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# Cadmium sensitivity, uptake, subcellular distribution and thiol induction in a marine diatom: Exposure to cadmium

Meng-Jiao Wang, Wen-Xiong Wang*

*State Key Laboratory in Marine Pollution, Section of Marine Ecology and Biotechnology, Division of Life Science, The Hong Kong University of Science and Technology (HKUST), Clear Water Bay, Kowloon, Hong Kong*

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ABSTRACT

The aims of this study were to (1) evaluate the changes in the Cd tolerance of a marine diatom after exposure under different Cd concentrations for various durations and (2) to explore the potential subcellular and biochemical mechanisms underlying these changes. The 72-h toxicity, short-term Cd uptake, subcellular Cd distribution, as well as the synthesis of phytochelatins (PCs) were measured in a marine diatom *Thalassiosira nordenskioeldii* after exposure to a range of free Cd ion concentrations ($[Cd^{2+}]$, 0.01–84 nM) for 1–15 days. Surprisingly, the diatoms did not acquire higher resistance to Cd after exposure; instead their sensitivity to Cd increased with a higher exposed $[Cd^{2+}]$ and a longer exposure period. The underlying mechanisms could be traced to the responses of Cd cellular accumulation and the intrinsic detoxification ability of the preconditioned diatoms. Generally, exposure to a higher $[Cd^{2+}]$ and for a longer period increased the Cd uptake rate, cellular accumulation, as well as the Cd concentration in metal-sensitive fraction (MSF) in these diatoms. In contrast, although PCs were induced by the environmental Cd stress (with $PC_2$ being the most affected), the increased intracellular Cd to PC-SH ratio implied that the PCs' detoxification ability had reduced after Cd exposure. All these responses resulted in an elevated Cd sensitivity as exposed $[Cd^{2+}]$ and duration increased. This study shows that the physiological/biochemical and kinetic responses of phytoplankton upon metal exposure deserve further investigation.



## 1. Introduction

Metal pollution with increasing anthropogenic inputs into aquatic environments has become a major environmental concern. Variability in the responses of aquatic organisms to metal stress has been documented for different species, and the underlying mechanisms are likely species dependent (Newman and McIntosh, 1991). Some species may develop specific and efficient strategies to cope with such stress. It has been well established that organisms (including phytoplankton) originating from metal contaminated water frequently show enhanced tolerance (Twiss, 1990; Klerks and Weis, 1987). Correspondingly, laboratory studies have also shown that some freshwater species that have been acclimated to metals become more tolerant than the non-acclimated ones (Soldo and Behra, 2000; Muyssen and Janssen, 2001; Zeng et al., 2009). Such type of response, which confers an advantage on the organism, has either a physiological or genetic basis (or both). The underlying mechanisms are hypothesized to include (but are not limited to) changes in metal uptake or excretion rate (Wang and Rainbow, 2005), ability to store or sequester the metal from the sensitive receptor (Wikfors et al., 1991), extracellular ligands production to sequester metals (Russel and Morris, 1970), and differences in enzyme sensitivity to metal inhibition (Newman and McIntosh, 1991).

On the other hand, the acquisition of tolerance after metal exposure is not a necessary consequence for other species (Muyssen and Janssen, 2005; Johnson et al., 2007). Indeed, Newman and McIntosh (1991) have interestingly argued that the evolution of tolerance was an exception since most species were excluded from the contaminated sites. Metal pollution often leads to high mortality and intense selection. If no tolerance can be obtained after exposure, or alternatively the organisms become even more sensitive, there must be a corresponding physiological basis underlying such responses. However, an increase in sensitivity after metal exposure has been less well reported in the previous studies.

Studies in the responses of marine phytoplankton to metal exposure and the underlying mechanisms have critical implications for marine ecological restoration and the assessment thereof. Among the many possible physiological effects, the induction of phytochelatins (PCs) to store metals, especially Cd, in a biologically inactive form is considered among the most important (Wright and Welbourn, 2002). PCs, a family of cysteine-rich peptides with the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n = 2–11$) (Grill et al., 1985) and $PC_{2-4}$ being the predominant structures in phytoplankton (Ahner

* Corresponding author.
*E-mail address:* wwang@ust.hk (W.-X. Wang).

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et al., 1995), are able to chelate metals through their thiol groups, and play an important role in metal detoxification and tolerance in plants (Vatamaniuk et al., 1999). Enhanced metal tolerance was achieved by induction of PCs through the introduction and/or overexpression of genes linked with the biosynthesis of PCs (Mejáre and Bülow, 2001; Tsuji et al., 2002; Kobayashi et al., 2005). Earlier, we have demonstrated that the marine diatom *Thalassiosira nordenskioeldii* responded to higher environmental [$Cd^{2+}$] with elevated PCs synthesis (Wang and Wang, 2008a). However, for this diatom, the induction of PCs under longer Cd exposure have not been vigorously tested, nor its role in the development of Cd tolerance.

On the other hand, we have previously shown that the subcellular distribution of Cd in this diatom could explain the Cd toxicity quantified under different irradiance and temperature conditions (Wang and Wang, 2008a,b). Additionally, earlier studies have suggested that metal uptake kinetics responded to metal exposure (Wang and Rainbow, 2005; Guan and Wang, 2006), and may be important in determining the intracellular metal concentrations as well as the resulting metal tolerance. Nevertheless, our understanding of the relationship among metal uptake, subcellular distribution, thiol production and the development of metal tolerance after exposure to metals in marine phytoplankton is rather limited. Therefore, this study was specifically designed to investigate the responses of the marine diatom *T. nordenskioeldii* to Cd exposure under different [$Cd^{2+}$] and durations. We probed any changes in Cd sensitivity based on Cd uptake kinetics, Cd cellular accumulation, Cd subcellular distribution as well as thiol induction.

## 2. Materials and methods

### 2.1. Diatom culture and experimental medium

Marine diatoms *T. nordenskioeldii* were isolated from the surface water of Port Shelter, eastern Hong Kong, and the diatom stock was maintained in the f/2 medium at 23.5 °C with a light intensity of 170 μmol photons $m^{-2}$ $s^{-1}$ under a 14:10 light:dark (LD) cycle. To better control the metal speciation, the Aquil artificial seawater (ASW, Price et al., 1988) was adopted for the exposure, uptake and toxicity experiments. The 0.22 μm filtered ASW was first passed through the chelex-100 ion-exchange resin before nutrients and other chemicals were added. The exposure medium was enriched with bacteria-free N, P, vitamins and Si at f/2 levels, and trace metals at the f/10 levels (free of EDTA to avoid its influence on the following uptake experiment). The medium for the toxicity experiment was similar, with the only difference being the addition of EDTA at the f/10 level with the trace-metal stock. No nutrient or EDTA was added into the uptake medium, to ensure that the radioactivity of the experimental samples was sufficiently high to be detected by γ detector. The pH of medium was kept at 8.2 ± 0.1 with suprapure NaOH. Cd speciation was adjusted by nitrilotriacetate (NTA, 0.05 mmol $L^{-1}$ for the uptake medium and 0.1 mmol $L^{-1}$ for the other medium) and calculated with the MINEQL+ software (version 4.5, Environmental Research Software, Hallowell, ME, USA). MINEQL+ is based on the Davis equation, which is applicable when the ionic strength is no more than 0.5 M (Stumm and Morgan, 1996), while Aquil's artificial seawater has an ionic strength of 0.7 M (Price et al., 1988). Although the activity coefficient goes down by roughly 0.02 with NaCl solution, it does not change too much between 0.5 M and 0.7 M ionic strength based on the Davis equation (Morel and Hering, 1993). Thus the calculated [$Cd^{2+}$] may only be slightly overestimated. Because our main objective was to generate a gradient of [$Cd^{2+}$], such a small deviation in the calculated [$Cd^{2+}$] should not affect the overall conclusion of our experimental results.

