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Thiol compounds induction kinetics in marine phytoplankton during and after mercury exposure

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ABSTRACT

We investigated the kinetics of Hg(II) and MeHg accumulation and the synthesis of phytochelatins (PCs), cysteine (Cys), glutathione (GSH), and γ-glutamylcysteine (γ-EC) in a marine diatom *Thalassiosira weissflogii* during a 3-h (short-term) and a 96-h (long-term) exposure period, and during a subsequent 96-h recovery period. MeHg induced the synthesis of a significant level of GSH, but it was Hg(II) that gave rise to significant levels of other non-protein thiol compounds. The thiol compounds Cys, γ-EC, and PC_{2-3} were induced in *T. weissflogii* within the first 30 min of exposure, followed by PC_4, but the concentrations of all six compounds returned to the control levels after the 96-h recovery period. The kinetics of these non-protein thiol compounds pointed to a rapid cellular response to environmental mercury pollution. After a first decrease, the molar ratio of PC-SH (sulfhydryl in PCs) to intracellular Hg increased slightly which demonstrated the role of PCs in Hg(II) detoxification. However, PC–SH was bound with Hg(II) at a stoichiometric ratio of 0.1–0.3, indicating the involvement of other detoxification mechanisms. Elucidating the effects of mercury on intracellular non-protein thiol pools may help us better understand the metal detoxification in phytoplankton.

1. Introduction

Since the beginning of the industrial age, human activities have greatly increased the amount of mercury released into the environment worldwide [1,2]. Mercury is distinguished from other metals in its tendency to biomagnify along the aquatic food chain, which eventually leads to disease and even deaths in humans. Indeed, due to its oxidative stress and specific interaction with sulfhydryls in enzymes, mercury can exert toxicity at all trophic levels [3–5]. Both plants and animals have developed defense strategies against mercury [6–8]. As the entry point of mercury into the aquatic ecosystem, phytoplankton has also adopted several mechanisms to resist mercury toxicity.

Previous studies have proved that algae could alleviate mercury toxicity by employing either extracellular or intracellular self-protection mechanisms. Through the release of the biogenic reducing factor, as well as cellular reduction, algae could transform Hg(II) into a more volatile and less bioavailable form, known as dissolved gaseous mercury (DGM) [9–11]. In addition, the immobilization of mercury on the cell surface was also able to lighten the metal toxicity [12,13], with up to 56% of the intracellular mercury accumulated being stored in the cellular debris fraction [14]. Furthermore, intracellular mercury sequestration through the production of metal-binding thiol peptides was another important mechanism for resisting the large amounts of mercury located in the cytoplasm. *In vitro* study has demonstrated that such thiol compounds could restore the function of enzymes inactivated by toxic metals [15].

When plants, algae or yeast are exposed to toxic metals such as mercury, the synthesis of thiol-rich peptides such as phytochelatins (PCs) with a general structure of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n = 2–11$) is induced. PCs are synthesized enzymatically from glutathione with a PC synthase [16]. This enzyme can be activated by a variety of metals, such as Cd, Cu, Pb, Ag, Zn and Hg, leading to the synthesis of PCs. PC_{2-4} are the primary species found in phytoplankton. The complex formation of PCs with metals abides by a sulfhydryl-group-to-metal ratio ranging from 2 to 4. However, the synthesis of PCs, the stoichiometry of their binding to metal, and the contribution of PCs to metal detoxification are specific to metal and algal species. Glutathione (GSH), on the other hand, is the main non-protein thiol pool involved in metal sequestration as well as in mitigating oxidative injury in cells [17]. Thus, the exploration of GSH, PC_{2-4} and their precursors (cysteine and γ-Glu-Cys (γ-EC)) upon metal exposure may help us gain a better understanding of the detoxification mechanism and tolerance in phytoplankton.

The synthesis of PCs in phytoplankton exposed to a variety of metals has been well investigated [18–20]. An attempt has been made to explore the possibility of PC-SH acting as a bioindicator of metal contamination or species sensitivity [21,22]. However, there

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are few studies on the synthesis of PCs in algae exposed to mercury [11], especially to its organic form. Furthermore, the responses kinetics of PCs under mercury exposure is still largely unknown, which makes it difficult to understand the metal responses and detoxification mechanisms in phytoplankton completely. In this study, we investigated (1) the kinetics of Hg(II)/MeHg accumulation in a marine diatom *Thalassiosira weissflogii* during a 3-h (short-term) and a 96-h (long-term) mercury exposure period, and during a subsequent 96-h recovery period; (2) the corresponding kinetic changes in Cys, GSH, γ-EC and PC_{2-4} during the exposure and recovery periods; and (3) whether PCs play an important role in Hg(II)/MeHg detoxification.

2. Materials and methods

2.1. Chemicals

Phytochelatin standards (PC_2, PC_3 and PC_4; purity >95%) were obtained from AnaSpec of the US. Most other chemicals used in this study were purchased from Sigma also of the US and were of the highest purity available. These chemicals were γ-glutamylcysteine (γ-EC), trifluoroacetic acid (TFA), methanesulfonic acid (MSA), 4-(2-hydroxyethyl)-piperazine-1-propane sulfonic acid (HEPPS), tris (2-carboxyethyl) phosphine hydrochloride (TCEP), N-acetylcysteine (NAC), L-cysteine (Cys), glutathione (GSH) and monobromobimane (mBBr). Diethylenetriamine-pentaacetic acid (DTPA) was obtained from Fluka of the US, HPLC-grade acetonitrile (ACN) from Duksan of Korea, and methanol from Merck of Germany. Water was filtered through a Milli-Q system (18.2 MΩ cm) before use.

