on the photochemical activity of PSI in *Chlamydomonas reinhardtii*, the reaction center P700 of PSI was analyzed by measuring absorbance changes at 830 nm (ΔA_{830}), which represent the oxidation and reduction of P700.[41] Fig. 3 shows the ΔA_{830} of PSI particles in CC125 and CC406 after 4.92 and 49.2 μg L^{-1} Cd exposure for short term and long term. The results reported that the magnitude of ΔA_{830} increased significantly after short-term Cd exposure in CC125, especially at the lower concentration of 4.92 μg L^{-1} Cd where ΔA_{830} increased by 57.6% compared to the control. In addition, there was no significant difference of ΔA_{830} between the long-term Cd exposure group and the control, indicating that short-term low dose Cd exposure exerted a hormetic effect on the photochemical activity of PSI in CC125, while hormesis is time-dependent and would disappear when the exposure time extends. Nevertheless, for the wall-less strain CC406, both short-term and long-term Cd groups decreased the ΔA_{830} significantly, and the latter was more effective, indicating that PSI in wall-less strain is more sensitive to Cd exposure compared to walled strain. In short, the present data confirmed that PSI was the target of the toxicity of Cd exposure in *C. reinhardtii*. Accordingly, as the concentration of Cd increases, high Cd limited the photochemical activity of PSI in the cyanobacterium.[38] Besides, PSI was also the target of other metals. Cu^{2+} showed an obvious increase of PSI quantum yield and electron transport and enhanced the PSI activity in *Microcystis aeruginosa*,[42] and Al^{3+} exposure changed the structure of PSI complex proteins and inhibited the electron transport through both acceptor and donor sides of PSI.[40]

3.4 Effects of Cd on the photosystem II (PSII) activity of *Chlamydomonas reinhardtii*

The fast fluorescence induction kinetics of algae were measured to obtain detailed information about the PSII photochemical activity of CC125 and CC406 exposed to Cd for short-term and long-term (Fig. 4a and b). The results showed that the fast fluorescence induction kinetics of algae in all treatment groups of the two algae were typical OJIP curves. When the donor side of PSII suffered damage, chlorophyll fluorescence intensity rose rapidly in a short time (before *J* point) and the *K* point appeared. Then, the polyphasic fast fluorescence induction O–J–I–P would turn into O–K–J–I–P or with even more inflection points.[43] However, there were no *K* points appeared on the OJIP curves of treatment groups, indicating that both short-term and long-term Cd exposure did not affect the donor side of the PSII of algae.

The OJIP curve was analyzed using the JIP-test (Table 1) to evaluate the variation of the photosynthetic apparatus (Fig. 4e and f). In CC125, after 4.92 and 49.2 μg L^{-1} Cd exposure, the performance index on absorption basis (PI_{ABS}), containing three parameters RC/ABS, Φ_{P0} and Ψ_O, increased by 73.7% and 59.2% in short-term tests, but decreased by 4.6% and 32.2% in long-term tests respectively compared to the control. Similar trends were observed in φ_{Eo} and φ_{Po}. However, ABS/RC, representing