$^{109}Cd$ was added as a radioactive tracer when necessary (see below). All polycarbonate flasks and beakers were rinsed with Milli-Q water (18.2 MΩ cm) after being soaked in 10% HCl for at least 24 h.

### 2.2. Diatom exposure

In the exposure experiment, diatoms in the mid-exponential growth phase were collected by centrifugation, rinsed with the Cd-free medium, and resuspended in exposure medium at three different [$Cd^{2+}$] (Control-Cd: $1.0 \times 10^{-11}$ mol $L^{-1}$, Low-Cd: $1.3 \times 10^{-8}$ mol $L^{-1}$, High-Cd: $8.4 \times 10^{-8}$ mol $L^{-1}$). The spiked Cd concentrations were nominal, and any potential Cd contamination in the artificial seawater was removed by the chelex-100 ion-exchange resin. Two replicated bottles were spiked with stable Cd only (used for uptake, toxicity and PC measurements), and another two replicated bottles were spiked with both stable Cd and radiotracer $^{109}Cd$ (14.1–370 kBq $L^{-1}$, in 0.1 mol $L^{-1}$ HCl, used for Cd accumulation and subcellular distribution measurements). The exposure lasted for 1, 7 and 15 days in order to quantify the influences of Cd exposure on diatom tolerance and kinetics. To ensure a constant exposure and to keep the diatom in the exponential growth phase (cell density was counted every 24 h by the Coulter Particle Counter to ensure exponential growth of the cells), the cells were harvested and resuspended in a freshly prepared exposure medium every 2 or 3 days, with an initial cell density of $1–7 \times 10^4$ cells $mL^{-1}$ for each renewal.

The temperature and irradiance during the exposure period were the same as the culture conditions. Cell density was recorded every 24 h to calculate the average growth rate. At the end of each exposure period, the cells were collected for the experiments described below.

### 2.3. Uptake, toxicity, and Cd accumulation

A 1-h short-term uptake experiment was conducted on the exposed diatoms. The tested [$Cd^{2+}$] for the uptake experiment were $1.2 \times 10^{-10}$, $2.1 \times 10^{-9}$, $2.1 \times 10^{-8}$ and $2.1 \times 10^{-7}$ mol $L^{-1}$. The medium was spiked with $^{109}Cd$ (14.1–2590 kBq $L^{-1}$) and equilibrated for 24 h before the experiment. At the end of each exposure period, the diatoms were collected by centrifugation, rinsed and resuspended into the uptake medium with an initial cell density of around $3 \times 10^5$ cells $mL^{-1}$. At 15, 30, 45 and 60 min, a 10 mL aliquot was gently filtered (<100 mm Hg) onto a 1 μm polycarbonate membrane, washed with 0.22 μm filtered seawater for 3 times and 10 mL diethylenetriaminepentaacetic acid (DTPA, 2 mmol $L^{-1}$) solution for 5 min (Lee et al., 1995). Afterwards, the membranes with cells were measured with a Wallac γ detector for the radioactivity of the intracellular Cd. Cd uptake rate was determined from the slope of the linear regression between intracellular Cd accumulation and time (h).

Simultaneously with the uptake experiment, a 72-h toxicity test was conducted at six different [$Cd^{2+}$] ($2.2 \times 10^{-11}$, $5.4 \times 10^{-8}$, $1.6 \times 10^{-7}$, $4.2 \times 10^{-7}$, $1.1 \times 10^{-6}$ and $2.8 \times 10^{-6}$ mol $L^{-1}$). The corresponding total dissolved Cd concentration for each treatment was $2.0 \times 10^{-9}$, $3.6 \times 10^{-6}$, $1.0 \times 10^{-5}$, $2.5 \times 10^{-5}$, $6.0 \times 10^{-5}$ and $1.4 \times 10^{-4}$ mol $L^{-1}$, respectively. The cell density of the diatoms was recorded every 24 h with the Coulter Particle Counter. The slope of the linear regression of the natural logarithmically transformed cell density versus time was calculated as the average growth rate, which was used to calculate the median inhibition concentration ($IC_{50}$) to compare the Cd sensitivity.

### 2.4. Subcellular Cd distribution and phytochelatin analyses

At the end of each exposure period, a 200 mL aliquot from each radioactive replicate was gently filtered onto a 1 μm polycarbonate membrane, rinsed three times with 0.22 μm filtered natural seawa-

ter, and resuspended in 10 mL of 8-hydroxyquinoline-5-sulfonic acid hydrate (HQS, 1 mmol $L^{-1}$ in 0.22 μm filtered seawater) for 10 min to remove any loosely surface-adsorbed Cd (Ettajani et al., 2001; Price and Morel, 1990). Afterwards, the cells were collected by centrifugation, rinsed by resuspending in 3 mL Tris-buffer (0.02 mol $L^{-1}$, with 0.15 mol $L^{-1}$ NaCl, pH = 8), followed by another round of centrifugation and resuspension in Tris-buffer, while 1 mL of the HQS supernatant was measured for radioactivity as surface-adsorbed Cd. The diatom cells were broken with the Constant Cell Disruption System (Constant Systems Limited, UK) before the differential centrifugation, with a breaking efficiency of over 97%. The broken cells were first centrifuged at 4000 × *g* (4 °C) for 15 min to separate the pellet (P1) and supernatant (S1). The S1 was further ultracentrifuged (100,000 × *g*, 1 h, 4 °C) to separate the organelles (pellet) and the proteins (supernatant, S2). After treatment in a 80 °C water bath for 10 min, tubes of S2 were then kept in ice for another 60 min before centrifugation at 50,000 × *g* for 10 min (4 °C), which further separated the S2 into the heat stable protein (HSP, supernatant) and heat denatured protein (HDP, pellet). Meanwhile, P1 was water bathed at 100 °C for 2 min, followed by a NaOH (0.5 mol $L^{-1}$) digestion at 70 °C for 1 h. Afterwards, it was centrifuged at 10,000 × *g* for 10 min to separate the cellular debris (supernatant) and the metal-rich granules (MRG, pellet) (Lavoie et al., 2009a,b; Wang and Wang, 2009). The radioactivity of each fraction was measured with the Wallac γ detector. The recovery rate, which was determined by comparing the sum of the radioactivity of all five fractions (cellular debris, MRG, organelles, HSP and HDP) with the total radioactivity measured before cell breakage, was greater than 85%.

