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PHOTOSYNTHETIC AND CELLULAR TOXICITY OF CADMIUM IN *CHLORELLA VULGARIS*

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Abstract: The toxic effects of cadmium (Cd) on the green alga *Chlorella vulgaris* were investigated by following the response to Cd of various toxicity endpoints (cell growth, cell size, photochemical efficiency of PSII in the light or Φ_{PSII}, maximal photochemical efficiency or Fv/Fm, chlorophyll *a* fluorescence, esterase activity, and cell viability). These toxicity endpoints were studied in laboratory batch cultures of *C. vulgaris* over a long-term 96-h exposure to different Cd concentrations using flow cytometry and pulse amplitude modulated fluorometry. The sequence of sensitivity of these toxicity endpoints was: cell yield $>>$ $\Phi_{PSII} \approx$ esterase activity $>$ Fv/Fm $>$ chlorophyll *a* fluorescence $\approx$ cell viability. It is shown that cell apoptosis or cell death only accounted for a minor part of the reduction in cell yield even at very high algistatic free Cd^{2+} concentrations, and other mechanisms such as blocked cell divisions are major contributors to cell yield inhibition. Furthermore, cadmium may affect both the electron donors and acceptors of the electron transport chain at high free Cd^{2+} concentration. Finally, the resistance of cells to cell death was size-dependent; medium-sized cells had the highest toxicity threshold. The present study brings new insights into the toxicity mechanisms of Cd in *C. vulgaris* and provides a detailed comparison of the sensitivity of various Cd toxicity endpoints. *Environ Toxicol Chem* 2013;32:2762–2770. © 2013 SETAC

Keywords: Cadmium Photosynthetic toxicity Cellular toxicity Flow cytometry *Chlorella vulgaris*

INTRODUCTION

Water polluted with metals is a worldwide environmental concern, particularly in China. The use of metals in China has increased significantly with the rapid growth of both industry and urban development, creating serious water-related environmental problems, such as the recent cadmium (Cd) spills observed in Guangxi province. Cadmium is usually a nonessential element for algae growth and is a potentially toxic metal. Because of its relatively high solubility and its relatively low tendency to adsorb on inorganic particulates in freshwater systems at pH ≤ 7 as well as its high toxicity, the presence of Cd can be particularly problematic in freshwater ecosystems [1,2]. This metal, even at low nanomolar concentrations, may affect a variety of physiological processes in algae, including growth, photosynthesis, and respiration [3–6]. Furthermore, Cd accumulated in algae might be biomagnified through the food chain [7], which could affect the entire food web. Thus, because of the wide range of potential negative impacts of Cd in nature, knowledge about its toxicity in algae is a priority in the ecological risk assessment of Cd in aquatic ecosystems.

Toxic effects of metals in algae have been traditionally studied in laboratory toxicity assays, in which several physiological and biochemical responses (e.g., cell biomass, growth rate, chlorophyll content, or enzyme activity) are followed over time with conventional instrumentation (e.g., electronic particle counter, fluorometer, spectrophotometer, etc.) [8,9]. Although these common measurement methods of metal toxicity have been major contributors for the derivation of more accurate water quality guidelines and the ecological risk assessments of metals, unraveling the complex mechanisms leading to metal toxicity in algal cells clearly needs the application of new, more powerful, rapid, and automated technologies, such as pulse amplitude modulated fluorometry (PAM) and flow cytometry [10].

The use of PAM fluorometry has allowed a number of studies to look more closely at metal effects on algal photosynthesis. Based on photosynthetic processes, PAM uses chlorophyll fluorescence as a probe to detect changes in photosynthetic efficiency in response to environmental stressors [11]. This technique can be used as a rapid, relatively sensitive and noninvasive probe to detect the photosynthetic toxicity of metals in algae [12]. For example, Juneau et al. [13] observed Cu toxicity on the photosynthesis of 2 freshwater algae, *Chlamydomonas reinhardtii* and *Selenastrum capricornutum*, after 5 h exposure to total Cu concentrations higher than 157 nM (metal speciation undefined). Miao et al. [14] also reported significant Zn, Cd, or Cu toxicity on photosynthetic efficiency of 4 marine phytoplankton species exposed to very low free metal ion concentrations ($<$10 nM).

Both the traditional techniques used in toxicity assays and PAM fluorometry show average toxic effects in algal population but do not provide information concerning individual cells. Contrary to other existing techniques, flow cytometry detects multiple parameters of a single algal cell simultaneously, including scatter signals representative of cell size and internal granular degree. Combined with specific dyes, flow cytometry also can be used to analyze many biochemical processes such as cell viability, enzyme activity, or membrane integrity [15,16]. Even though flow cytometry is frequently used in the biomedical field [17,18], this technology has only been recently applied to algal toxicology studies. Researchers can now study several metal toxicity mechanisms (e.g., enzymes implicated in reactive

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oxygen species detoxification, esterase activity, membrane damaged) more efficiently [19–22] and even in more complex systems containing several algae species [21,23]. The application of flow cytometry in toxicological studies of algae provides a better understanding of metal toxicity pathways and toxic effects, because observations can be made from the population to the cellular level. Although an increase has occurred in the use of flow cytometry as an assessment tool for metal toxicity in the last decade, most of the assessments have been focused on Cu rather than Cd.

In the present study, an algal toxicity test was used to evaluate Cd toxicity on cell biomass. In parallel, PAM fluorometry and flow cytometry technologies were used to investigate the toxicity of Cd to various photosynthetic and cellular processes in *C. vulgaris* and provide a more theoretical basis on which to judge the toxic effects of Cd.

MATERIALS AND METHODS

Culture conditions

Chlorella vulgaris was provided from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences, and cultured in sterile BG11 medium (pH adjusted to 7.1 with HCl) at (25 ± 1) °C under 14 h light at 54 μE/m^2/s followed by 10 h in the dark in a GXZ-280B culture cabinet. The cultures were agitated manually 2 to 3 times per day. The chemical components of BG11 medium are summarized in Supplemental Data, Table S1, and the medium recipe is presented in Supplemental Data, Table S2. The culture medium was sterilized by autoclaving the culture medium with all the constituents listed in Supplemental Data, Table S2, except calcium chloride and ferric ammonium citrate, which were autoclaved separately and aseptically added to the medium after cooling.