Both radioactive and stable Hg(II) (added as $HgCl_2$) and MeHg were used in the experiments. ^{203}Hg(II) ($t_{1/2}$ = 46.6 day, in 0.1 M HCl) was purchased from Eckert & Ziegler of the US. Me^{203}Hg was synthesized based on the method of Rouleau and Block [23]. Stable Hg(II) powder was obtained from Sigma and prepared in 1 M ultrapure HCl purchased from BDH of the UK. The stable MeHg stock solution was obtained from Brooks Rand of the USA.

2.2. Algal cultures and exposure conditions

The stock of *T. weissflogii* (CCMP 1587, Provasoli-Guillard National Center, US) was grown at 23.5 ± 1 °C under 55 μmol photons m^{-2} s^{-1} with a 14:10 light:dark (L:D) cycle. The seawater, collected from 10 km off the eastern shore of Hong Kong, was filtered through a 0.22 μm GP Express PLUS Membrane (Sericup, Millipore Corporation) before use. The algal cells were acclimated to the experimental conditions by transferring them from the culture medium to the experimental medium, which was enriched with N, P, vitamins and Si at *f*/2 levels, and trace metals at *f*/20 levels (free of EDTA, Cu and Zn). Afterwards, diatoms in the mid-exponential growth phase were harvested by centrifugation (3000 rpm, 24 °C, 5 min) and then resuspended in the exposure medium with three different levels of Hg(II) (control: 0 μg L^{-1}, Hg(II)-L: 8.4 μg L^{-1}, Hg(II)-H: 222 μg L^{-1}) and MeHg (control: 0 μg L^{-1}, MeHg-L: 0.1 μg L^{-1}, MeHg-H: 5 μg L^{-1}). In our preliminary experiments, the growth rate of diatoms had not been inhibited in the low concentration treatment, but it had decreased significantly in the high concentration treatment. The pH of the medium was maintained at 8.2 ± 0.1. Two replicated bottles were spiked with stable Hg only (used for the growth and PCs measurements), and another two replicated bottles were spiked with both stable Hg and radiotracer ^{203}Hg (used for the Hg accumulation analysis). The initial cell density for all experiments was 4–5 × 10^4 cells mL^{-1}. Temperature and irradiance conditions during the exposure period were kept the same as the culture conditions.

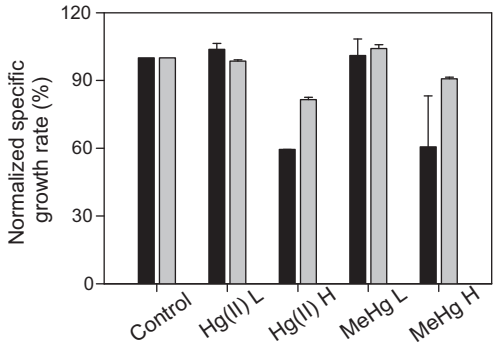

Fig. 1. The changes in the normalized specific growth rate of *T. weissflogii* during (black bars) and after (grey bars) exposure to different levels of Hg(II) and MeHg. Control: no mercury addition, Hg(II)-L: 8.4 μg L^{-1}, Hg(II)-H: 222 μg L^{-1}, MeHg-L: 0.1 μg L^{-1}, and MeHg-H: 5 μg L^{-1}. Data are mean ± SD (n = 2).

2.3. Uptake, accumulation and recovery

A 3-h (short-term) uptake experiment was conducted both for Hg(II) and MeHg. Radioactive ^{203}Hg(II) and Me^{203}Hg were added to the exposure medium and equilibrated overnight to trace the metal uptake by *T. weissflogii*. Either a 10 mL or a 20 mL aliquot was sampled from each bottle at 0.5, 1, 2 and 3 h. The cells were collected by gentle filtration (<50 mm Hg) onto a 3 μm polycarbonate membrane, and then rinsed with 0.22 μm filtered seawater several times and 5 mL of cysteine (8 mM) solution for 1 min to remove the loosely surface-bound mercury [24]. Afterwards, the radioactivity of the cells was measured with a Wallac γ detector (279 keV) to quantify the intracellular Hg(II)/MeHg levels.

Simultaneously, a 96-h (long-term) accumulation experiment with the same exposure dosage as that in the short-term experiment was carried out. At each time point (12, 24, 48, 72 and 96 h), the cells were harvested to determine the intracellular metal concentration in the same way as was done in the short-term experiment. The cell density was recorded every 24 h with a haemocytometer and the specific growth rate of population was calculated from the slope of the linear regression between the natural logarithm of cell density and exposure time.

Following the 96 h of exposure to Hg(II)/MeHg, the diatoms were collected, rinsed, and resuspended immediately in a fresh medium with no mercury in it. The recovery experiment also lasted 96 h, and the cells were harvested to measure the intracellular metal contents at 0, 19, 48, 72 and 96 h. During the recovery process, the cell density was also measured every 24 h to calculate the specific growth rate.

2.4. Non-protein thiols analysis

For each treatment, two replicates spiked with stable mercury were set up for non-protein thiols analysis. At the same time points as those in the uptake, accumulation and recovery experiments, a 200 mL algal sample was collected by vacuum filtration and then rinsed twice with 0.22 μm filtered seawater. No measurement was made for recovered cells after MeHg exposure, since no apparent thiol compounds were induced under MeHg stress. The concentrated cells were immediately transferred into a microtube containing 1 mL of extraction buffer (6.3 mM DTPA with 0.1% TFA). Samples were stored at −80 °C until HPLC analysis.