A 200 mL aliquot from each stable replicate was filtered onto a pre-combusted GF/F membrane, rinsed three times with 0.22 μm filtered seawater, and stored in liquid nitrogen until the analysis of PCs and related peptides. The analysis followed the procedures described in previous studies (Wei et al., 2003; Wang and Wang, 2009), which was based on earlier studies showing that PCs are heat stable (Wagner, 1984; Price and Morel, 1990). Briefly, the frozen cells were transferred into 2 mL of methanesulfonic acid (MSA, 10 mmol $L^{-1}$, 70 °C) for enzyme denaturation before being homogenized with the Constant Cell Disruption System. Afterwards, the mixture of broken cells was centrifuged (13,800 × *g*, 10 min, 4 °C) to separate the supernatant for pre-column derivatization. After pH adjustment with borate buffer (100 mmol $L^{-1}$, with 10 mmol $L^{-1}$ diethylenetriaminepentaacetic acid) and reaction with dithiothreitol (DTT, 15 mmol $L^{-1}$), fluorescence tag monobromobimane was added to the extract for cysteine and peptides labeling. The excess monobromobimane was further reacted with DTT, after which the whole mixture was stabilized with MSA (1 mol $L^{-1}$) and injected into HPLC for cysteine and peptides (γ-EC, GSH and $PC_{2-4}$) analysis. The Discovery RP Amide C16 reverse phase column and the chemical standards of $PC_{2-4}$ were purchased from Supelco and AnaSpec, respectively. A gradient elution mode with 95% acetonitrile and $KH_2PO_4$ (25 mmol $L^{-1}$, pH = 3.2) was adopted.

Simultaneously, a 10 mL aliquot from each stable replicate was gently filtered onto a pre-combusted (450 °C, 5 h) GF/F membrane, rinsed with 0.22 μm filtered seawater, and measured with a CHNS/O analyzer 2400 (Series II, Perkin Elmer Instrument) for particulate organic carbon (POC) analysis.

### 2.5. $IC_{50}$ calculation and data analysis

The ICPIN software (version 2.0, USEPA, Duluth, MN, using the linear interpolation method) was adopted to calculate the median inhibition concentration ($IC_{50}$) during either the exposure or 72-h toxicity experiment. If the *p* value was lower than 0.05 by one-way ANOVA with the Tukey post hoc multiple comparisons (SPSS 11.0), a significant difference was accepted. Error propagation calculation was adopted where applicable.

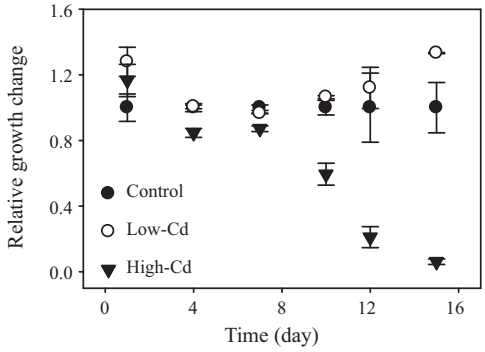


**Fig. 1.** The relative change in the average growth rate of the diatom *T. nordenskioeldii* during different periods of exposure to Cd. Control: $10^{-11}$ M [$Cd^{2+}$], Low-Cd: $1.3 \times 10^{-8}$ M [$Cd^{2+}$], and High-Cd: $8.4 \times 10^{-8}$ M [$Cd^{2+}$]. Data are shown as mean ± semi-range ($n = 2$).

## 3. Results

### 3.1. Toxicity during and after Cd exposure

The relative change in the average growth rate ($\mu$) during 15-day Cd exposure was specific to the medium [$Cd^{2+}$] (Fig. 1). During these 15 days, cells in the Low-Cd treatment ($1.3 \times 10^{-8}$ mol $L^{-1}$) did not show any inhibition of $\mu$, or even exhibited a slight increase on Days 12–15. In contrast, cells in the High-Cd treatment ($8.4 \times 10^{-8}$ mol $L^{-1}$) began to show a significant ($p < 0.05$) decrease in $\mu$ after 1 day of exposure, which further deteriorated after 7 days of exposure. The average $\mu$ of High-Cd diatoms was 0.59 ± 0.06, 0.11 ± 0.06, and 0.06 ± 0.01 on Days 7–10, 10–12, and 12–15, respectively.

Fig. 2 shows the decrease in the average growth rate ($\mu$) with increasing tested [$Cd^{2+}$] in a 72-h Cd toxicity test after different durations of Cd exposure. After 1 day of Cd exposure, the cells did not show any significant difference in $\mu$ inhibition among the three pre-conditioned treatments. After 7 days of exposure, however, *T. nordenskioeldii* exposed at High-Cd levels showed a more precipitous decrease in $\mu$ than the cells in the other two treatments. With a further extension of exposure to 15 days, the Low-Cd cells began to show a slightly accelerated decrease in $\mu$ compared to the control treatment when tested [$Cd^{2+}$] was lower than $1.1 \times 10^{-6}$ mol $L^{-1}$. Simultaneously, Cd inhibition on $\mu$ for the High-Cd cells became so drastic that the growth of cells at [$Cd^{2+}$] of $1.6 \times 10^{-7}$ mol $L^{-1}$ was already completely inhibited. Besides, at the lowest [$Cd^{2+}$], the $\mu$ of the High-Cd cells showed a much lower value than those of the other two treatments after 15 days of exposure. Such an increase in Cd sensitivity with higher exposed [$Cd^{2+}$] and longer duration was supported by the $IC_{50}$ values listed in Table 1.

### 3.2. Cd short-term uptake

In all treatments, a linear relationship between the intra-Cd concentration and the exposure time was observed during the 1-h uptake period (data not shown), and the slope of the linear regression was calculated as the Cd uptake rate, which was plotted against uptake [$Cd^{2+}$] (Fig. 3). However, it is worth noting that the Cd uptake rate was surprisingly low for the 15-day control diatoms (about 2.6–4.8 times lower than that of the 1-day or 7-day control diatoms). Therefore the average Cd uptake rate of the control diatoms after 1 day and 7 days of exposure was calculated and plotted in the bottom panel of Fig. 3 for reference.

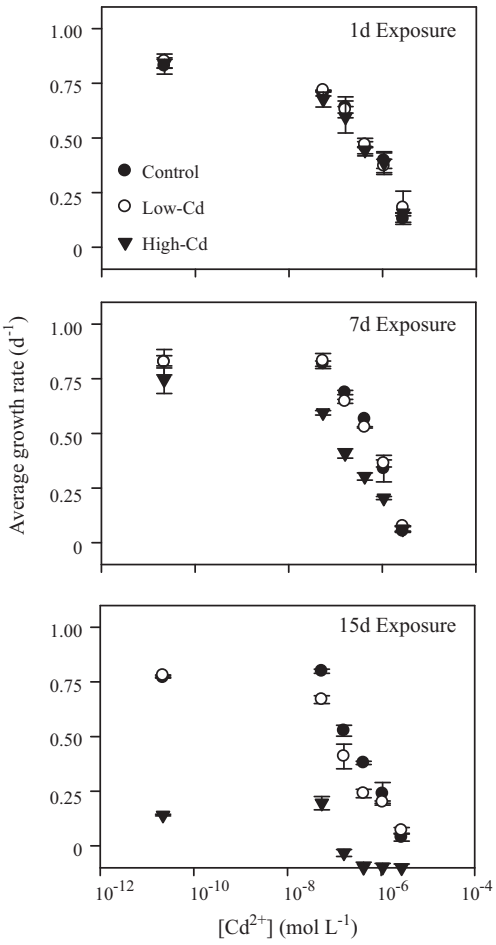


**Fig. 2.** The average growth rate of the diatom *T. nordenskioldii* in 72-h Cd toxicity tests following Cd exposure for 1, 7, and 15 days. Control: $10^{-11}$ M [$Cd^{2+}$], Low-Cd: $1.3 \times 10^{-8}$ M [$Cd^{2+}$], and High-Cd: $8.4 \times 10^{-8}$ M [$Cd^{2+}$]. Data are shown as mean ± semi-range ($n = 2$).

