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Studies of biouptake and transformation of mercury by a typical unicellular diatom *Phaeodactylum tricornutum*

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Mercury (Hg) is a toxic heavy metal with its biogeochemical cycling in the ocean depending on the type and behavior of the oceanic microalgae. The present work aimed to evaluate bioaccumulation and transformation of Hg by *Phaeodactylum tricornutum*, a typical unicellular diatom, when exposed to the extremely high level of Hg in order to understand the possible mechanisms of acute stress response. *P. tricornutum* can accumulate Hg (its bioaccumulation factor is at 10^4 level), and the 96 h EC_{50} was estimated to be 145 μg L^{-1}. The amounts of surface-bound Hg being about 1.2 to 4.8 times higher than those of intracellular Hg under exposure to $HgCl_2$ (from 20 to 120 μg L^{-1} concentrations) suggested that the cell wall of *P. tricornutum* is an important "fence" towards Hg. After entering the *P. tricornutum* cell, Hg underwent transformation in its chemical form via interactions with high molecular weight sulfur-containing proteins (accounting for 68% of the intracellular Hg), and glutathione as well as the induced phytochelatins (PCs) (24% Hg) which alleviated the toxicity of $HgCl_2$. In addition, the existence of organic ligands greatly influenced the uptake and transformation behavior of *P. tricornutum* towards $HgCl_2$, especially in the case of cysteine (Cys), which increased the uptake of Hg, but alleviated the toxicity of Hg towards *P. tricornutum* due to the fact that Cys is an important precursor for the synthesis of PCs inside the cell. The uptake process of Hg by *P. tricornutum* was in agreement with the Freundlich isotherm, suggesting a typical heterogeneous sorption process. More importantly, we observed the conversion of $HgCl_2$ into methylmercury inside the *P. tricornutum* cells and its release into the culture solution using HPLC/CVG-AFS and GC-MS, although the mechanism needs to be further investigated.

mercury, methylmercury, *P. tricornutum*, species transformation, toxicity, phytochelatin

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Microorganisms are able to accumulate heavy metals via both ways of surface-bound sorption and intracellular involvement [1]. The cell walls of microorganisms contain many different functional groups such as amine, carboxyl, hydroxyl, sulfates and phosphates, which interact with heavy metals. Microorganisms can also produce proteins and/or polypeptides such as metallothioneins and other cysteine-rich peptides which complex heavy metals and thus detoxify them in the cells [2]. On the other hand, many kinds of natural and anthropogenic ligands are always present in the aquatic environment, and heavy metals may form complexes with such existing ligands resulting in various chemical species, determining significantly the modes and amounts of the heavy metals to enter into the cells of microorganisms. In addition, some heavy metals may be transformed to methylated compounds by microorganisms [3]. All these accumulation and transformation processes affect the fate and transport of heavy metals in the environment.

As one of the typical toxic heavy metals, mercury (Hg) pollution is a global problem because of its persistence, bioaccumulation and toxicity in the environment [4,5], and

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therefore is a risk factor for the health of people [6,7]. Hg can take a myriad of pathways to enter the environment, but one of the main sources of Hg to most aquatic ecosystems is the atmospheric deposition of Hg through long-distance atmospheric transport from anthropogenic and natural sources. Generally, the concentration of total Hg is less than 0.12 μg L^{-1} in seawater [5], however, the Hg concentration is as high as 2.3 μg L^{-1} or even 260 μg L^{-1} in some seriously polluted areas [8,9]. Many marine organisms take part in and may play an important role in its biogeochemical cycling in the ocean. *Phaeodactylum tricornutum* is a unicellular diatom distributed widely in oceanic and fresh waters. It is at the bottom of aquatic food chains, and may be consumed by the next higher level. It represents a major entry point of toxic heavy metals to the organisms at higher trophic levels, leading to their accumulation and bioamplification in higher organisms along the food chains [10,11]. Additionally, because of its known structure and genome [12], it has frequently been used as a model diatom in studies of algal physiology and ecology as well as studies of heavy metal transportation and transformation in the oceanic ecosystem [13]. However, previous studies on *P. tricornutum* were mainly focused on the accumulation, transformation and toxicity of cadmium and copper, those concerning Hg are scarce [14].