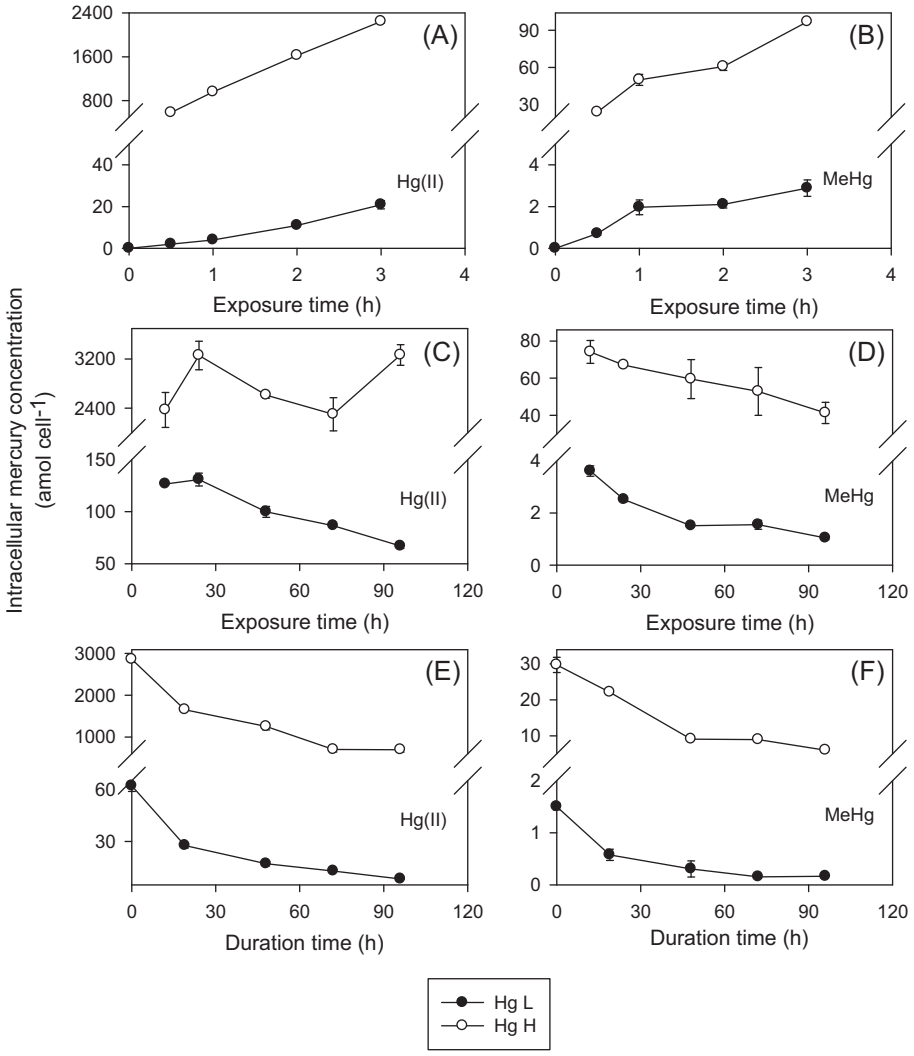

Fig. 2. Mercury kinetics in *T. weissflogii* during exposure and recovery processes. (A) Hg(II) 3-h (short-term) exposure experiments; (B) MeHg 3-h (short-term) exposure experiments; (C) Hg(II) 96-h (long-term) exposure experiments; (D) MeHg 96-h (long-term) exposure experiments; (E) Hg(II) 96-h recovery experiments; (F) MeHg 96-h recovery experiments. Hg(II)-L: 8.4 μg L^{-1}, Hg(II)-H: 222 μg L^{-1}, MeHg-L: 0.1 μg L^{-1}, and MeHg-H: 5 μg L^{-1}. Data are mean ± SD (n = 2).

After thawing, the cells were homogenized using ultrasonic instrument. Microscopic examination revealed that their breakage efficiency was higher than 98%. The broken cells were then centrifuged (13,000 × *g*, 10 min, 4 °C) to collect the supernatant for subsequent analysis. Pre-column derivatization was conducted based on the methods of Rijstenbil and Wijnholds [25] and Sneller et al. [26]. Briefly, 615 μL of HEPPS (200 mM in 6.3 mM DTPA, pH 8.2) and 25 μL of TCEP (20 mM) were mixed together to act as a disulfur reductant. To this solution was added either 250 μL of a standards mixture (five working standards: Cys, GSH and γ-EC at the concentrations of 4, 8, 16, 32 and 40 pmol μL^{-1} and PC_{2-4} at the concentrations of 0.8, 1.6, 3.2, 6.4 and 8.0 pmol μL^{-1} respectively) or the separated supernatant. Ten μL of NAC (0.5 mM) was added to each sample as an internal standard to trace the performance of HPLC analysis. Afterwards, the mixture was incubated in a 45 °C water bath for 10 min which was aimed at reducing the disulfide bonds to sulfhydryls. The fluorescent labeling was then started by the addition of 10 μL of mBBr (50 mM). After another 30 min of incubation in the 45 °C water bath, the reaction was ceased by adding 100 μL of MSA (1 M). Analysis of the derivatized samples followed the procedures described in Minocha et al. [27] which adopted a gradient elution mode with two solvents: (A) 99.9% ACN + 0.1% TFA and (B) 89.9% water + 10% ACN + 0.1% TFA by volume. The column used was a Phenomenex Synergi 4u Hydro-RP C_{18} column (100 mm × 4.6 mm).