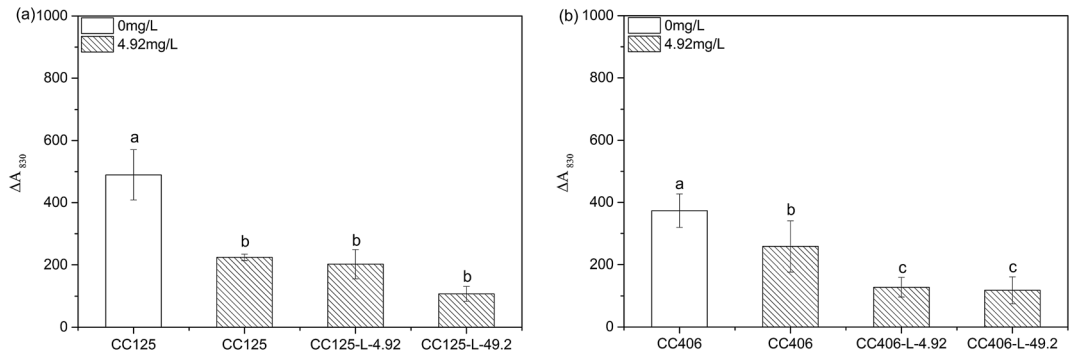

Fig. 5 Amplitudes of far-red-light-induced P700 absorbance changes at approximately 830 nm (ΔA_{830}) of long-term low dose Cd exposure of selection walled (CC125, a) and wall-less (CC406, b) *Chlamydomonas reinhardtii* strains after exposure to a high Cd concentration of 4.92 mg L^{-1}. The selection algae cells were treated with 4.92 (L-4.92 groups) and 49.2 μg L^{-1} (L-49.2 groups) Cd separately for about 1000 generations. Values represent mean ± s.d ($n = 3$). Those with different letters on error bars are significantly different ($p < 0.05$), where the letter "a" is assigned to the groups with the highest values.

the light energy photosynthetic pigments absorbed per reaction center, decreased by 15.0% and 15.5% in short-term tests, but increased by 1.4% and 16.4% in long-term tests after 4.92 and 49.2 μg L^{-1} Cd exposure respectively compared to the control. Similar trends were observed in other specific fluxes (per RC) as well as M_o and φ_{Do}. In addition, there was no significant difference in the variation of the photosynthetic apparatus between CC125 and CC406. These data indicated that long-term