The purpose of our present study was to investigate the bioaccumulation and transformation of Hg by *P. tricornutum*. The accumulation behavior and toxicity of various Hg species to *P. tricornutum* as well as the conversion of Hg species inside the cell were studied when *P. tricornutum* was exposed to the extremely high level of Hg species in order to understand the possible mechanisms of acute stress response and the role of microalgae during the biogeochemical cycling of Hg in the marine environment.

1 Experimental

1.1 Reagents and chemicals

3-*N*-morpholino-propane-sulfonic acid (MOPS) purchased from Sangon Biological Engineering Technology & Services Co. Ltd. (SBETS, Shanghai, China) was used to control the acidity of seawater culture solutions at pH 7.5. Chymotrypsin, β-lactoglobulin, insulin, vitamin B_{12} and glutathione from SBETS were used as molecular weight standards to calibrate the molecular weight of Hg associated with proteins and peptides expressed in *P. tricornutum* using size-exclusion chromatography. $HgCl_2$, humic acid (HA), tryptophan (Trp), methionine (Met), cysteine (Cys), nitrilotriacetic acid (NTA) and ethylenediamine tetraacetic acid disodium salt (EDTA), which were used as model ligands during Hg exposure experiments towards *P. tricornutum*, were obtained from Sinopharm Chemical Reagent Co. Ltd. (SCR, Shanghai China). Sodium borohydride ($NaBH_4$), tributyl phosphine (TBP), acetonitrile (ACN), trifluoroacetic acid (TFA) as well as 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), used for the reduction and derivatization of phytochelatins (PCs) during the extraction, HPLC separation and determination of PCs, were purchased from Sigma-Aldrich (St. Louis, MO, USA); dichloromethane (CH_2Cl_2), methanol, mercaptoethanol (β-ME), dithioerythreitol (DTE), ammonium acetate (CH_3COONH_4) and formic acid (HCOOH), used for selective methylmercury (MeHg) extraction and HPLC-AFS determination with photo-induced cold vapor generation (CVG) [15–17], were purchased from Merck (Darmstadt, Germany); sodium tetraphenylborate ($NaBPh_4$), also from SCR, was used to derivatize MeHg for GC-MS analysis; and ultrapure water (18 MΩ) was prepared with a Milli-Q system (Millipore, Bedford, MA, USA), and was used throughout this study. Other reagents used in this study were at least of analytical grade.

1.2 Instrumentation and optimum conditions

Hg determination and speciation were performed on a non-dispersive atomic fluorescence spectrometer (AFS, Beijing Rayleigh Analytical Instrument Corporation, China) with a UV-HCOOH induced CVG unit coupled with a Shimadzu LC-2010A system (Kyoto, Japan). An Hg lamp (253.7 nm) was used as an excitation source (Beijing Institute of Vacuum Electronics Research, China) [15–17]. A lamp current (30 mA), voltage (–310 V), observation height (7 mm) and Ar flow rate (300 mL min^{-1}) were optimized and adopted for AFS determination. An Agilent C_{18} column (particle size, 5 μm; 4.6 mm I.D. × 250 mm in length), and an isocratic elution with 2% CH_3OH-H_2O containing 0.1% β-ME and 0.06 mol L^{-1} CH_3COONH_4 buffer (pH 6.8) at flow rate of 1.0 mL min^{-1} were employed for the separation of Hg species, and a low-pressure Hg lamp (40 W) and 1.9 mol L^{-1} HCOOH were used for Hg CVG. Fourier transform infrared spectroscopy (FT-IR) (Nicolet Avatar 360, Thermo Fisher Scientific, USA) was used to investigate the interaction between Hg and cell walls. Reverse-phase HPLC for the separation and determination of the Hg- and Cd-induced PCs was carried out on an Agilent 1100 series chromatographic system (Agilent Technologies, Palo Alto, CA) equipped with a UV-Vis detector (190–600 nm). A C_{18} reverse phase column (particle size, 5 μm; 2.0 mm I.D. × 250 mm in length; Shimadzu, Japan) was used for the separation of glutathione (GSH) and PC variants, during which two mobile phases 0.02% TFA-H_2O (A) and 0.02% TFA-ACN (B) were used for programmed elution (0–25 min, B 2%–25%) at a flow rate of 0.15 mL min^{-1}. On-line GSH and PCs derivatization and determination were performed using 1.8 mmol L^{-1} DTNB containing 15 mmol L^{-1} EDTA and 0.3 mol L^{-1} K_2HPO_4 buffer (pH 7.88) delivered to join in the HPLC effluent via a T-joint using an isocratic pump (LC-20AD, Shimadzu, Kyoto, Japan) at a flow rate of 0.05 mL min^{-1} and determination at 410 nm. Electrospray ion trap mass spectrometry (ESI-IT-MS) (ESQUIRELC, Bruker