3. Results

3.1. Diatom sensitivity during and after mercury exposure

The change in the specific growth rate during and after different levels of mercury exposure was normalized to that of the control treatments (Fig. 1). During the 96-h exposure and recovery periods, neither Hg(II) nor MeHg showed any inhibition effects in the low-concentration treatments. In contrast, when *T. weissflogii* was exposed at the high-Hg(II)/MeHg levels, a significant decrease in the relative growth rate (40 ± 0% and 39 ± 23% for Hg(II) and MeHg, respectively) was observed. This inhibition effect continued even after the diatom had recovered from high mercury exposure (Hg(II): 18 ± 1%; MeHg: 9 ± 1%). It should be noted that although *T. weissflogii* was able to recuperate after Hg(II)/MeHg-H exposure (the specific growth rate after mercury exposure was higher than that during mercury exposure), the diatoms showed a greater potential to recover from MeHg stress than from Hg(II), indicating that the toxic mechanisms were mercury species related.

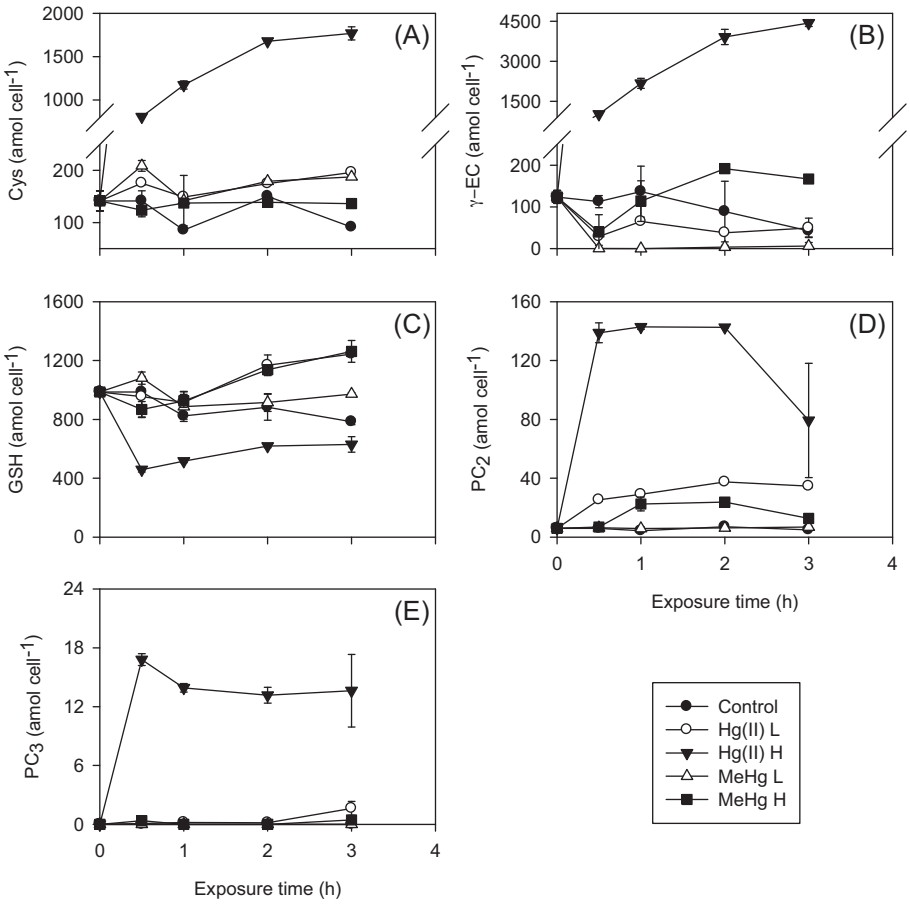

Fig. 3. Kinetics of non-protein thiols induction (A: Cys; B: γ-EC; C: GSH; D: PC_2; E: PC_3) in *T. weissflogii* during 3-h of exposure to various mercury levels. Control: no mercury addition, Hg(II)-L: 8.4 μg L^{-1}, Hg(II)-H: 222 μg L^{-1}, MeHg-L: 0.1 μg L^{-1}, and MeHg-H: 5 μg L^{-1}. Data are mean ± SD (n = 2).

3.2. Mercury accumulation in *T. weissflogii*

Fig. 2A shows the change in intracellular mercury concentration with the exposure time. A linear relationship was observed during the 3-h uptake period in all treatments. The mercury uptake rate was then calculated from the slope of the concentrations of the newly accumulated mercury *versus* exposure time (data not shown). The high-mercury treated cells displayed a 126 times higher Hg(II) uptake rate and a 31 times higher MeHg uptake rate than the low-mercury treated cells. Furthermore, the MeHg uptake rate was obviously higher than the Hg(II) uptake rate (33 ± 1 amol $cell^{-1}$ h^{-1} for 5 μg L^{-1} MeHg, while 6 ± 0 amol $cell^{-1}$ h^{-1} for 8.4 μg L^{-1} Hg(II)).

The mercury accumulation in *T. weissflogii* cells during long-term exposure is shown in Fig. 2B. Different from the pattern observed in the short-term exposure experiments, the intracellular mercury concentrations decreased as exposure went on, except for the Hg(II)-H treatment. A similar but more pronounced trend in intracellular mercury concentrations was observed once the algal cells had recovered from mercury exposure (Fig. 2C), even in the Hg(II)-H treatment. The decrease (after 96 h exposure with respect to time 0 h) was more intensive under lower mercury levels: Hg(II)-H (76 ± 1%); Hg(II)-L (86 ± 1%); MeHg-H (80 ± 1%); MeHg-L (89 ± 0%).