Experimental design

Chlorella vulgaris were cultivated in 150-mL flasks in 50 mL BG11 medium and exposed to Cd ($CdCl_2$ $2.5H_2O$, analytical grade, Sinopharm Chemical Reagent) at nominal total concentrations of 1 μM, 5 μM, 10 μM, 50 μM, and 100 μM for 96 h. The measured total Cd concentrations by atomic absorption spectrometry were within 10% of the target nominal total Cd concentrations. Each exposure concentration and blank control (no $CdCl_2$ added) was set up in triplicate. The initial cellular density was 10^5 cells/mL after inoculation ($t = 0$). The cultures were shaken 2 to 3 times daily to allow the algae to be homogeneously distributed in solution and to prevent the algae from sticking to the flask.

Cadmium speciation modeling

The initial concentrations of the free Cd^{2+} ions in the exposure media were computed with the MINEQL+ chemical speciation program (Ver 4.6) [24] with updated equilibrium constants [25], using the total concentrations of each component (Supplemental Data, Table S1), a fixed input pH of 7.1, and a CO_2 partial pressure at equilibrium with the atmosphere ($pCO_2 = 10^{-3.41}$ atm). All calculations take into account the adsorption of Cd on (possible) ferrihydrite at equilibrium, which was modeled using the 2-layer surface complexation model [26] embedded in MINEQL +.

Cell yield inhibition

The cell biomass was estimated every 24 h by measuring the optical density at 450 nm with a microplate reader (Model-680, Bio-Rad). The cell yield relative to control in percentage (P) for each treatment was calculated using the following formula

$$P = 100 \times (N_{t\ treatment} - N_0)/(N_{t\ control} - N_0) \quad (1)$$

where $N_{t\ control}$ and $N_{t\ treatment}$ represent the optical density of the blank control and the treatment, respectively, at $t = 24$ h, 48 h, 72 h, or 96 h. The N_0 was the initial optical density in either the control or the Cd treatment. The median effective Cd concentration (EC50) inhibiting the cell yield after 96 h of exposure was calculated by the nonlinear regression using a 4-parameter curve fitting approach (Equation 2).

$$P = P_{min} + \frac{P_{max} - P_{min}}{1 + ([Cd]/EC50)^{hillslope}} \quad (2)$$

where P is the cell yield relative to control for a given Cd exposure concentration; P_{max} and P_{min} is the modeled maximum and minimum cell yield relative to control; [Cd] is the total Cd or free Cd^{2+} concentration, and hill slope the fitted parameter to the curve. Note that for the 48-h exposures, because of the absence of highly toxic Cd concentrations, the nonlinear regressions did not yield statistically significant EC50 values. In these cases, the 48-h EC50 was estimated using a linear regression within the linear portion of the plot of P as a function of the total Cd or free Cd^{2+} concentration.

Chlorophyll fluorescence analysis

After 96 h exposure, the chlorophyll fluorescence parameters of *C. vulgaris*, previously adapted in the dark for 25 min, were measured using a MAXI-Imaging-PAM fluorometer (WALZ, Germany). The maximal photochemical efficiency of PSII in the dark (Fv/Fm) and the actual photochemical efficiency of PSII in the light (Φ_{PSII}) were selected as indicators of photosynthetic capacity in *C. vulgaris* [11]. The Fv/Fm or [(Fm–F_0)/Fm] was computed using F_0 (the minimal level of fluorescence) and Fm (the maximal level of fluorescence in the dark) [11]. The Φ_{PSII} or [(Fm′–Fs)/Fm′)] was calculated using Fm′ (the maximal level of fluorescence in the light) and Fs (the steady level of fluorescence in the light) [11].

Flow cytometric analysis

After exposure of 48 h and 96 h, the esterase activity, cell viability, autofluorescence (chlorophyll *a* fluorescence), and cell size were analyzed on a FACSCalibur flow cytometer (Becton Dickinson) equipped with an argon laser (excitation at 488 nm).

Fluorescein diacetate (FDA) was selected to assess esterase activity [23,27]. Fluorescein diacetate is not inherently fluorescent, whereas the product formed through esterase-mediated hydrolysis is. Therefore, FDA fluorescence is a measure of metabolic activity, but it is also affected by changes in cell membrane permeability or integrity [10]. Fluorescein diacetate staining was conducted according to the protocol of Yu et al. [23]. More precisely, FDA (dissolved in acetone) was added to algae cultures at a final concentration of 25 μM, and the cells were incubated for 8 min in darkness. The FDA fluorescence emission was then collected in the FL1 channel (515–545 nm). Acetone controls (without FDA) were also performed, and no toxicity was observed.

Propidium iodide was selected as a fluorescence probe to assess algal cell viability [23,27]. Propidium iodide cannot pass through intact cell membrane. Thus, low fluorescence intensity is observed when propidium iodide is incubated with normal

healthy cells. When the algal cell membrane is damaged, propidium iodide enters the cell and stains the nucleic acids, resulting in high fluorescence intensity. Therefore, propidium iodide can be used to differentiate between live and dead cells. Propidium iodide was added to algal cultures at a final concentration of 10 μM, and the cells were incubated for 15 min in darkness. Subsequently, propidium iodide fluorescence emission was collected in the FL2 channel (564–606 nm). Heat-treated cells (100 °C for 10 min) were tested as negative controls, and a right shift of FL2 fluorescence was observed.

Another physiological parameter, cell autofluorescence (chlorophyll *a* fluorescence) can be directly measured as an indicator of chlorophyll *a* content and electron transport inhibition [22,28,29]. Chlorophyll *a* fluorescence emission of unstained cells was collected in the FL3 channel (>650 nm). The samples were also used to analyze cell size, which was determined by forward scatter signal (FSC) and side scatter signal (SSC) [19].