Daltonics, Germany) was used for identifying PCs and the operational parameters were: nebulizer, 75 psi; dry gas, 8 L min^{-1}; dry temperature, 300°C; capillary voltage, –3500 V; end plate offset, –500 V. A DRC II inductively coupled plasma mass spectrometer (ICP-MS) (PerkinElmer, Sciex, Canada) was used for online simultaneous determination of ^{202}Hg and $^{32}S^{16}O$ in the effluent from the size-exclusion chromatography (SEC) (Superdex peptide 10/300 GL 10 I.D. × 300 mm in length, 13 μm particle size, optimum separation range 100–7000 Da, GE Healthcare, USA). The ICP-MS operational parameters were: Ar nebulizer gas, 0.18 L min^{-1}; Ar auxiliary gas, 1.0 L min^{-1}; Ar plasma gas, 15 L min^{-1}; RF power, 1200 W; dwell time, 100 ms; lens voltage, 7.2 V; DRC O_2 inlet, 0.6 mL min^{-1}. The SEC fractions containing Hg and S were also collected and analyzed using a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF-MS) (microflex, Bruker Daltonics, Germany). The instrument was equipped with an N_2 laser emitting at 337 nm; spectra were acquired in the reflector positive-ion mode; ion source 1 (19.01 kV), ion source 2 (15.96 kV), laser power (20 μJ), reflector (19.99 kV) and pulsed ion extraction (140 ns) were adopted, and 2, 5-dihydroxybenzoic acid was used as the matrix. After derivatization using $NaBPh_4$, MeHg identification was performed using a Shimadzu GC-MS QP2010 (Shimadzu, Japan) equipped with an electron impact ion source and an Agilent DB-XLB (15 m × 0.25 mm × 0.25 μm) capillary column. Injection temperature was set at 250°C, and column oven temperature was programmed as 120°C increased to 230°C at 10°C min^{-1} held for 5 min, then increased to 280°C at 25°C min^{-1} held for 2 min; column flow was 0.8 mL min^{-1} He; and the *m*/*z* scan range was from 60 to 310.

1.3 *P. tricornutum* culture and exposure

P. tricornutum was obtained from the Center for the Collection of Marine Bacteria and Phytoplankton in the State Key Laboratory of Marine Environmental Science, Xiamen University, China. Stock cultures of *P. tricornutum* were grown in axenic conditions at (20 ± 1)°C under illumination with fluorescent daylight (100 μmol (photon) m^{-2} s^{-1} with a 14∶10 h light∶dark cycle). The culture medium was natural seawater, which was passed through a Millipore filter of pore size 0.45 μm and sterilized at 120°C for 20 min containing the modified f/2 enrichment solution [18]. The cultures were manually shaken daily in order to increase the growth rate of *P. tricornutum* and collect larger amounts of biomass. Stock cultures (100 mL) were used to inoculate 1 L of the culture medium. After 4 days culture and before the addition of $HgCl_2$ and/or the corresponding ligands of HA, Trp, Cys, Met, NTA and EDTA, respectively, the acidity of the culture solutions was adjusted and buffered using 0.01 mol L^{-1} MOPS at pH 7.5. The exposed *P. tricornutum* suspension (4 mL) was collected daily and its optical density was measured using a spectrophotometer at 680 nm wavelength (which is related to the absorption of chlorophyll *a*). The number of *P. tricornutum* in the 4 mL suspension was obtained based on the linear correlation between the optical density and the cell numbers counted using microscopy.