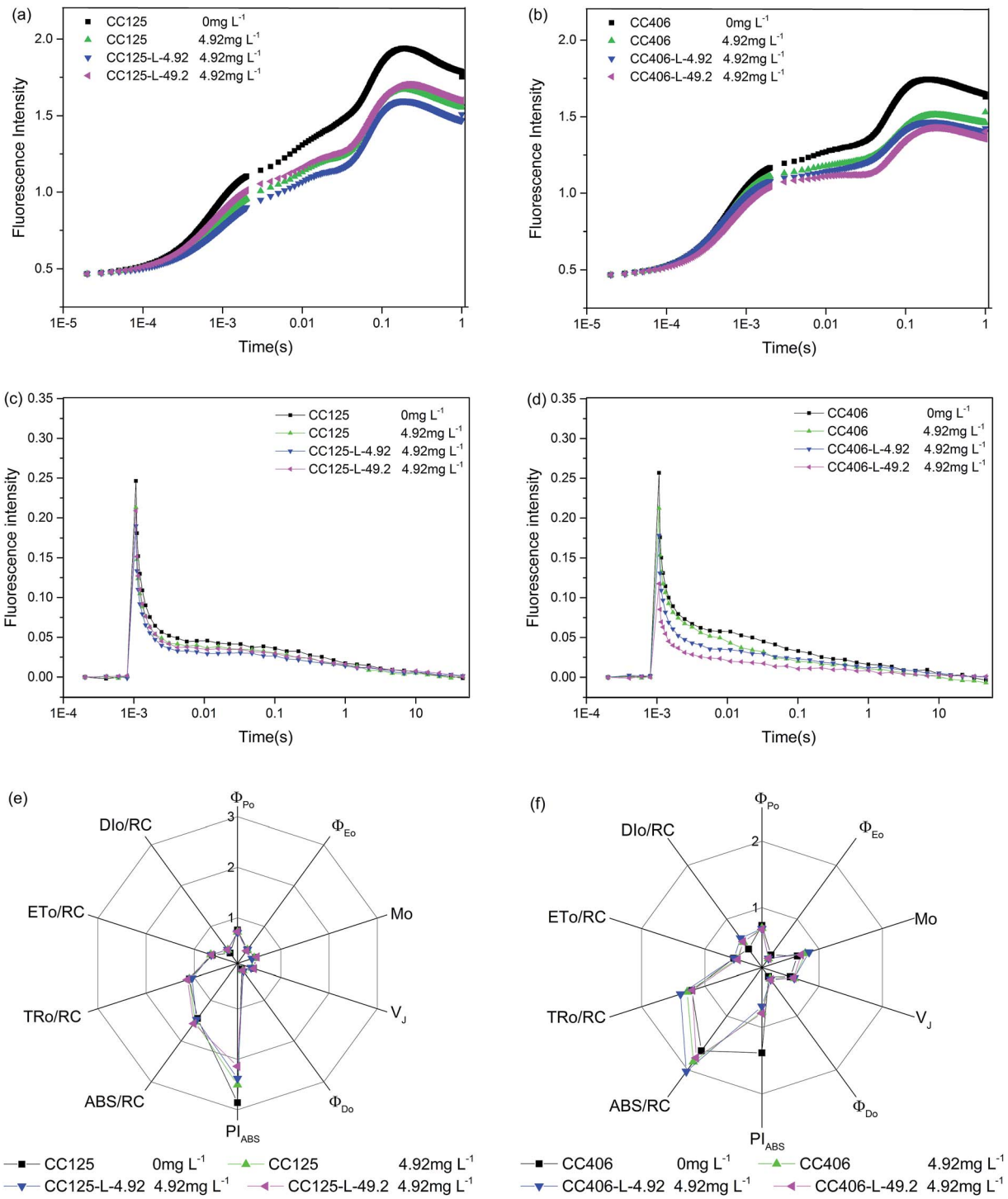

Fig. 6 Effects of a high Cd concentration of 4.92 mg L^{-1} on the photosystem II (PSII) activity of long-term low dose Cd exposure of selection walled (CC125, a, c, and e) and wall-less (CC406, b, d, and f) *Chlamydomonas reinhardtii* strains. The selection algae cells were treated with 4.92 (L-4.92 groups) and 49.2 μg L^{-1} (L-49.2 groups) Cd separately for about 1000 generations. (a and b) Chlorophyll a fluorescence (OJIP) transients; (c and d) the Q_A^- reoxidation kinetics; (e and f) photosynthetic parameters deduced with the JIP test analysis of OJIP transients. Values represent mean ± s.d ($n = 3$).

Cd exposure at low doses decreased the PSII photochemical activity of CC125 and CC406, while short-term Cd treatment had the opposite effect. The acceptor side of PSII of the two algae cells suffered damage after long-term Cd treatment, with less light energy used for electron transfer and more for reducing Q_A, leading to the fast Q_A reduction and suppression of electron transfer from Q_A to the next electron acceptor. It was confirmed by the Q_A^- reoxidation kinetics (Fig. 4c and d) which represents the efficiency of electron transfer on the acceptor side of PSII. Besides, the stimulation of short-term Cd stress to PSII at low doses was probably due to the hormetic effects discussed above. However, the reason for the Cd-induced increase of photochemical activity remains unclear and Ying and others reported that this might be related to the activities of rubisco.[44]

In addition, the results presently demonstrated that the photochemical activities of both PSI and PSII of *Chlamydomonas reinhardtii* were affected after Cd exposure (Fig. 3 and 4), which was in accordance with the previous report observed in Cd treated *cyanobacterium* where both the photosystem I acceptor side and photosystem II electron transport were disturbed.[38] Differently, studies about the toxicity of other heavy metals confirmed that PSI is the major target of manganese toxicity within the photosynthetic apparatus of *Arabidopsis* plants,[45] and PSI showed higher tolerance to Sb(v) and Cu^{2+} than PSII in *Microcystis aeruginosa*.[39,42]

3.5 Response of *Chlamydomonas reinhardtii* to high Cd concentration after long-term low dose cadmium exposure

After about 1000 generations, selection cells after long-term low dose Cd exposure were transferred to a high Cd concentration of 4.92 mg L^{-1} to investigate whether they evolved specific adaptation to Cd stress. After 72 h exposure, the photochemical activities of both PSI and PSII in selection cells were measured and are depicted in Fig. 5 and 6 separately. Fig. 5 shows that after exposure to a high Cd concentration of 4.92 mg L^{-1}, the ΔA_{830} values of both CC125 and CC406 normal cells decreased and the selection cells of CC125 (CC125-L-4.92 and CC125-L-49.2) had the same ΔA_{830} as the normal cells. Besides, the selection cells of CC406 (CC406-L-4.92 and CC406-L-49.2) had lower ΔA_{830} than the normal cells. This can be explained from the results of Fig. 3 that the PSI of CC406 was disturbed after long-term low dose Cd exposure and became more sensitive to Cd stress. Fig. 6 shows that after exposure to a high Cd concentration of 4.92 mg L^{-1}, PI_{ABS}, φ_{Eo} and φ_{Po} in both CC125 and CC406 normal cells decreased, while the specific fluxes (per RC) as well as M_o and φ_{Do} increased significantly. Besides, there was no significant difference between the selection cells and normal cells, indicating that the PSII of both the selection cells and normal cells suffered the same damage after high Cd concentration exposure. In conclusion, the PSI and PSII of CC125 and CC406 after long-term low dose Cd exposure failed to evolve specific adaptation to Cd stress.