Three samples were prepared for each treatment. A total of 10 000 florescent events were recorded per sample at a low flow rate of 12 μL/min. Forward scatter signal and chlorophyll *a* fluorescence were used to exclude non-algal particles. The data analysis was performed using Summit 5.0 Software. The mean fluorescence intensity (MFI) for each fluorescence channel (FL1, FL2, or FL3) was measured in the control and the Cd-exposed treatments. Control regions of fluorescence were set based on the fluorescence signals obtained with control algal cultures. The cells in the control regions were considered as normal cells. The percentage of normal cells in the control region of FL1, FL2, and FL3 for each Cd-exposed sample was then computed.

Statistical analysis

Because of the low sample size ($n = 3$) and the frequent nonhomogeneity of variances in the data, significant effects of Cd or exposure time on various toxicity endpoints were conservatively evaluated with nonparametric statistical analyses (i.e., tests of Kruskal-Wallis and Mann-Whitney), using the SPSS 16.0 software. Results were considered to be significant at $p < 0.05$. Unless otherwise mentioned, all values are reported as the means ± 1 standard deviation.

RESULTS

Free Cd^{2+} concentration

The modeled initial free Cd^{2+} concentrations were 0.23 μM, 2.4 μM, 5.8 μM, 35 μM, and 72 μM for initial total Cd concentrations of 1 μM, 5 μM, 10 μM, 50 μM, and 100 μM, respectively (Table 1). The proportion of Cd adsorbed on ferrihydrite was minor, between 0.6% and 1.2% of total Cd, and thus Cd adsorption on ferrihydrite had a negligible effect on free Cd^{2+} concentrations. The free Cd^{2+} concentration was controlled by the presence of 2 metal ligands, ethylenediaminetetra-acetic acid (EDTA) and citrate. The initial modeled concentration of Cd $(Citrate)^{-1}$ represented 10% to 12% of the free Cd^{2+} concentrations across all the Cd treatments from 1 μM to 100 μM.

Table 1. Modeled initial free Cd^{2+} concentrations in the BG11 medium calculated with MINEQL+

Total Cd concentration (μM)	1	5	10	50	100
Free Cd^{2+} concentration (μM)	0.23	2.4	5.8	35	72

Because the free Cd^{2+} concentration was buffered by both a low-affinity ligand, citrate, and a strong-affinity ligand, EDTA, the free Cd^{2+} concentration was not very sensitive to changes in total Cd and total EDTA concentration (e.g., pipetting variability) when the total Cd concentration approaches the total EDTA concentration (i.e., when Cd may saturate the EDTA ligand; Supplemental Data, Figure S1).

Cell yield inhibition

After 24 h exposure to Cd concentrations ranging from 1 μM to 100 μM, total Cd (free Cd^{2+} 0.23–72 μM), the optical densities in the Cd-exposed cultures relative to these in the control cultures (i.e., the cell yields), were not significantly affected (Figure 1). After 48 h exposure to 50 μM total Cd (35 μM free Cd^{2+}), the cell yield began to significantly decrease by approximately 46% to 68%, with respect to the control cultures. After 72 h or 96 h of exposure, the cell yield was significantly inhibited at all of the Cd concentrations tested. At Cd concentrations equal to or lower than 5.8 μM free Cd^{2+} (or 10 μM total Cd), the percentage of inhibition on cell yield did not change significantly over the last 48 h of exposure. However, at higher Cd concentration (35 μM and 72 μM free Cd^{2+} equivalent to 50 μM and 100 μM total Cd), the degree of cell yield inhibition progressively increased from 48 h to 96 h of exposure. The calculated 48-h EC50 values as approximated by linear regression were 70.2 μM total Cd or 50.5 μM free Cd^{2+}, whereas the mean 96-h EC50 values (± standard errors [SE]) were 21.3 ± 8.91 μM total Cd or 13.8 ± 6.92 μM free Cd^{2+}.

Photosynthetic toxicity

The mean measured photosynthetic efficiencies for algae exposed for 96 h to various free Cd^{2+} concentrations are pictured in Figure 2A to display the changes in Fv/Fm or Φ_{PSII} as a function of free Cd^{2+} concentrations. The percentages of inhibition of Fv/Fm or Φ_{PSII} at different free Cd^{2+} exposure concentrations relative to control are also presented in the histogram of Figure 2B. The Fv/Fm and Φ_{PSII} normalized to control decreased significantly only for a 96-h exposure to high free Cd^{2+} concentrations (≥35 μM Cd^{2+}). At 35 μM and 72 μM free Cd^{2+}, Fv/Fm relative ratios decreased significantly by 14.6% and 38.9%, respectively, whereas the Φ_{PSII} relative ratios showed a significant 21.0% and 62.1% reduction, respectively. The differences in the relative inhibition of Fv/Fm and Φ_{PSII} indicated that the Φ_{PSII} of *C. vulgaris* was more sensitive to Cd than Fv/Fm.

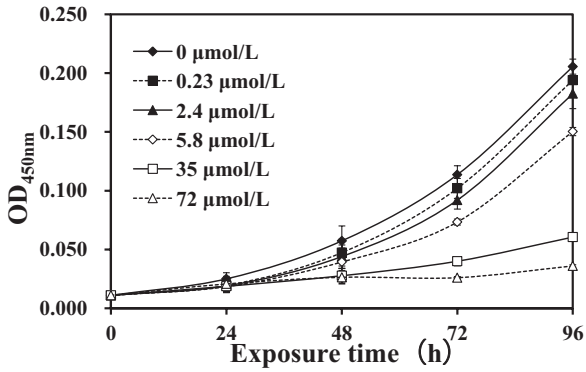

Figure 1. Optical density measured at 450 nm, approximating cell biomass, in *Chlorella vulgaris* cultures exposed for 96 h to various free Cd^{2+} concentrations. The values represent the means ± 1 standard deviation, $n = 3$.