1.4 Hg toxicity towards *P. tricornutum* and total, surface-bound and intracellular Hg accumulated by *P. tricornutum*

The cell density of *P. tricornutum* was adopted as an indicator of Hg toxicity. After washing with natural seawater (filtered and sterilized prior to use) for three times and centrifuging at 4000×*g*, the obtained *P. tricornutum* cells from 25 mL culture solution were subjected to digestion with concentrated HNO_3 overnight, then diluted with ultrapure water for CVG-AFS to obtain the total amount of Hg accumulated. Surface-bound and intracellular Hg amounts were obtained as follows: the *P. tricornutum* cells were collected from 25 mL culture solution via centrifugation at 4000×*g* for 10 min, and the Hg remaining in the supernatant was determined using CVG-AFS. The *P. tricornutum* cell pellets obtained were washed three times using the natural seawater to remove any substrate and then resuspended for 10 min in 10 mL 0.01 mol L^{-1} DTE solution to remove the Hg adsorbed on the cell walls [19], the Hg in the supernatant after centrifugation at 5000×*g* for 15 min and digestion with HNO_3 was finally determined using CVG-AFS to obtain the amount of surface-bound Hg. The *P. tricornutum* cells obtained after washing by DTE were subjected to HNO_3 digestion and then determined using CVG-AFS to obtain the amount of intracellular Hg. The amounts of total, surface-bound and intracellular Hg in the control group were determined to be (0.25 ± 0.2), (0.14 ± 0.1) and (0.11 ± 0.1) μg g^{-1}, and were used to correct the experimental results.

1.5 Hg EC_{50} towards *P. tricornutum*

To investigate the toxicity of $HgCl_2$ to *P. tricornutum*, the alga was cultured under exposure to $HgCl_2$ at different concentrations (20, 40, 80, 100, 120, 140, 160, 180 and 200 μg L^{-1}). The 96 h cell numbers were measured to obtain a concentration-inhibition curve of the growth inhibition rate, which was calculated from the cell density determined against $HgCl_2$ concentration. The 96 h EC_{50} value for *P. tricornutum* was estimated to be 145 μg L^{-1} $HgCl_2$.

1.6 Hg-containing fractions and phytochelatins (PCs) inside *P. tricornutum*

An intracellular NH_4HCO_3-extract (N_2 saturated 50 mmol L^{-1} NH_4HCO_3 under ice-cold, pH 7.8) was obtained after ultrasonication for 2 min and centrifugation at 20000×*g* for 15 min. The Hg-containing fractions were analyzed using

SEC/ICP-MS, in which 50 mmol L^{-1} NH_4HCO_3 solution (pH 7.8) was used as a mobile phase at a flow rate of 0.75 mL min^{-1}. In addition, the fractions containing Hg and S were collected; after lyophilization, and were analyzed using MALDI-TOF-MS.

P. tricornutum cells exposed to different concentrations of $HgCl_2$ were collected, resuspended in ice-cold 1 mol L^{-1} NaOH-0.5% (w/w) $NaBH_4$ for ultrasonic extraction of their PCs in an ice bath [20]. The homogenates were centrifuged at 20000×*g* for 15 min at 4°C, and then a 500 μL aliquot of the supernatant was subjected to reaction with 50 μL TBP for 10 min to further reduce the disulfide-linkage of the PCs. An appropriate amount of 6 mol L^{-1} HCl was then added to adjust the pH to 1, and finally the homogenate was centrifuged again at 20000×*g* at 4°C. The supernatant obtained was used for the analysis of PCs using RP-HPLC with postcolumn DTNB derivatization. The assignments of the respective peaks were performed by directly infusing the HPLC effluents into ESI-IT-MS without postcolumn DTNB derivatization.