3.3. Kinetics of thiol compounds induction

The synthesis of six thiol compounds during the 3-h (short-term) and 96-h (long-term) Hg(II)/MeHg exposure are shown in Figs. 3 and 4, respectively. All these species remained almost unchanged while the cells were growing under the control conditions. Comparable concentrations of these compounds were detected in *T. weissflogii* under Hg(II)-L, MeHg-L and MeHg-H treatments, with a few exceptions. For the Hg(II)-H treatment, significant amounts of Cys (808 ± 1 amol $cell^{-1}$), γ-EC (1024 ± 15 amol $cell^{-1}$), PC_2 (139 ± 7 amol $cell^{-1}$) and PC_3 (17 ± 0 amol $cell^{-1}$) were synthesized within 30 min of exposure. The amount of PC_4 was detectable after 3 h of exposure and reached nearly 16 ± 3 amol $cell^{-1}$ at 12 h under Hg(II)-H treatment. The Cys content in *T. weissflogii* under Hg(II)-H condition increased with exposure time, reaching a concentration of 1769 ± 76 amol $cell^{-1}$ at 3 h. It then began to decrease from 12 to 96 h. Similar curves were observed for γ-EC, PC_2 and PC_3, although when their respective maximum values appeared varied. GSH concentrations in algal cells remained almost stable during the short-term exposure, or even decreased under Hg(II)-H stress, and then showed a 1.4–1.7 times increase after 96 h of exposure in Hg(II)/MeHg-H treatments compared with the control cultures.

Since MeHg was only able to weakly induce the synthesis of thiol complexes, we only monitored the kinetics of thiol compounds recovering from Hg(II) exposure. During the 96-h recovery period, the levels of Cys and PCs decreased as time went on under Hg(II)-H condition (Fig. 5). GSH showed a slight increase under Hg(II)-L condition. Its quantity in *T. weissflogii* was the highest among all the thiol compounds resulting from Hg(II) exposure under any condition.

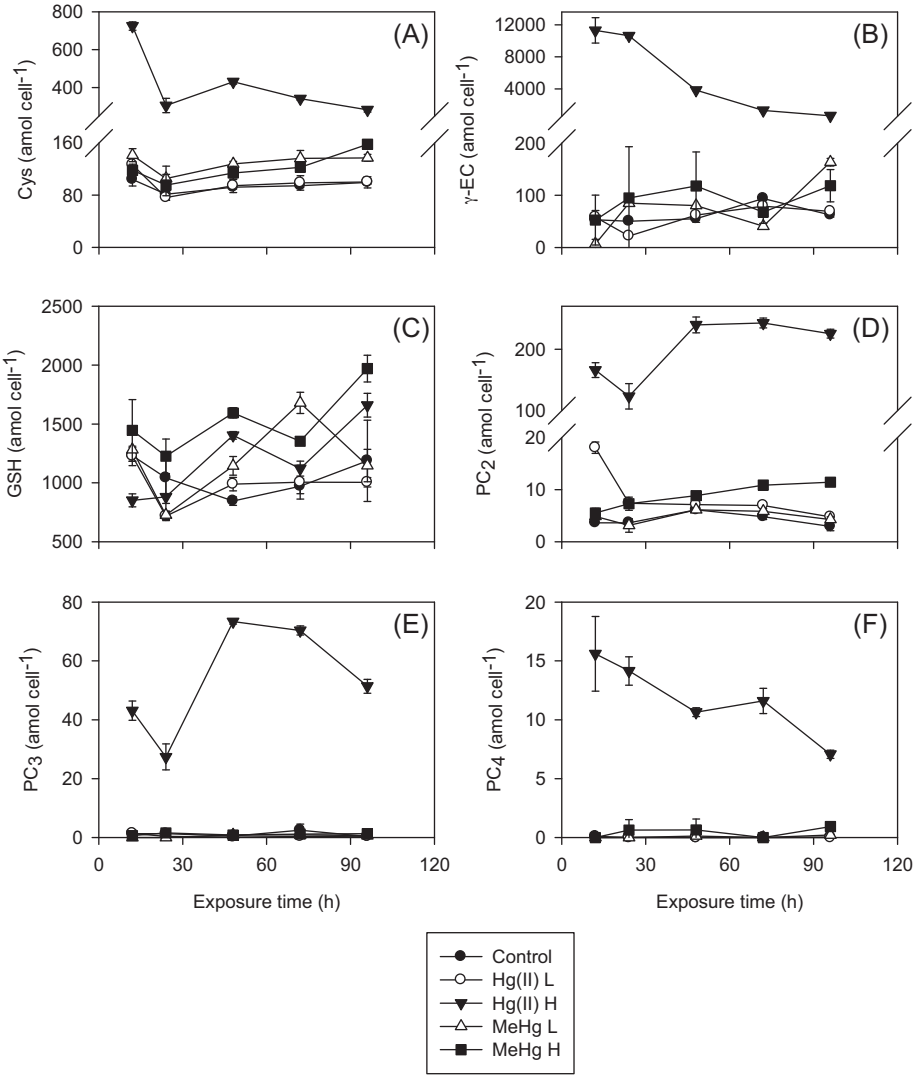

Fig. 4. Kinetics of non-protein thiols induction (A: Cys; B: γ-EC; C: GSH; D: PC_2; E: PC_3; F: PC_4) in *T. weissflogii* during 96-h of exposure to various mercury levels. Control: no mercury addition, Hg(II)-L: 8.4 μg L^{-1}, Hg(II)-H: 222 μg L^{-1}, MeHg-L: 0.1 μg L^{-1}, and MeHg-H: 5 μg L^{-1}. Data are mean ± SD (n = 2).