In addition, Cd contents in selection cells after transferring to a high Cd concentration of 4.92 mg L^{-1} for 72 h were measured (Fig. 7). The results showed that CC125 accumulated higher Cd contents than CC406 in control groups, which was probably because of the Cd retention effect of proteins and

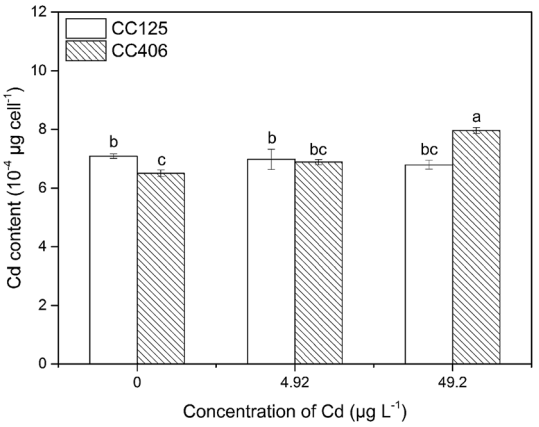

Fig. 7 Cd contents in walled (CC125) and wall-less (CC406) *Chlamydomonas reinhardtii* strains when transferred into 4.92 mg L^{-1} Cd after long-term low dose Cd exposure selection. Values represent mean ± s.d (n = 3). Those with different letters on error bars are significantly different ($p < 0.05$), where the letter "a" is assigned to the groups with the highest values.

polysaccharides in the cell wall. The results were in accordance with the reports of Macfie and Welbourn.[46] Besides, the selection cells of CC406 accumulated more Cd than the control, especially in 49.2 μg L^{-1} Cd-treated groups, which showed a 22.3% increase compared to the control. However, for CC125, there was no significant difference between the selection cells and control. Cd speciation prediction indicated that the free Cd ion concentration in 4.92 and 49.2 μg L^{-1} Cd-treatment medium was only 6.7×10^{-6} and 7.1×10^{-5} μM L^{-1} respectively (ESI Table S2†). But, long-term exposure to low dose Cd exerted a toxic effect on CC406 (Fig. 2) and destroyed the cytomembrane,[47] leading to the change of membrane permeability and an increase of Cd accumulation. Nevertheless, due to the protection of the cell wall, long-term exposure to low dose Cd had a lower toxic effect on CC125 than CC406 and had no significant effect on the accumulation of Cd in CC125. These data confirmed that long-term exposure to low dose Cd had a toxic effect on *C. reinhardtii* and *C. reinhardtii* failed to evolve specific adaptation to Cd stress on physiological indexes after 1000 generations of selection of low-dose cadmium. In agreement with other abiotic stresses, *C. reinhardtii* failed to evolve specific adaptation to elevated CO_2 concentrations of 1050 parts per million after about 1000 generations of selection.[48] Differently, *C. reinhardtii* showed adaptation to high salt stress by down-regulating the genes involved in the stress response after 1255 generations.[49] These data suggested that *C. reinhardtii* showed different adaption strategies to different abiotic stresses after long-term exposure selection. And it's necessary to study the genetic changes of *C. reinhardtii* after 1000 generations of selection of low-dose cadmium in the future.

4. Conclusion

In conclusion, short-term Cd exposure at environmentally relevant concentrations did not cause significant intracellular

lipid peroxidation in *Chlamydomonas reinhardtii*, but presented hormetic effects with stimulating the growth rate, chlorophyll contents and photochemical activity of both PSI and PSII in algae. However, when the exposure time extended, after 1000 generations, algae showed higher intracellular lipid peroxidation and lower photochemical activities of the photosystem compared to the control, though the growth rate and chlorophyll contents were not affected. Besides, *C. reinhardtii* failed to evolve specific adaptation to a high Cd concentration of 4.92 mg L^{-1} after long-term exposure to low dose cadmium selection. Selection experiments in more realistic systems will be necessary to validate the physiological response of aquatic organisms to long-term Cd exposure at environmentally relevant concentrations.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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