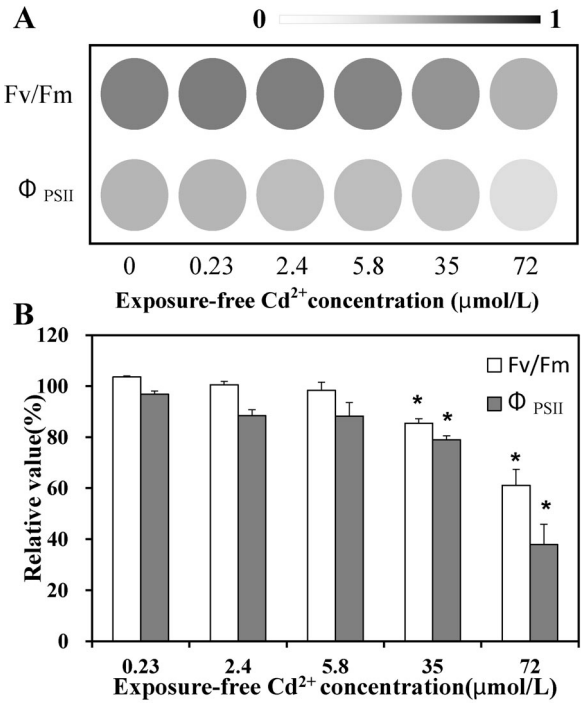

Figure 2. Effects of a 96-h exposure to various free Cd^{2+} concentrations on the maximal photochemical efficiency (Fv/Fm) and the actual photochemical efficiency in the light (Φ_{PSII}). The mean values are represented by (**A**) a black and white color scale or (**B**) a histogram. The values represent the means ± 1 standard deviation, $n = 3$. The asterisks represent a significant difference in photosynthetic parameters ($p < 0.05$) for the Cd-exposed algae compared with control.

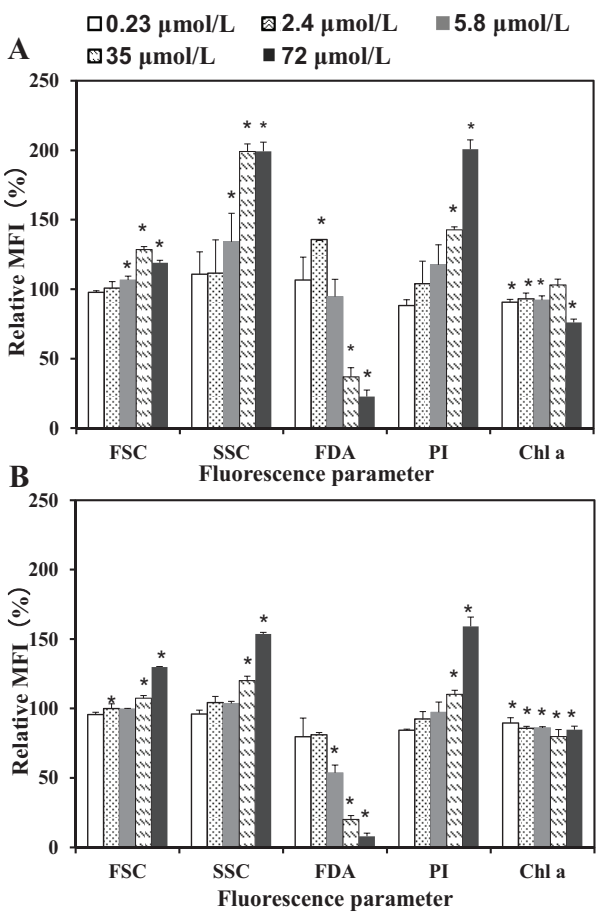

Figure 3. Effects of a (**A**) 48-h or (**B**) 96-h exposure of *Chlorella vulgaris* to various free Cd^{2+} concentrations on the mean fluorescence intensity (MFI) of several fluorescence signals (FSC = forward scatter signal; SSC = side scatter signal; FDA = fluorescein diacetate; PI = propidium iodide; Chl a = Chlorophyll *a*). The values represent the means ± 1 standard deviation, $n = 3$. The asterisks represent a significant difference in MFI ($p < 0.05$) for the Cd-exposed algae compared with the control.

Cellular toxicity

Figure 3 illustrates the MFI of each parameter, measured using flow cytometry, and the results are represented as a relative ratio with the blank control. After a 48-h exposure, *C. vulgaris* cells exposed to 5.8 μM, 35 μM, and 72 μM free Cd^{2+} showed significantly higher FSC-MFIs than those of the blank control, and the effects became more important with increasing Cd^{2+} concentration. After 96 h, only treatments with 35 μM and 72 μM free Cd^{2+} showed higher FSC-MFIs, whereas a slight reduction in the FSC-MFIs were observed under treatment with 0.23 μM free Cd^{2+} compared with the blank control. The differences in the FSC-MFIs reflect differences in cell size; therefore, the increase in FSC fluorescence intensities revealed a larger cell size in Cd-exposed cells of *C. vulgaris*. Moreover, an increase of SSC-MFIs was observed under exposure to increasing Cd^{2+} concentrations for 48 h and 96 h, which also indicated cell size differences.

As shown in Figure 3, 0.23 μM free Cd^{2+} had little effect on the MFI of FDA fluorescence. As the free Cd^{2+} concentration increased to 2.4 μM, an increase in the FDA fluorescence was observed after a 48-h exposure, but the signal returned to the baseline intensity after 96 h. As the free Cd^{2+} concentration and exposure time increased, the esterase activity of *C. vulgaris* sharply declined. The MFIs were only 8% of the blank controls for the algae exposed to 72 μM free Cd^{2+} during 96 h.

The MFIs of propidium iodide fluorescence for the cells exposed to 35 μM and 72 μM free Cd^{2+} were significantly higher than the controls, indicating that a high free Cd^{2+} concentration resulted in cell membrane damage. However, the effect became weaker as the exposure time increased. Because the fluorescence intensity of chlorophyll *a* is an indicator of the chlorophyll *a* content and electron transport inhibition [19,20], the significant inhibition relative to control in the chlorophyll *a* MFIs at all free Cd^{2+} concentrations (except at 35 μM Cd^{2+} for 48 h) indicates that Cd exposure also affects chlorophyll *a* content or photosynthetic electron transport.