4. Discussion

4.1. Mercury toxicity and accumulation

In this study, the growth of diatom cells was inhibited at higher levels of mercury exposure. Since Hg(II) is less toxic, its concentration needed to be 44 times higher than that of MeHg to achieve the same inhibition effect. The growth rate of *T. weissflogii* when the cells were recovering from exposure was obviously higher than that when the cells were under high mercury exposure. However, the specific growth rate during recovery in the MeHg-H treatment (0.44 d^{-1}) was somewhat higher than that in the Hg(II)-H treatment (0.40 d^{-1}), although the specific growth rates during exposure to Hg(II)-H and MeHg-H were similar. The quicker restoration of population growth after MeHg exposure may be due to the toxicity patterns of MeHg being different from those of Hg(II). Previously, we have shown that the inhibition on growth by MeHg was mainly resulted from the reduced cell division rate [28]. In contrast, the growth reduction caused by Hg(II) was closely related to the increasing number of injured or dead cells [28], which do not usually recover even when the metal stress is removed.

Metal content in phytoplankton cells can be controlled by metal uptake, efflux and growth dilution. In the Hg(II)-H treatment, uptake was important in the initial intracellular Hg(II) accumulation during long-term exposure. Afterwards, the interaction among growth dilution, cellular direct reduction of Hg(II) to DGM [29], and enhanced membrane permeability (disrupted by Hg(II)) [30] may lead to an observed fluctuation in intracellular mercury concentration. As for the other three treatments, any decrease in metal content with time was determined mainly by population growth (*e.g.*, growth dilution). In comparison, a more obvious decline occurred when the algal cells were recovering from Hg(II) exposure, which was probably related to the Hg(II) efflux.

4.2. Thiol compounds induction kinetics

Non-protein thiol compounds are cys-containing peptides which are involved in cellular essential metal homeostasis, nonessential metal detoxification [31] and sulfur incorporation [32]. Therefore, it is beneficial for plants or algae to ride out the environmental metal fluctuation if such molecules exist continually in the cells. Previous studies have demonstrated that PCs were always present in many algal species without metal stress [19,33]. For example, the concentrations of PC_2 and PC_3 in *Chlamydomonas reinhardtii* were found to be 5.4 amol/cell and 0.5 amol/cell respectively [33]. Similar concentrations were found

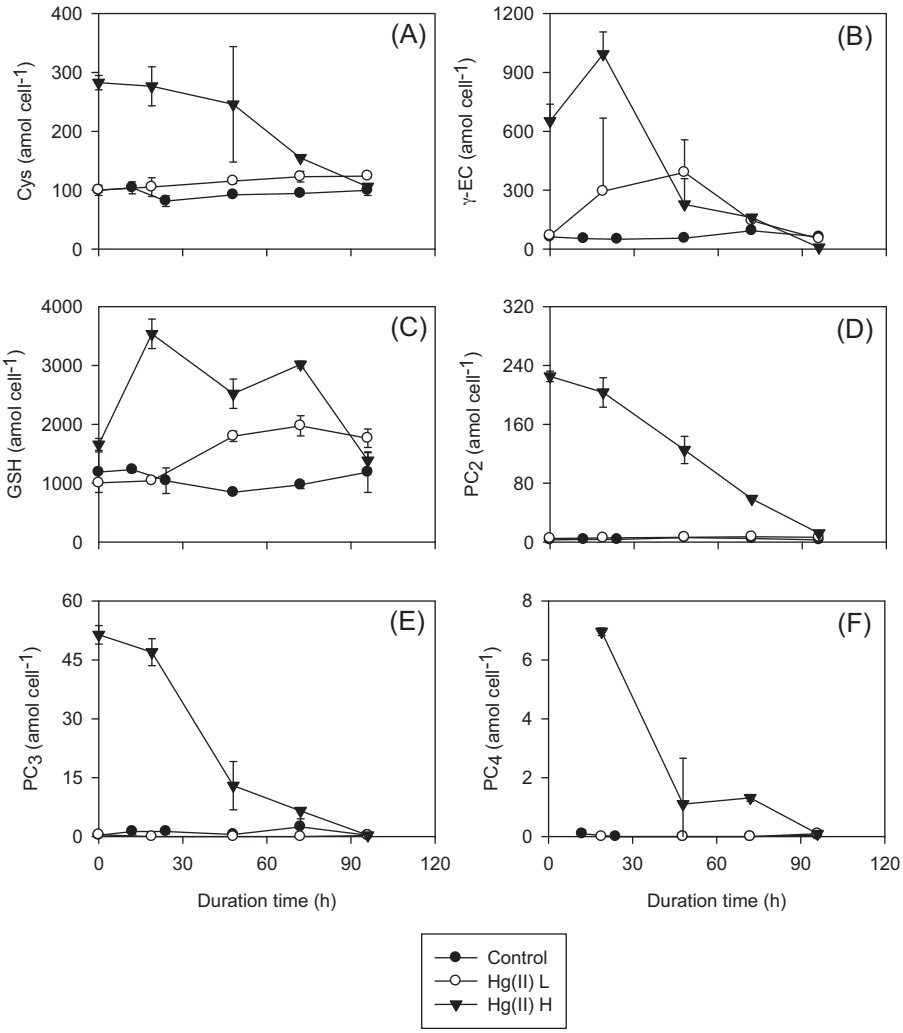

Fig. 5. Kinetics of non-protein thiols induction (A: Cys; B: γ-EC; C: GSH; D: PC_2; E: PC_3; F: PC_4) in *T. weissflogii* recovering from different levels of mercury exposure. Control: no mercury addition, Hg(II)-L: 8.4 μg L^{-1}, Hg(II)-H: 222 μg L^{-1}. Data are mean ± SD (n = 2).

in *T. weissflogii* (PC_2 = 5.5 amol/cell; PC_3 = 0.2 amol/cell) in the controls in this study.