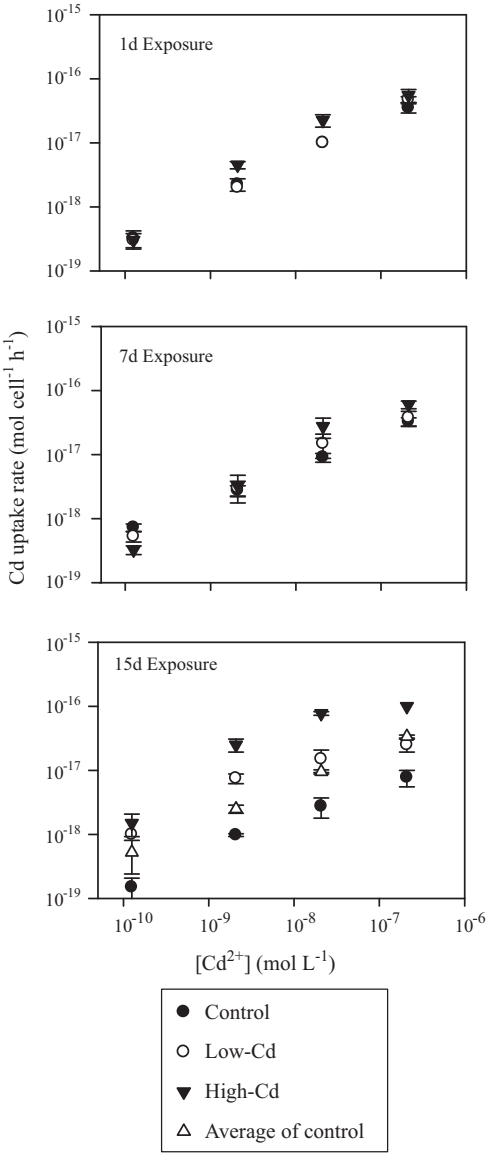


**Fig. 3.** The Cd short-term (1 h) uptake rate of the diatom *T. nordenskioeldii* quantified at different [$Cd^{2+}$] after different periods of Cd exposure (1 day, 7 days, 15 days). In the bottom panel, the average uptake rate of the control diatom after exposure for 1 and 7 days was also plotted for reference. Control: $10^{-11}$ M [$Cd^{2+}$], Low-Cd: $1.3 \times 10^{-8}$ M [$Cd^{2+}$], and High-Cd: $8.4 \times 10^{-8}$ M [$Cd^{2+}$]. Data are shown as mean ± semi-range ($n = 2$).

After the first day of exposure, the High-Cd cells exhibited a significantly ($p < 0.01$) higher uptake rate compared with the other two treatments when the tested [$Cd^{2+}$] was between $1.2 \times 10^{-10}$ and $2.1 \times 10^{-7}$ mol $L^{-1}$. However, no significant difference of Cd uptake rate was observed between the Low-Cd exposed cells and the control cells after 1 and 7 days of exposure. When the exposure period was extended to 15 days, the elevated uptake rate with higher exposed [$Cd^{2+}$] was rather obvious, i.e., cells in the Low-Cd treatment exhibited a significantly ($p < 0.01$) higher Cd uptake than cells in the control (3.2–7.7×), but it was still significantly ($p < 0.01$ when the tested [$Cd^{2+}$] was >$1.2 \times 10^{-10}$ mol $L^{-1}$) lower than the Cd uptake of cells in the High-Cd treatment. At tested [$Cd^{2+}$] > $1.2 \times 10^{-10}$ mol $L^{-1}$, the uptake rates of the High-Cd cells were 9–27.2 times higher than the control treatment.

**Table 1**
The calculated median inhibition [$Cd^{2+}$] ([$Cd^{2+}$]-based-$IC_{50}$) in diatom *T. nordenskioeldii* in the 72-h Cd toxicity experiment after different exposure durations (1 day, 7 days and 15 days) to different [$Cd^{2+}$] (Control: $10^{-11}$ M [$Cd^{2+}$], Low-Cd: $1.3 \times 10^{-8}$ M [$Cd^{2+}$], and High-Cd: $8.4 \times 10^{-8}$ M [$Cd^{2+}$]). Data are shown as mean ± semi-range ($n = 2$).

| Exposure duration | Exposed Cd concentration | $IC_{50}$ (μmol $L^{-1}$) |
|---|---|---|
| 1-day exposure | Control | 0.832 ± 0.176 |
| | Low-Cd | 0.728 ± 0.071 |
| | High-Cd | 0.791 ± 0.211 |
| 7-day exposure | Control | 0.736 ± 0.121 |
| | Low-Cd | 0.728 ± 0.067 |
| | High-Cd | 0.307 ± 0.045 |
| 15-day exposure | Control | 0.596 ± 0.045 |
| | Low-Cd | 0.282 ± 0.110 |
| | High-Cd | 0.108 ± 0.000 |

### 3.3. Cd intracellular accumulation and subcellular distribution

The cell carbon content increased with the exposed [$Cd^{2+}$], especially after 15 days of exposure (Control: 20.4 ± 0.4 pg C $cell^{-1}$, Low-Cd: 19.4 ± 0.8 pg C $cell^{-1}$, High-Cd: 31.1 ± 0.7 pg C $cell^{-1}$). Therefore the Cd concentration was expressed on the cell carbon content basis instead of on a per cell basis. Following the POC normalization, a linear log–log relationship between [$Cd^{2+}$] and either total cellular-Cd concentration (total-Cd) or intracellular-Cd concentration (intra-Cd) was observed for the diatoms (the left panel of Fig. 4 is plotted on a log-linear scale for clearer comparison). Both the total cellular-Cd and intracellular-Cd accumulation of the