Esterase activity. In Figure 4A, the number of cells exposed to different free Cd^{2+} concentrations during 96 h is plotted against the FDA fluorescence intensities collected with the FL1 fluorescence channel. A 96-h exposure to 35 μM or 72 μM free Cd^{2+} caused a left shift in the FL1 fluorescence relative to control (Figure 4A). Increasing the free Cd^{2+} concentration reduced the number of cells exhibiting normal esterase activity. The percentages of cells with normal esterase activity status were then calculated after setting the FDA control region based on the FL1 fluorescence of the cells from the blank control (Figure 4A). As shown in Figure 5A, after 48 h and 96 h of exposure, the percentage of cells in the FDA control region was similar to the blank control when the free Cd^{2+} concentration was below or equal to 5.8 μM, but the percentage rapidly decreased when the free Cd^{2+} concentration reached 35 μM. When the free Cd^{2+} exposure concentration attained 72 μM, the inhibition was even greater, reducing the FDA fluorescence signal in the control region from 40.5% after 48 h to 23.2% after 96 h.

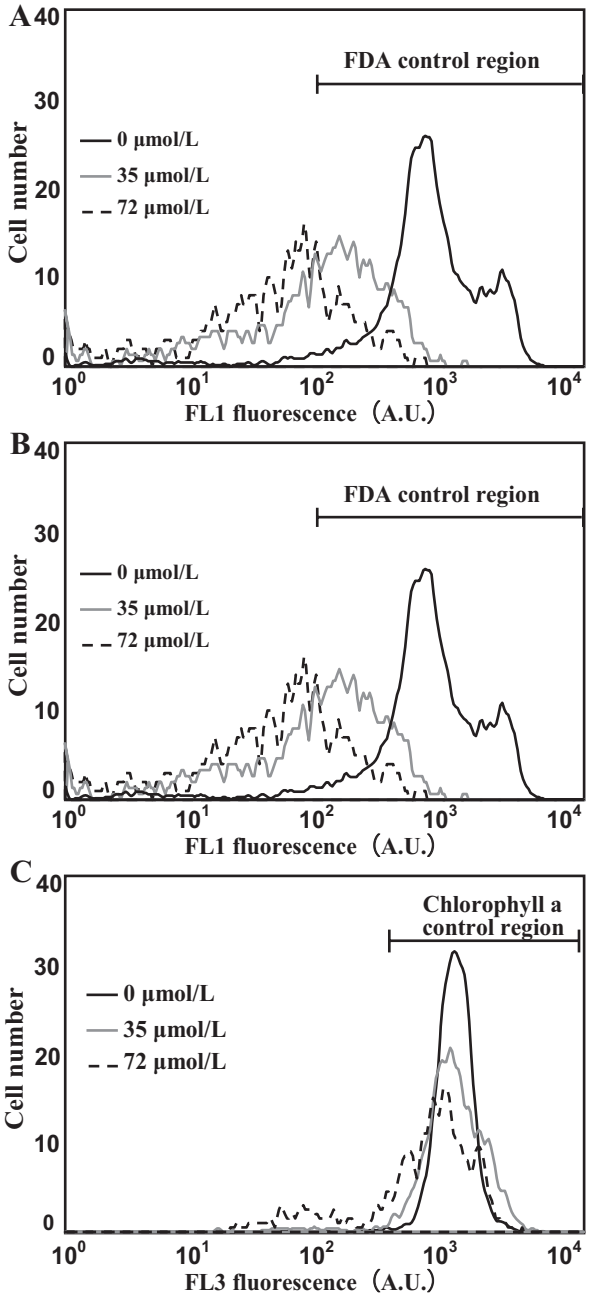

Figure 4. Plot of the cell number as a function of the fluorescence signal harvested in the (**A**) FL1 channel (FDA fluorescence), (**B**) FL2 channel (propidium iodide fluorescence), or (**C**) FL3 channel (chlorophyll *a* fluorescence). The control fluorescence region of each channel is shown on the plots. The *Chlorella vulgaris* cells were exposed to free Cd^{2+} concentrations of 0 μM, 35 μM, and 72 μM for 96 h.