1.7 Characterization of MeHg converted by *P. tricornutum* under $HgCl_2$ exposure

All the culture solutions were sterilized at 120°C for 20 min, and stock cultures of *P. tricornutum* were grown in axenic conditions. Inoculation and other operation were performed in a sterile operating room. Cells were collected from 400 mL *P. tricornutum* suspensions under $HgCl_2$ exposure (20, 80 and 120 μg L^{-1}, respectively) via centrifugation at 4000×*g*. The obtained cell pellets and corresponding culture solutions were subjected to the following treatments: the cell pellets were resuspended in 10 mL 0.01 mol L^{-1} DTE for 10 min to wash off any surface-adsorbed Hg, then the cells were collected via centrifugation at 5000 g; after being resuspended in 2 mL KOH/CH_3OH (25%, w/v) solution [21], the cells were ultrasonicated then the homogenized suspension was extracted three times using 3 mL CH_2Cl_2 on a SKY-200B rocker (SUKUN, Shanghai, China) at 250 r/min for 1 h each time. After the CH_2Cl_2 phases obtained were combined and concentrated under a N_2-blow, they were subjected to HPLC/CVG-AFS. While the culture solutions obtained were acidified using 20 mL concentrated HCl then extracted using 30 mL CH_2Cl_2 mL and shaking for 1 h. The obtained CH_2Cl_2 phase of the three extracts were combined and concentrated followed the same procedures as in the case of the intracellular extracts. Moreover, parts of all the above samples were derivatized using $NaBPh_4$ after the addition of 0.1 mL 0.1% sulfuric acid, and then the derivatives were extracted using 2 mL hexane for GC-MS measurements. The results obtained from the control group using identical treatments were determined to be (0.08 ± 0.06) μg g^{-1} for *P. tricornutum* and (0.07 ± 0.04) ng L^{-1} for culture solution, and were used to correct all the results under $HgCl_2$ exposure.

2 Results and discussion

2.1 Growth status of *P. tricornutum* and distribution of accumulated Hg

The growth of *P. tricornutum* under exposure to various Hg species was evaluated in terms of cell density, which was determined using microscopic counting and visible spectrometry at 680 nm. The relationship of the cell density and exposure time under different concentrations of $HgCl_2$ is shown in Figure 1(a), indicating that the growth of *P. tricornutum* was inhibited when $HgCl_2$ was added to the culture medium. After 6 d of exposure, the density of *P. tricornutum* decreased by 3.6%, 7.6%, 14.5%, 27.6% and 34.3% under the stress of 20, 40, 80, 100 and 120 μg L^{-1} $HgCl_2$, respectively, when compared with that of the control, suggesting that the toxicity of $HgCl_2$ becomes greater with increase in $HgCl_2$ concentration. It should be noted that the growth rates of *P. tricornutum* under exposure to 100 and/or 120 μg L^{-1} $HgCl_2$ was remarkably decreased just after one day exposure, and then tended to a gently ascending plateau, implying that the *P. tricornutum* might have started possible self-detoxifying mechanisms. The 96 h EC_{50} value for *P. tricornutum* was estimated to be 145 μg L^{-1} $HgCl_2$ based on the concentration-inhibition curve of the cell density against $HgCl_2$ concentration from 20 to 200 μg L^{-1}. The total Hg accumulated by *P. tricornutum* increased from 196 to 2229 μg g^{-1} dry weight (DW) after 6 d of exposure alongside the increase in $HgCl_2$ concentration from 20 to 120 μg L^{-1} (Figure 1(b)). The amounts of Hg adsorbed on the surface of the cell wall (surface-bound Hg) increased from 108 to 1847 μg g^{-1} DW while those inside the cell (intracellular Hg) from 88 to 382 μg g^{-1} DW (Figure 1(c)). Bioaccumulation factors of Hg by *P. tricornutum* were 7.27×10^4 (intracellular Hg), 8.86×10^4 (surface-bound Hg) and 1.61×10^5 (total Hg) under the exposure of 20 μg L^{-1} $HgCl_2$; 2.04×10^4, 4.73×10^4 and 6.77×10^4 under 40 μg L^{-1} $HgCl_2$; 8.82×10^3, 3.87×10^4 and 4.75×10^4 under 80 μg L^{-1} $HgCl_2$; 7.66×10^3, 4.23×10^4 and 5.00×10^4 under 100 μg L^{-1} $HgCl_2$; and 7.44×10^3, 3.59×10^4 and 4.34×10^4 under 120 μg L^{-1} $HgCl_2$. The amounts of surface-bound Hg were about 1.2 to 4.8 times higher than those for intracellular Hg, suggesting that the cell wall played a more important role in accumulating and/or scavenging Hg. The interactions of cell walls with Hg are also suggested by FT-IR studies (Figure 2) showing that the transmittance at 3437 cm^{-1} was shifted to 3433 cm^{-1} after adsorption. This change might result from the chemical interaction of Hg with N–H and/or O–H groups present on the cell wall. After Hg adsorption, the C=O stretching vibration band shifted from 1647 to 1652 cm^{-1}, indicating that the *N*-acetyl group I band of proteins was involved in the cell-adsorption process of Hg. A significant shift was observed from 1068 to 1006 cm^{-1} after the cell-adsorption of Hg, suggesting that the chemical interactions take place between Hg and the Si–O or C–O of the saccharide on the