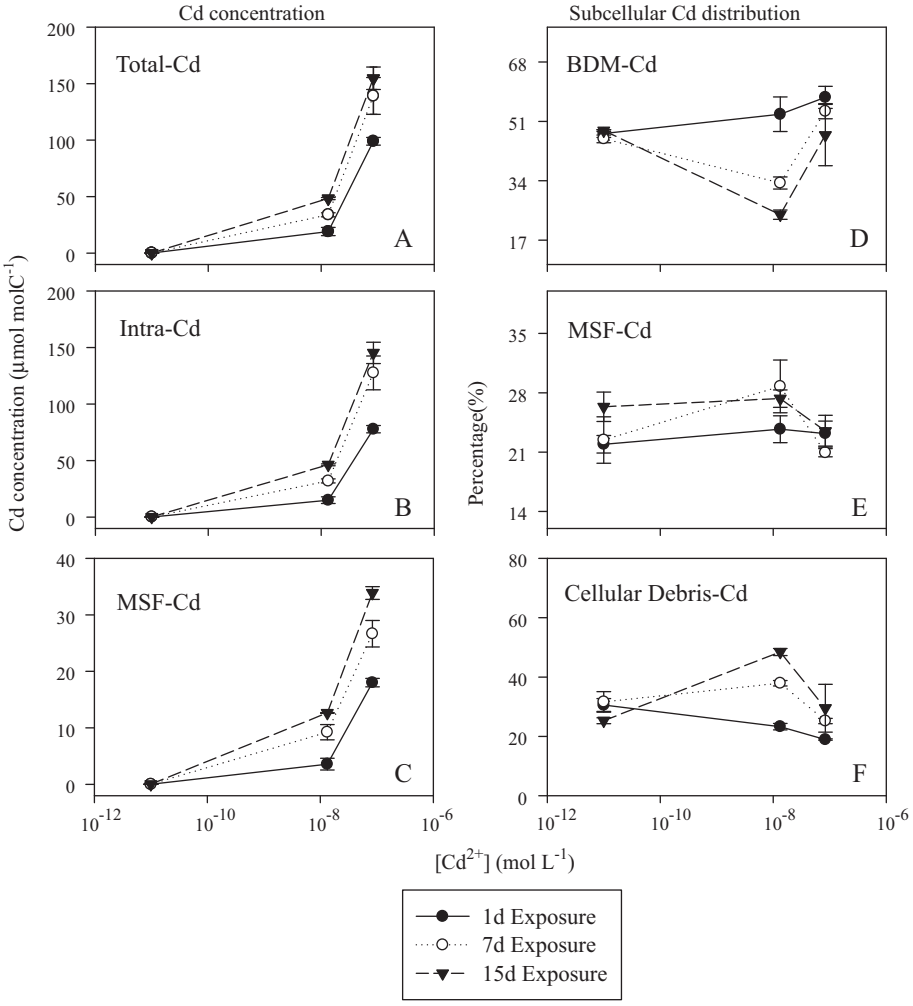


**Fig. 4.** The change in (A) total cellular-Cd concentration (total-Cd), (B) intracellular-Cd concentration (intra-Cd), (C) metal sensitive fraction Cd concentration (MSF-Cd), (D) the percentage of Cd in biologically detoxified metal fraction (BDM-Cd), (E) the percentage of Cd in MSF (MSF-Cd), and (F) the percentage of Cd in cellular debris, with exposed $[Cd^{2+}]$ in the diatom *T. nordenskioeldii* after different periods of Cd exposure (1 day, 7 days, 15 days). Data are shown as mean ± semi-range ($n = 2$).

Low-Cd cells showed significant ($p < 0.01$) increases with a longer exposure duration. A similar pattern was observed for cells in the High-Cd treatment, except that the increase in both total-Cd and intra-Cd became insignificant when the exposure duration was extended from 7 to 15 days (Fig. 4A and B).

The 1-day exposed diatoms showed a different Cd subcellular distribution pattern compared with those exposed for 7 or 15 days in some of the subcellular pools (Fig. 5). For example, the percentage of surface-adsorbed Cd increased and then leveled off with increasing $[Cd^{2+}]$ after 1 day of exposure, and was much higher than those of the 7-day and 15-day exposed cells. It was possible that the equilibrium of Cd distribution among different cellular fractions was not achieved within the first day of exposure. Besides, the HSP-Cd% increased with the exposed $[Cd^{2+}]$ after the first day of exposure, while it showed an initial decrease and subsequent increase with $[Cd^{2+}]$ when the exposure period was extended to 7 and 15 days (Fig. 5). The subcellular Cd pools were then grouped into more ecotoxicologically meaningful compartments, i.e., MSF (organelle + HDP), and biologically detoxified metal fraction (BDM, as HSP + MRG). The MSF-Cd% did not show any significant difference among treatments. On the other hand, for both Low-Cd and High-Cd exposed diatoms, the percentage of Cd in the detoxified metal fraction (BDM-Cd%) decreased while the cellular debris-Cd% increased with a longer exposure period (Fig. 4D–F).

The Cd concentrations in the MSF fraction (Fig. 4C) as well as in organelles and cellular debris (data not shown) increased with the exposed $[Cd^{2+}]$ and duration. An increase in Cd accumulation with higher exposed $[Cd^{2+}]$ was also observed for the other subcellular pools, but not necessarily with longer exposed durations. For example, the Low-Cd cells did not show any significant increase in HDP-Cd, HSP-Cd and BDM-Cd concentration when the exposure period was extended from 1 day to 15 days (data not shown).

### 3.4. Induction of cysteine and low molecular weight (LMW) thiols

The synthesis of L-cysteine and five LMW peptides after Cd exposure is shown in Fig. 6. The concentrations of these compounds were normalized on the POC basis, and their changes were dependent on both exposed $[Cd^{2+}]$ and duration. For the High-Cd treatment, a significant induction of γ-EC ($3.13 \pm 0.35$ μmol mol $C^{-1}$, $p < 0.01$) and $PC_2$ ($4.02 \pm 0.01$ μmol mol $C^{-1}$, $p < 0.001$) was observed after the first day of exposure. With increasing exposure period, the synthesis of γ-EC increased continuously ($12.82 \pm 3.27$ μmol mol $C^{-1}$ after 7 days and $22.87 \pm 0.72$ μmol mol $C^{-1}$ after 15 days), while the concentration of $PC_2$ remained at a relatively constant level ($4.50 \pm 1.36$ μmol mol $C^{-1}$ after 7 days and $4.34 \pm 0.19$ μmol mol $C^{-1}$ after 15 days). In contrast, L-cysteine and $PC_3$ showed a significant ($p < 0.01$ for L-cysteine and $p < 0.05$ for $PC_3$) increase only after 15 days of exposure. In addition, the