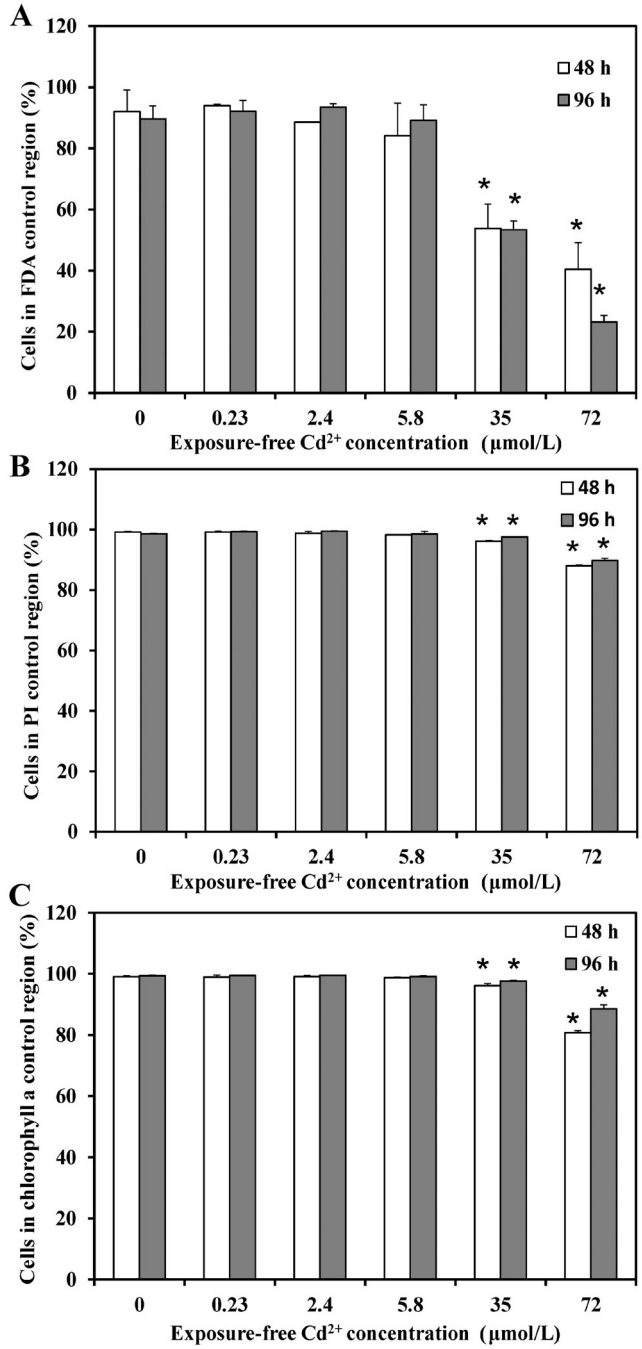

Figure 5. Percentage of algal cells in the (**A**) fluorescein diacetate or FDA, (**B**) propidium iodide or PI, and (**C**) chlorophyll *a* control regions at different free Cd^{2+} exposure concentrations. Panels **(A)**, **(B)**, and **(C)** show esterase activity, PI, and chlorophyll *a* fluorescence, respectively. The values represent the means ± 1 standard deviation, $n = 3$. The asterisks represent a significant difference ($p < 0.05$) in toxicity endpoints for Cd-exposed algae compared with the blank control.

Cell viability. Figure 4B shows the number of cells exposed to different Cd^{2+} concentrations for 96 h as a function of the fluorescence intensities collected in the FL2 channel. Exposure to 35 μM or 72 μM free Cd^{2+} caused a right shift in FL2 fluorescence relative to blank control (Figure 4B). These result were similar to those obtained for the heat-treated cells, showing a significant right shift in the FL2 fluorescence (data not shown), thus suggesting that Cd exposure leads to cell membrane damage and cell apoptosis. The percentages of live cells were calculated after setting the propidium iodide control region based on the FL2 fluorescence of the cells from the blank control (Figure 4B). As shown in Figure 5B, an increase in the free Cd^{2+} concentration to 35 μM free Cd^{2+} resulted in a significant reduction in the number of live cells after 48 h or 96 h of exposure, and the percentage of live cells was significantly further reduced when the free Cd^{2+} concentration exceeded 35 μM.

Chlorophyll a fluorescence. Figure 4C shows the number of cells exposed to different free Cd^{2+} concentrations for 96 h as a function of the chlorophyll *a* fluorescence collected with the FL3 fluorescence channel. A 96-h exposure to 35 μM and 72 μM free

Cd^{2+} caused a left shift in FL3 fluorescence relative to control (Figure 4C), indicating that the chlorophyll *a* content or the photosynthetic electron transport was inhibited in some algal cells. The percentages of cells with normal chlorophyll *a* signal were calculated after setting chlorophyll *a* control region based on the FL3 fluorescence of the cells from the blank control (Figure 4C). As shown in Figure 5C, concentrations of 35 μM and 72 μM free Cd^{2+} decreased significantly the percentages of cells in the control region of FL3 fluorescence, and this effect tended to be weaker at 96 h than at 48 h of exposure.

DISCUSSION

Cadmium speciation and bioavailability

Several laboratory experiments performed using a range of aquatic organisms from unicellular algae to fishes have shown that metal uptake and (acute) toxicity depends very strongly on metal speciation and is usually proportional to the concentration of the free ion metal concentrations [30,31] although some exceptions exist [32]. In chronic long-term metal exposure, this relationship is further complicated by the presence of cellular exudates and physiological regulation of metal uptake, even though the free metal ion does play an important role in controlling metal toxicity even in these conditions [33,34]. Throughout the present study, we thus decided to focus on the modeled initial free Cd^{2+} concentration for the interpretation of the toxicity endpoints.

Cadmium toxicity on cell yield

The cell yield was by far the most sensitive endpoint tested in the present study. We obtained a significant reduction in cell yield of $20 \pm 8\%$ after 72 h and 96 h of exposure to 230 nM free Cd^{2+} (1 μM total Cd), whereas Cd concentrations equal to or higher than 35 μM free Cd^{2+} (50 μM total Cd) were needed to significantly inhibit all of the other cellular processes after 48 h or 96 h.

The 96-h EC50 on the cell yield of *C. vulgaris* (21.3 ± 8.91 μM total Cd or 13.8 ± 6.92 [SE] μM free Cd^{2+}) was comparable to the mean 96-h EC50 of 9.1 μM total Cd and 4.7 μM (speciation undefined) on the cell yield of *C. vulgaris* reported by Lam et al. [35] and Qian et al. [3], respectively. These EC50s are, however, considerably higher than the EC50s calculated in other studies using different green algae species, but the same metal (Cd). Lavoie et al. [36] had shown that the mean EC50s on the cell yield of *Chlamydomonas reinhardtii* (72-h EC50) and *Pseudokirchneriella subcapitata* (96-h EC50) were 273 and 127 nM initial free Cd^{2+}. Furthermore, the data of Le Faucheur et al. [37] indicate that the 72-h EC50 on the cell yield of another green alga, *Scenedesmus vacuolatus*, is lower than 100 nM initial free Cd^{2+}. The much higher EC50 reported here for *C. vulgaris* compared with EC50s measured in other green algae could indicate that *C. vulgaris* is more tolerant to Cd than other closely related species or might be linked to differences in experimental conditions among studies. For example, the studies of Lavoie et al [36] and Le Faucheur et al. [37] have used culture media with relatively low concentrations of competing essential metals (e.g., Cu, Zn, Co, Mn) and at a well-buffered pH of 7 [36] or 7.45 [37] throughout the long-term exposure, whereas the present study and other reports of Lam et al. [35] and Qian et al. [3] used culture media with relatively high trace metal concentrations and no pH buffer. Thus, one possibility would be that high trace metal concentrations as well as unbuffered pH during Cd toxicity assays have contributed to decrease Cd toxicity (and increase EC50) in the present study as well as in the studies of Lam et al [35] and Qian et al [3].