Significant synthesis of non-protein thiol compounds was observed upon Hg(II)-H exposure. As the precursors of GSH and PCs, Cys and γ-EC were synthesized rapidly in *T. weissflogii* (within 30 min) and their concentrations increased continually during the 3-h exposure. A rapid synthesis of PC_2 and PC_3 was also detected within 30 min, after which their intracellular content reached a plateau caused by the limited availability of their substrate GSH (which decreased first and then increased ever so slightly). Morelli et al. [11] also concluded that Hg(II) could lead to a rapid increase in PC_2 and a slightly less rapid increase in PC_{3-4}. The synthesis of PC_4 was recorded after 3 h of exposure to Hg(II)-H in this study. The lagging effect appearing with a higher degree of polymerization could prove the biosynthesis mechanisms of the PCs again, *i.e.*, PC_n is the substrate for PC_{n+1} [34]. When Hg(II)-H stress was removed, the concentrations of all these six thiol compounds decreased dramatically and returned to normal levels within 96 h, indicating a process of rapid reduction and/or export as reported earlier by others [35–37]. Therefore, both the synthesis and reduction of Cys, γ-EC and PCs displayed a rapid intracellular response to Hg(II) stress. The shorter half times of γ-EC and PC_{2-4} than that of intracellular Hg(II) (Table 1) suggested that other pathways, such as proline [38] or other thiols [31,39,40], may be involved in the detoxification.

However, when *T. weissflogii* cells were exposed to MeHg, very small amounts of Cys, γ-EC and PCs were induced compared to the control treatments, suggesting that the induction capability of MeHg was much lower than that of Hg(II). This could be explained by two possible reasons: (1) the chelate efficiency of two cysteine molecules bound with monovalent MeHg was lower than that of two cysteine molecules bound with the divalent Hg(II), and (2) the only way for PCs to be synthesized upon MeHg exposure was with the help of the Hg(II) generated by the demethylation of MeHg [41]. In addition, the concentration of GSH after 96 h of Hg(II)-H or MeHg-H exposure was respectively 1.4 or 1.7 times that of the

Table 1
Loss rate constant k (h^{-1}) and half time (h) of intracellular Hg(II) and non-protein thiol compounds in the diatom *Thalassiosira weissflogii* during a 96-h recovery period which followed on the heels of Hg(II) exposure at 222 μg L^{-1}.

Hg/thiols	k (h^{-1})[a]	$t_{1/2}$ (h)
Hg(II)	0.015 ± 0.001	46.3 ± 2.6
Cys	0.010 ± 0.000	66.4 ± 3.1
γ-EC	0.044 ± 0.006	15.9 ± 2.0
PC_2	0.029 ± 0.000	23.8 ± 0.3
PC_3	0.057 ± 0.010	12.5 ± 2.2
PC_4	0.045 ± 0.003	15.5 ± 1.1

[a] Loss rate constant k (h^{-1}) was calculated from the slope of the natural log of the percentage of intracellular mercury or non-protein thiols and the time of depuration.