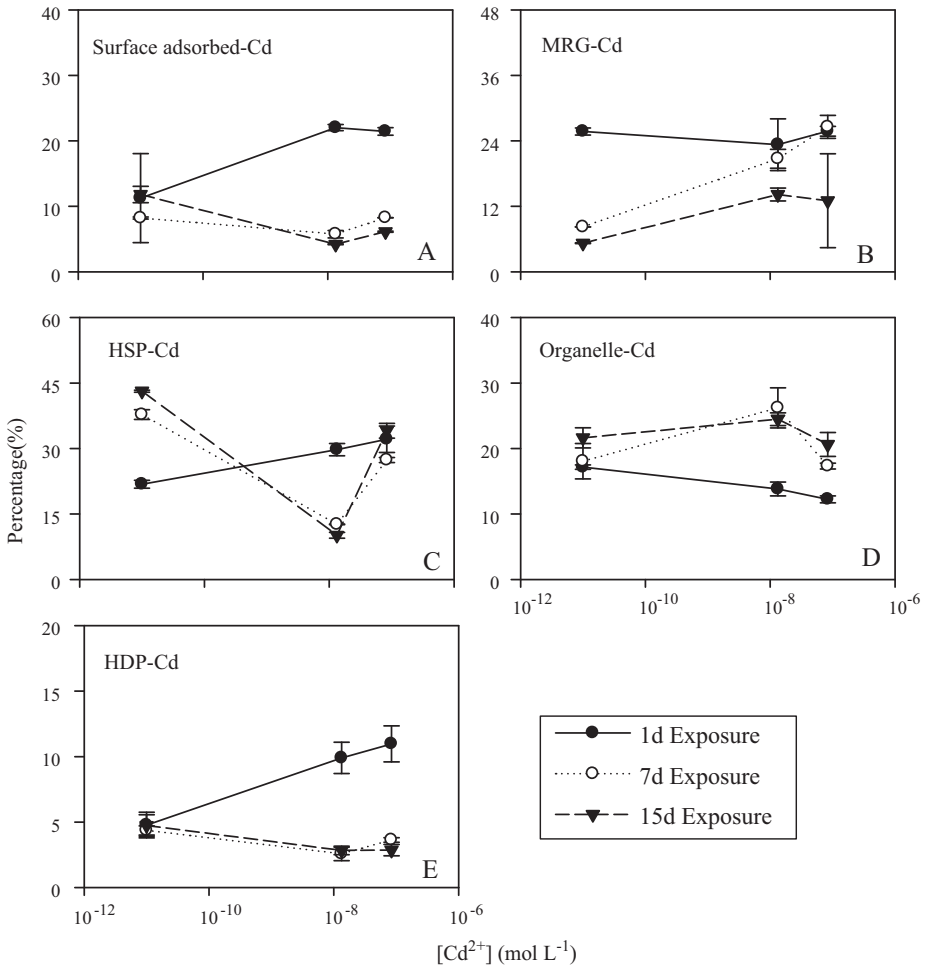


**Fig. 5.** The percentage change in (A) surface-adsorbed-Cd with respect to total cellular Cd (i.e., surface-adsorbed + intracellular Cd), and (B–F) subcellular Cd fractions, as a function of $[Cd^{2+}]$ after Cd exposure for 1, 7 and 15 days. Data are shown as mean ± semi-range ($n = 2$).

$PC_4$ concentration in all treatments was under the detection limit ($2 \times 10^{-8}$ mol $L^{-1}$), except for the High-Cd cells, after 15 days of exposure ($0.15 \pm 0.01$ μmol mol $C^{-1}$).

The cells in the Low-Cd treatment bore a similar pattern of temporal changes in these compounds as those in the High-Cd treatment, except for a few differences. Briefly, the concentration of $PC_2$ in the Low-Cd treatment was significantly ($p < 0.05$) higher than that in the control after 1 day of exposure ($1.39 \pm 0.13$ μmol mol $C^{-1}$), yet much lower than that in the High-Cd treatment. Its concentration was comparable from 1 day to 15 days of exposure. The concentration of γ-EC increased significantly ($p < 0.05$) after 7 days of exposure ($6.24 \pm 0.97$ μmol mol $C^{-1}$), and then leveled off. A significant ($p < 0.01$) increase in the concentration of L-cysteine was only observed after 15 days of exposure ($2.57 \pm 0.24$ μmol mol $C^{-1}$). The total PC-SH concentration was also calculated as ($2\times$ $PC_2 + 3\times$ $PC_3 + 4\times$ $PC_4$), and the intra-Cd/PC-SH ratio was computed correspondingly. The intra-Cd/PC-SH ratio increased with the exposed $[Cd^{2+}]$ and duration, except for the Low-Cd diatoms; the ratio of which was only significantly ($p < 0.001$) elevated after 15 days of exposure (Fig. 7A). A linear regression between the intra-Cd/PC-SH and the relative change in $IC_{50}$ was conducted ($p = 0.002$, Fig. 7C).

## 4. Discussion

Previous studies in a few freshwater organisms (cyanobacteria, periphyton, zooplankton) showed increased tolerance to Cd after exposure as a result of either physiological acclimation or genetic adaptation (Soldo and Behra, 2000; Muyssen and Janssen, 2001; Zeng et al., 2009). Contrary to these earlier studies and our expectation, no enhanced Cd tolerance in the marine diatom *T. nordenskioeldii* was found following sublethal or lethal Cd stress. Instead, an increased sensitivity with higher exposed $[Cd^{2+}]$ and longer exposure was documented in our study. Such change in Cd sensitivity could be traced to the responses of Cd cellular accumulation (which is related to Cd uptake kinetics and subcellular distribution) and the intrinsic detoxification ability (PCs synthesis) of the diatoms to Cd exposure, as discussed below.

### 4.1. Cd uptake, cellular accumulation, subcellular distribution and sensitivity

Metal accumulation in phytoplankton is determined by metal uptake, efflux and growth dilution. Assuming that the efflux of the metal is much lower than the growth rate, the metal concentration in cells under steady-state conditions can be simply calculated as the uptake rate divided by the growth rate constant (Sunda and Huntsman, 1998). In previous study, an elevated uptake after metal exposure was observed for some other organisms (McGeer et al., 2007; Guan and Wang, 2006). Consistently, after exposure for different durations, the Cd uptake rate in our High-Cd diatoms was significantly elevated compared to the other two treatments, and the average growth rate was also inhibited (except for the 1-day exposure batch). Both responses led to an increase in Cd cellu-

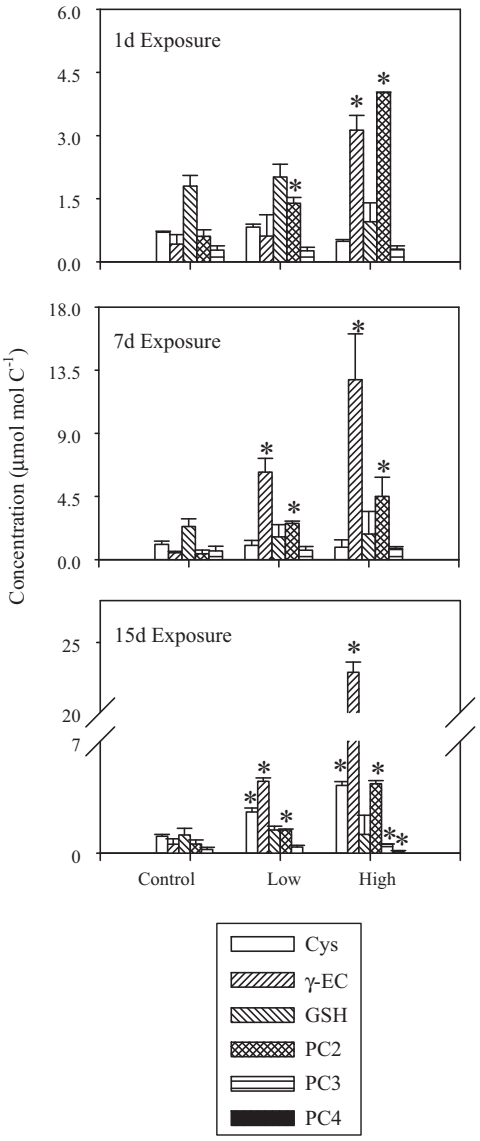


**Fig. 6.** Concentrations of L-cysteine, γ-EC, GSH and $PC_{2-4}$ in the diatom *T. nordenskioeldii* after exposure at different $[Cd^{2+}]$ for 1 day, 7 days, and 15 days. Control: $10^{-11}$ M $[Cd^{2+}]$, Low: $1.3 \times 10^{-8}$ M $[Cd^{2+}]$, High: $8.4 \times 10^{-8}$ M $[Cd^{2+}]$. Any significant difference from the control treatment is marked with *. Data are shown as mean ± semi-range ($n = 2$).