Cadmium toxicity on photosynthetic and cellular processes

Both Fv/Fm and Φ_{PSII} were significantly reduced only after 48 h or 96 h of exposure to free Cd^{2+} concentrations equal to or higher than 35 μM, indicating that Cd may significantly affect photosynthesis of *C. vulgaris* only at very high free Cd^{2+} concentrations, unlikely to be found even in the most metal-contaminated environments [38–40].

Similarly, the toxicity of Cd on cellular processes (i.e., esterase activity, phospholipidic membrane or cell viability, chlorophyll *a* fluorescence) was only observed when the free Cd^{2+} exposure concentration was equal to or higher than 35 μM. The cellular toxicity of Cd reduced the number of cells in control regions, emitting fluorescence in the FL1, FL2, or FL3 fluorescence channels (cells in normal status), and the percentage of normal cells was negatively correlated with the Cd exposure concentration, suggesting that the toxic effects of Cd were not apparent in all cells. Yu et al. [41] showed similar results regarding Cu toxicity to esterase activity, but in the cyanobacteria *Microcystis aeruginosa*.

Cadmium toxicity over time

Cadmium toxicity on cell yield, esterase activity, cell viability, and chlorophyll *a* fluorescence after 48-h and 96-h exposure periods were statistically compared, and the results are summarized in Table 2. No significant differences in Cd toxicity were found on all studied cell parameters between 48 h and 96 h of exposure to free Cd^{2+} concentrations lower than or equal to 5.8 μM. However, a significant reduction in Cd toxicity over time (between 48 h and 96 h) on cell viability or chlorophyll *a* fluorescence was observed at 35 μM and 72 μM free Cd^{2+}, whereas a significant increase in Cd toxicity on cell yield and esterase activity was observed at 72 μM free Cd^{2+}.

The reduction over time in Cd toxicity on cell viability and chlorophyll *a* fluorescence could be explained by various hypothetical mechanisms. One possible cause would be a decrease in Cd uptake fluxes over time because of depletion in the total dissolved Cd concentration, which would be caused by intense Cd bioaccumulation in algae. Other cellular resistance mechanisms could also be induced slowly during Cd exposure and hence could contribute to protecting the cells from Cd toxicity.

Contrary to the decreased Cd toxicity on cell viability and chlorophyll *a* fluorescence over the last 48 h of exposure, Cd toxicity on esterase activity increased significantly in the same period. The contrasting apparent modulation of Cd toxicity over

Table 2. Variations in Cd toxicity over time[a]

	Exposure-free Cd^{2+} concentration (μM)				
Toxicity endpoints	0.23	2.4	5.8	35	72
Cell yield	0[b]	0	0	0	+[c]
Esterase activity	0	0	0	0	+
Cell viability	0	0	0	–[d]	–
Chlorophyll *a* fluorescence	0	0	0	–	–

[a]Comparison of the responses of various toxicity endpoints relative to control after 96 h exposure with respect to that obtained after 48 h of exposure.
[b]No significant difference.
[c]Significant increase in Cd toxicity over time.
[d]Significant decrease in Cd toxicity over time.

time on different cell parameters might be explained by a possible underestimation of Cd toxicity on esterase activity after a 48-h exposure period using the FDA technique. Because FDA uptake is a function of both the esterase activity and cell membrane permeability, the percentage of normal cells exhibiting a normal esterase activity might be overestimated after 48 h Cd exposure because of a hypothetical increase in membrane permeability to FDA, which partially compensates for an eventual decrease in esterase activity (caused by Cd toxicity) after 48 h. Franklin et al. [10] also reported a transitory increase in FDA fluorescence of *Pseudokirchneriella subcapitata* exposed to low Cu concentrations (<200 nM total Cu) during 24 h or less.

Contrary to the reduction over time in Cd toxicity on chlorophyll *a* fluorescence and cell viability, the inhibitory effect of Cd on cell yield apparently increased in the last 48 h exposure to high Cd concentrations. This increase in Cd toxicity over time is partially attributable to the exponential growth of algae. Indeed, because the algae are growing exponentially in control conditions, one should expect that, for a rapid and constant inhibition of Cd on growth rate, the apparent Cd toxicity on cell yield increases over time. However, the data also show that no Cd concentrations were high enough to significantly decrease the cell yield measured after 24 h. At 35 μM and 72 μM free Cd^{2+} (50 μM and 100 μM total Cd), the growth rate slowly decreased up to the point at which no significant cell growth occurred during the last 48 h exposure to 72 μM free Cd^{2+}. These results demonstrate the slow kinetics of Cd toxicity in *C. vulgaris* and bring new knowledge on Cd toxicity processes in algae.

Comparisons of toxicity endpoints

Cell yield and photosynthesis. The cell yield was already inhibited after 72 h exposure to the lowest free Cd^{2+} concentration used (0.23 μM Cd^{2+}), whereas photosynthesis began to be significantly reduced only at free Cd^{2+} concentrations equal to or higher than 35 μM. This large difference in sensitivity between these 2 toxicity endpoints strongly suggests that Cd may affect cell division processes before negatively affecting photosynthesis, which generates energy for algal growth. Our results support the current paradigm stating that cell division processes are more sensitive than photosynthesis processes for assessing long-term sublethal metal toxicity in algae [5,37,42–44] although Miao et al. [14] also reported that photosynthetic efficiency may be as sensitive as growth rate in evaluating Cd, Cu, or Pb toxicity in 4 marine microalgae.