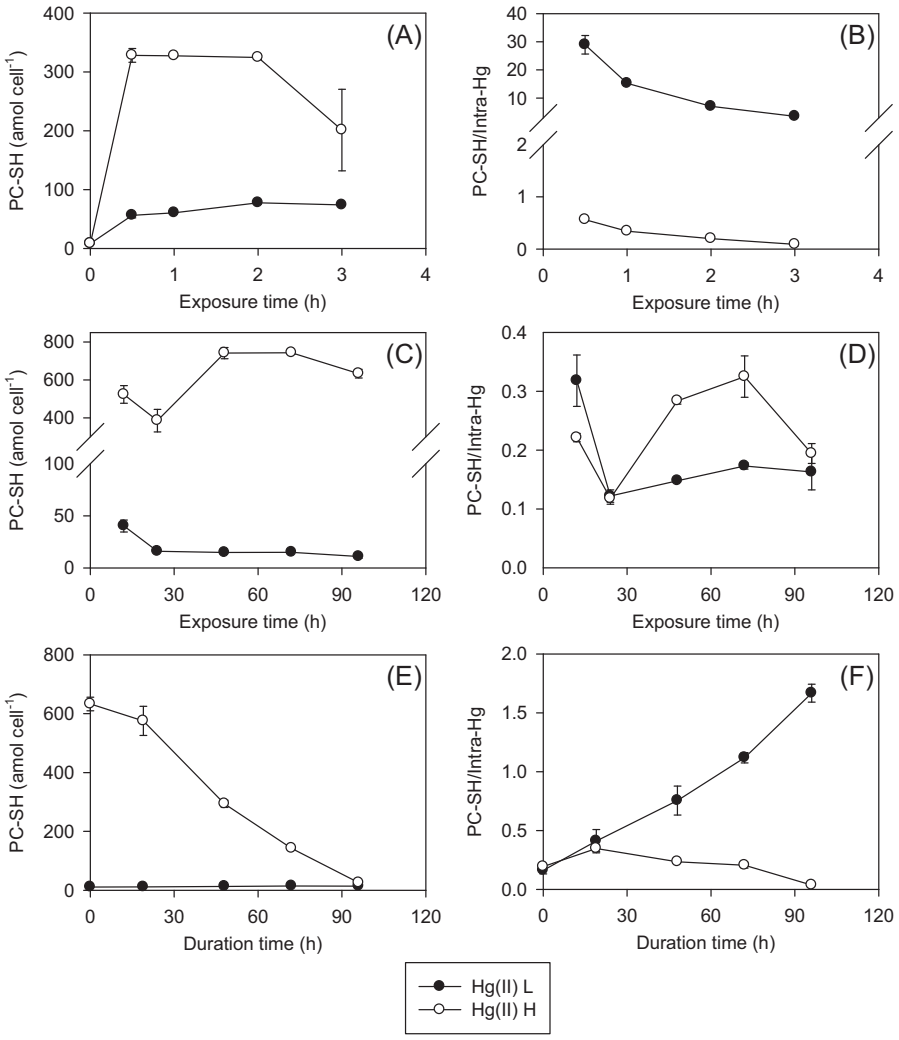

Fig. 6. Kinetics of PC-SH ($2 \times PC_2 + 3 \times PC_3 + 4 \times PC_4$) concentration (A) and the ratio of PC-SH to intracellular Hg (B) in *T. weissflogii* during a 3-h (short-term) exposure period. Kinetics of PC-SH concentration (C) and the ratio of PC-SH to intracellular Hg (D) in *T. weissflogii* during a 96-h (long-term) exposure period. Kinetics of PC-SH content (E) and the ratio of PC-SH to intracellular Hg (F) in *T. weissflogii* recovering from different levels of mercury exposure. Hg(II)-L: 8.4 μg L^{-1}, Hg(II)-H: 222 μg L^{-1}. Data are mean ± SD ($n = 2$).

controls. As the major intracellular thiol pool, GSH may play a role in intracellular mercury sequestration together with PCs.

4.3. The role of PCs in mercury detoxification

By chelating and reducing the intracellular free metal, PCs could alleviate the toxic effects caused by toxic metals. Consequently, a higher ratio of PC-SH to Hg in cells means more efficiency in detoxifying the metals. In this study, we calculated the ratio of PC-SH to Hg for Hg(II) (Fig. 6). The synthesis of PC_{2-4} was not significantly induced by MeHg, thus its ratio of PC-SH to MeHg could not be calculated. During the 3-h (short-term) exposure, PC-SH concentration increased first and then leveled off, while the level of intracellular Hg(II) showed a continually increasing trend, thus, the ratio of PC-SH to Hg decreased for both the Hg(II)-L and Hg(II)-H treatments. Although large amounts of PCs were synthesized in the Hg(II)-H treatment, the rate of synthesis still was not able to keep pace with the increase in intracellular Hg(II), which led to a much lower ratio of PC-SH to Hg and correspondingly more sensitivity in the Hg(II)-H treatment. The downward trend in the ratio of PC-SH to Hg continued for the first 24-h of exposure, and then reversed slightly from 24 h to 72 h, indicating the start of resistance from this diatom to mercury stress. Mehra et al. [42] proposed that the stoichiometry of PC-SH binding with Hg(II) was 2. This ratio was only 0.1–0.3 in this study, which may have resulted from the limited exposure time and/or other detoxification mechanisms involved. Apart from PCs (in cytoplasm), cellular debris (including cell walls or membranes) also played an important role in sequestering the intracellular mercury [14]. Thiol groups in non-PC pools, such as Cys, γ-EC and GSH, could also bind with free Hg(II) in cells. Due to all these mechanisms, no toxicity was exhibited by cells undergoing the Hg(II)-L treatment.

While the cells were recovering from Hg(II) exposure, the PC-SH concentrations restored to the control level quickly. Since PCs are essential for maintaining cellular metal homeostasis, and intracellular Hg(II) concentration decreased with recovery from Hg stress, the ratio of PC-SH to Hg in the Hg(II)-L treatment increased sharply. Interestingly, this ratio had initially increased while the *T. weissflogii* cells were recovering from Hg(II)-H exposure, indicating the protection mechanism of PCs might be involved.

In conclusion, the ability of MeHg to induce the formation of non-protein thiol compounds was much lower than that of Hg(II) (except for GSH), although MeHg had been significantly accumulated in the diatom cells. The response of *T. weissflogii* to environmental Hg(II) was very quick. Cys, γ-EC and PC_{2-3} were synthesized the quickest, followed by PC_4. All of these compounds

restored to the control levels within 96 h removing the metal stress. The rapid change of these non-protein thiol compounds during and after Hg(II) exposure makes them good bioindicators of environmental mercury pollution. Dynamic changes of the ratio PC-SH to intracellular Hg showed a decrease within the first 24 h and then an increase slightly, suggesting the role of PCs in Hg(II) detoxification. Due to other detoxification mechanisms, the stoichiometry of PC–SH binding with Hg(II) found in this study (0.1–0.3) was much lower than those found in *in vitro* studies. Future studies should pay more attention to the turnover of PCs, including their synthesis, complexation, recycle, degradation and excretion, which may be more important than their concentrations for our understanding of the metal detoxification.

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