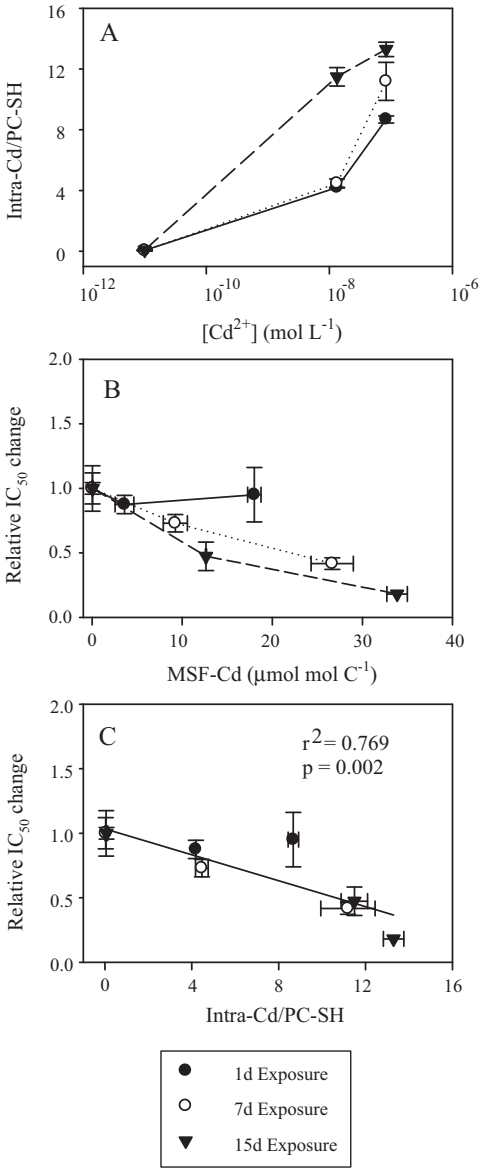


**Fig. 7.** (A) The change in the intra-Cd/PC-SH ratio with exposed $[Cd^{2+}]$ and duration. The relative change in the median inhibition $[Cd^{2+}]$ ($IC_{50}$, measured during the 72-h toxicity experiment) with (B) MSF-Cd concentration, and (C) the intra-Cd/PC-SH ratio, after periods of exposure. Data are shown as mean ± semi-range ($n = 2$).

lar accumulation. In addition, as the exposure period extended, the relative increase in the uptake rate and the corresponding growth inhibition became more obvious. Hence it was observed that Cd cellular accumulation in the High-Cd treatment increased with a longer exposure time. On the other hand, cells in the Low-Cd treatment did not show any growth inhibition during the 15 days of exposure, and a significant increase in uptake rate was only observed after 15 days of exposure. However, elevated Cd cellular accumulation in the Low-Cd treatment compared with the control was observed after 1 day of exposure, which may be primarily due to the higher exposed $[Cd^{2+}]$. Therefore, the concentration of total-Cd and intra-Cd increased with both exposed $[Cd^{2+}]$ and duration, corresponding to the increase in Cd sensitivity during and after exposure.

One may argue that after 15-day of exposure, the significant elevation in uptake rate of the pre-exposed diatoms may have been due to the unusually low uptake rate of the corresponding control treatment. However, the exposed diatoms still had a significantly higher Cd uptake rate than the control cells after 1-day and 7-day of exposure. Additionally, it was surprising to find the increase in Cd short-term uptake with higher exposed $[Cd^{2+}]$ after 15-day of exposure. In addition to the possible increase in internal binding ligands (e.g., PCs) available for Cd, the cell membrane permeability may have been greatly disrupted by Cd stress (especially for High-Cd diatoms), which may lead to an easier internalization of medium Cd.

After entering the cells, metals can bind with different subcellular compartments with different ecotoxicological significances, among which the organelles and HDP are considered as the metal sensitive fraction (MSF), while the MRG and HSP are collectively considered as the biologically detoxified metal fraction (BDM). The methodology of metal subcellular fractionation was originally based on animal models (Wallace et al., 2003; Giguère et al., 2006), but has been optimized for algal homogenates (Lavoie et al., 2009a,b). However, the interpretation of the subcellular data should be treated with caution, considering that various potential artifacts may affect the fractionation results. For example, the

organelles (e.g., vacuoles and chloroplasts) might be disrupted during harsh homogenization, and metals originally associated with the organelles may leak into HSP and HDP fractions (Lavoie et al., 2009a). In addition, low homogenization efficiency may lead to an overestimation of the metals in MRG and cellular debris. Therefore, in our study we chose a low pressure to achieve satisfactory breakage efficiency for the diatoms (>97% as verified under microscope) and tried to minimize the impact on the integrity of organelles. On the other hand, the cells after HQS extraction were rinsed with Tris-buffer to minimize the influence of the potentially 'carried over' HQS on the subcellular Cd distribution (e.g., extracting Cd in each fraction into the final HSP fraction). However, the differential centrifugation has some inevitable artifacts, e.g., overlaps among subcellular fractions (De Duve, 1975; Graham, 2001); and the presence of some free Cd cations in the HSP pool which are highly toxic yet considered as in the BDM compartment. Despite these potential problems, this method has been widely used in metal partitioning studies and can provide useful information if the results are interpreted with caution.

In our study, after 1–15 days of exposure, the percentage of BDM-Cd for both Low-Cd and High-Cd cells did not show any significant increase compared with that for the control (the only exception was for the High-Cd cells after 1 day of exposure). Besides, as the exposure period extended, the cells of both Low-Cd and High-Cd treatments exhibited a gradual decrease in BDM-Cd%. Although such decrease was partly compensated by the increased cellular debris-Cd%, no significant difference in MSF-Cd% was observed among cells exposed for different durations. Both Low-Cd and High-Cd cells also exhibited a MSF-Cd% comparable with the controls. None of these results provided any clear evidence for the expected involvement of the BDM fraction in effective Cd detoxification. The constant MSF-Cd% resulted in a similar pattern of change in MSF-Cd concentration as in intra-Cd concentration, i.e., the MSF-Cd concentration increased with both exposed [$Cd^{2+}$] and duration, corresponding to the similarly increasing Cd sensitivity during and after exposure.