Cell viability and cell yield. The slight 25% inhibition of cell yield after 72 h exposure to 0.23 μM free Cd^{2+} (1 μM total Cd) was not associated with any sign of cell death based on the propidium iodide assay, indicating, as expected for weak sublethal Cd toxicity, that cell apoptosis or death is not a quantitatively significant process leading to cell yield inhibition. More surprisingly, the results also show that after 48 h or 96 h of exposure to the highest Cd concentration tested (72 μM free Cd^{2+} or 100 μM total Cd), only approximately 10% of the algal cells were declared dead based on the propidium iodide fluorescence assay, even though no significant cell growth occurred during the last 48 h exposure at 100 μM total Cd or 72 μM free Cd^{2+}. This reasoning implies that Cd firstly affect cell division processes even at very high algistatic Cd concentrations and that at very high Cd concentrations totally inhibiting cell growth, most cells are able to survive for at least 96 h. This finding is consistent with the strong tendency of Cd to inhibit DNA synthesis and blocked cell division [45], but also raised a fascinating question: How does *C. vulgaris* escape cell death at such high Cd concentrations?

Chlorophyll a fluorescence and Φ_{PSII}. A proportional relationship exists between electron transport rate ($J = \Phi_{PSII} \times$ [absorbed light intensity] × [0.5]) and Φ_{PSII} [46]. The results from MAXI-Imaging-PAM demonstrated that the Φ_{PSII} was reduced by 62.1% after 96 h exposure to 72 μM free Cd^{2+} compared with the blank control, indicating that the electron transport rate of PSII was inhibited. The results obtained by flow cytometry showed that approximately 10% of the cells exhibited reduced chlorophyll *a* fluorescence under the same conditions. Although the electron transport was strongly reduced in the cells with reduced chlorophyll *a* fluorescence, the inhibition of the average transport rate did not reach 62.1%. Thus, cells showing normal fluorescence status might also suffer from the inhibition of electron transport. Samson et al. [29] showed that the inhibition of the electron acceptor increases fluorescence, whereas the inhibition of the electron donor reduces fluorescence. Our results thus suggest that the influence of Cd on electron transport affects both the electron acceptor and donor, providing an offset effect on chlorophyll *a* fluorescence.

Cell size and resistance to Cd toxicity. After a 48-h exposure at the highest free Cd^{2+} concentration (72 μM), propidium iodide fluorescence was used to identify normal and nonviable cells. The cells were divided into 8 groups based on FSC fluorescence (rank from 0 to 256 a.u.). Cells with FSC fluorescence of 105 a.u. or less were divided into 7 equal intervals, and the remaining cells were included in the eighth group. The percentage of normal cells and the mean value of FSC fluorescence in each group were calculated (Figure 6).

Our results demonstrate that the proportion of normal cells significantly varies as a function of cell size and motivate the study of Cd toxicity in individual cells. If the first group contains the smallest cells and the eighth group the biggest (from left to right on Figure 6), then the cells of very small (first group) and intermediate (fourth, fifth, sixth, and seventh groups) size had a higher Cd tolerance, but cells of small (second and third groups) or large (eighth group) size were more easily affected by Cd. The small sensitive cells might be relatively young cells with physiological functions that are not completely developed, resulting in reduced resistance to Cd toxicity. The very small resistant cells might be very young algal cells derived from cell division before analyses, which were not affected with Cd treatment, resulting in a high percentage of normal cells. In contrast, the largest algal cells might be composed of relatively old cells with more potential membrane defects than younger

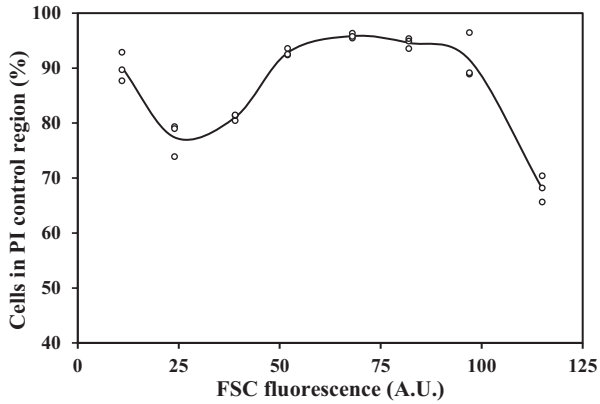

Figure 6. Percentage of cells in the control region of PI fluorescence at various signals of forward scatter signal (FSC) fluorescence. The cells were exposed to 72 μM free Cd^{2+} for 48 h.

cells. We also hypothesize that these large sensitive cells might have a slower metabolism and turnover rates of damaged biomolecules caused by Cd toxicity.

CONCLUSIONS

The toxic effects of Cd in *C. vulgaris* included the inhibition of cell growth, photochemical efficiency, chlorophyll *a* fluorescence, esterase activity, and cell viability. Here we show that the rate at which these toxic effects were induced by Cd depends on the given toxicity endpoints; both a recovery and an increase in Cd toxicity over time were demonstrated. Cadmium toxicity on cell yield and cell viability shows that cell apoptosis or cell death only accounted for a minor part of the reduction in cell yield even at very high algistatic Cd concentrations and that other mechanisms such as blocked cell divisions also contributed to cell yield inhibition.

Of all the toxicity endpoints measured in the present study, the inhibition of cell yield was by far the most sensitive, with a 96-h EC50 of 21.3 ± 8.9 (SE) μM total Cd or 13.8 ± 6.9 (SE) μM free Cd^{2+}, whereas the estimated 96-h EC50 values for other endpoints were close to or higher than 100 μM total Cd or 72 μM free Cd^{2+}. The sensitivity sequence of the toxicity endpoints was: cell yield $>>$ $\Phi_{PSII} \approx$ esterase activity $>$ Fv/Fm $>$ chlorophyll *a* fluorescence $\approx$ cell viability. The present study brings new insights into the toxicity mechanisms of Cd and the sensitivity to Cd of various cellular processes in a green alga.

SUPPLEMENTAL DATA

Figure S1.
Tables S1–S2. (282 KB DOC).

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