Cd bound to the MSF may have more important toxicological implications than the intracellular Cd cellular burden (Wang and Rainbow, 2006). Previous studies proved that MSF-Cd best indicated the Cd toxicity in the marine diatoms under different environmental conditions (Wang and Wang, 2008a,b). To better understand the relationship between MSF-Cd and Cd sensitivity, we plotted the relative change in [$Cd^{2+}$]-based-$IC_{50}$ versus MSF-Cd concentration (Fig. 7B). It was obvious that $IC_{50}$ decreased (i.e., Cd sensitivity increased) with increasing MSF-Cd concentration, but the pattern was dependent on the exposure period. At a fixed MSF-Cd concentration, a longer exposure was associated with a lower $IC_{50}$ (i.e., becoming more sensitive). This observation indicated that the effect of Cd depends on both exposure intensity and duration (Piotrowski and Buchanan, 1982). It is also worthy to mention that in the 72-h toxicity experiment after 15-day of exposure, the average growth rate of the High-Cd diatoms was much lower than the control cells even at the lowest tested [$Cd^{2+}$] (Fig. 2), indicating that Cd caused some irreversible damages and delayed toxic effects. Thus, without an effective protection of BDM, both the physical accumulation of MSF-Cd and functional damage in MSF were responsible for the increased Cd sensitivity with higher exposed [$Cd^{2+}$] and longer exposure period.

### 4.2. Phytochelatin-related peptides induction and sensitivity

As an important detoxification mechanism in higher plants and phytoplankton (Rauser, 1995), substantial amounts of PCs (especially $PC_2$) were induced after Cd exposure as expected. Nevertheless, the response of each PC oligomer was highly dependent on the level of exposed [$Cd^{2+}$] and duration. In our study, $PC_2$ showed a quick response to environmental Cd stress within the first day of exposure, and kept at the first day level when the exposure period was extended to 7 and 15 days, indicating the role of $PC_2$ as a rapid response to and defense against elevated Cd stress (both environmental and intracellular). In contrast, significant increases in $PC_3$ and $PC_4$ were only observed for the High-Cd diatoms after 15 days of exposure. Similarly, Morelli and Scarano (2001) found that $PC_2$ increased quickly as a response to Cd exposure and remained constant during the following exposure period, while other more polymerized PCs ($PC_{3-5}$) increased continuously with exposure time. The increase in the chain length of PCs implies an increase in the stability of Cd-PC complexes. Therefore, the responses of PC oligomers strongly suggested the tight regulation of intracellular Cd by PCs (Le Faucheur et al., 2005). A few studies proposed that microalgae that are able to synthesize PC oligomers of longer chains are more tolerant to metals (Torres et al., 1997; Pérez-Rama et al., 2001). However, the elevated PC-SH concentration as well as the increased importance of PC oligomers of longer chains ($PC_3$ and $PC_4$) could not account for the increased Cd sensitivity after Cd exposure in this study.

Since PCs are thought to protect organisms against the toxic effects of metals through binding to and immobilization of intracellular free metal ions (Ahner et al., 1995), the intra-Cd/PC-SH ratio (or intra-Cd to PC-SH difference), instead of the PC-SH concentration, can be used to indicate the intrinsic detoxification ability of organisms and explain the increased Cd sensitivity after Cd exposure. In this study, although PC-SH generally increased with exposed [$Cd^{2+}$] and period, it did not keep pace with the elevation in intracellular Cd accumulation. Therefore, the intra-Cd/PC-SH ratio increased with exposed [$Cd^{2+}$] and duration, indicating the decrease in intrinsic detoxification ability, in correspondence with the elevated sensitivity. The relative change in [$Cd^{2+}$]-based-$IC_{50}$ also showed a linear decreasing relationship with the intra-Cd/PC-SH ratio (Fig. 7C), which suggested that the overwhelming intra-Cd instead of the PC-SH concentration should be responsible for the subsequent Cd sensitivity.

The binding stoichiometry of thiol groups to Cd is expected to be 4–1, or at least 2–1 (Le Faucheur et al., 2005). However, no growth inhibition was documented for diatoms in the Low-Cd treatment after 15 days of exposure, during which the intra-Cd/PC-SH ratio was higher than 4. Ahner et al. (1995) also found that the intra-Cd concentration exceeded the PC-SH concentration in the dinoflagellate *Heterocapsa pygmaea* by many folds while no significant growth inhibition was observed. Possible explanations included additional mechanisms to protect the diatoms, such as the starch granule deposits, proline, and other unknown thiols (Nishikawa et al., 2006; Wu et al., 1998; Rauser, 1995). Additionally, the turnover of PCs, including their synthesis, complexation with metals, formation of intracellular complexes and transportation, recycle, degradation and excretion (Rauser, 1995), may be more crucial than the concentration of PCs in understanding the role of PCs in detoxification. This certainly deserves more thorough and systematic study.

GSH has a number of important functions other than detoxification, e.g., metabolism, catalysis, and transport (Meister, 1995). The consumption of GSH for PCs synthesis would result in a low GSH concentration and may potentially account for the increased Cd sensitivity. Previous studies found that GSH concentration decreased rapidly within the first few hours of Cd exposure because of induction of PCs, but then increased and remained constant afterwards (Le Faucheur et al., 2005; Morelli and Scarano, 2001). It is interesting to note that the GSH concentrations in the control treatments (about $3–5 \times 10^{-18}$ mol cell$^{-1}$) were much lower than those measured in previous studies (80 amol cell$^{-1}$ in the marine diatom *Phaeodactylum tricornutum*, Morelli and Scarano, 2001; 600 and 400 amol cell$^{-1}$ in the freshwater green algae *C. reinhardtii* and *P. subcapitata*, Lavoie et al., 2009a). Ahner et al. (2002)

found that the GSH concentrations of marine phytoplankton varied from $3.0 \times 10^{-17}$ mol cell$^{-1}$ (*Emiliania huxleyi*) to $2.4 \times 10^{-15}$ mol cell$^{-1}$ (*Thalassiosira weissflogii*), which were closer to our present measurements Additionally, in our study, the GSH levels did not show any significant difference among different pre-conditioned cells despite the formation of its precursor, γ-EC, and of PCs. These evidences indicated that the elevated sensitivity after Cd exposure was probably not due to GSH depletion as a result of phytochelatin synthesis in this study.

## 5. Conclusion

The Cd sensitivity of the marine diatoms *T. nordenskioeldii* increased with exposed [$Cd^{2+}$] and duration, in contrast to many of the previous studies on other aquatic organisms. Such an increase in sensitivity was likely caused by the increase in Cd cellular burden and the decrease in the detoxification ability (as quantitatively indicated by the change in the intra-Cd/PC-SH ratio) of the diatoms. With increasing exposed [$Cd^{2+}$] and exposure period, the Cd uptake rate was elevated, accompanied by an inhibition of average growth rate (only for the High-Cd diatom), both of which resulted in an increase in Cd cellular accumulation. Without the effective protection of BDM, both the physical accumulation of MSF-Cd and functional damage could be responsible for the increased Cd sensitivity. PCs were induced under Cd stress, with $PC_2$ being the most rapid, but not being able to keep pace with the increase in intracellular Cd. Therefore, the intra-Cd/PC-SH ratio increased with exposed [$Cd^{2+}$] and exposure duration, representing a decrease in the intrinsic detoxification ability, and thus may account for the elevated sensitivity. This study also indicates that the physiological and kinetic responses of phytoplankton upon metal exposure deserve further study.

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