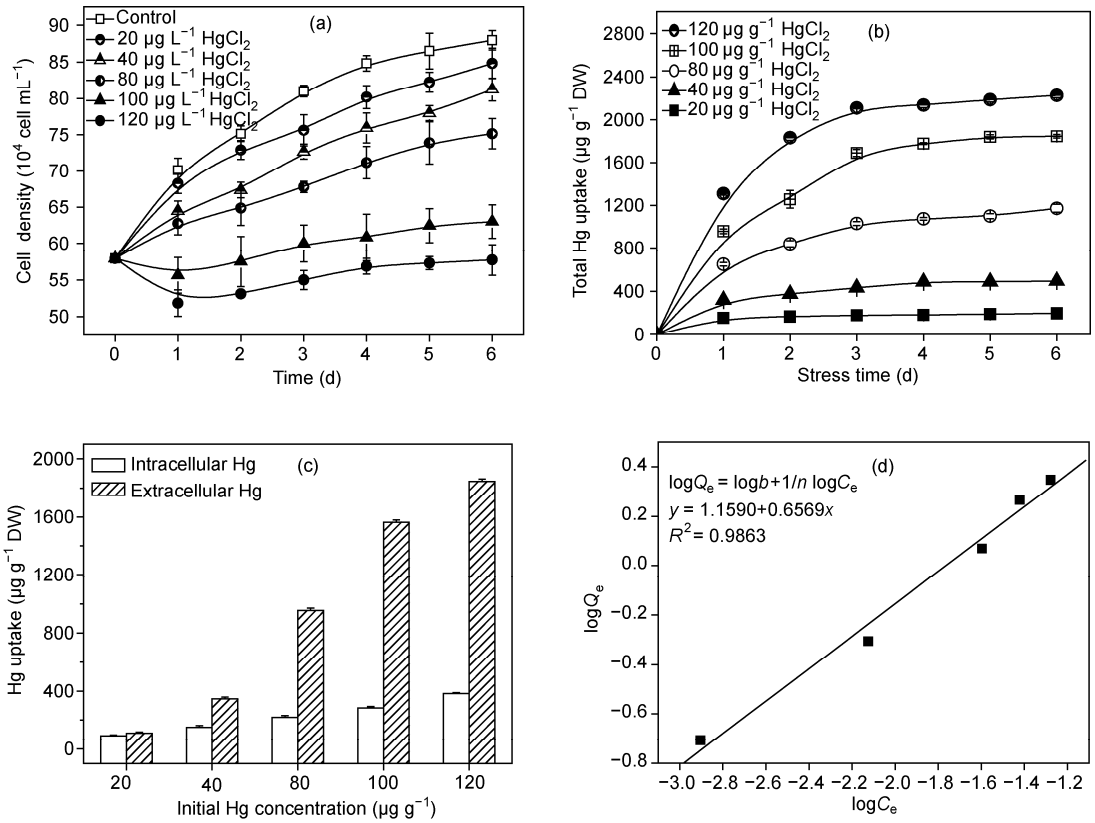

Figure 1 (a) Growth curves of *P. tricornutum* cells exposed to $HgCl_2$. (b) Total Hg accumulated by *P. tricornutum* cells exposed to $HgCl_2$ as a function of exposure time. (c) The intracellular and surface-bound distribution of Hg on *P. tricornutum*. (d) Freundlich isotherm of Hg adsorbed by *P. tricornutum* where log C_e is the logarithm of the equilibrium concentration of the Hg (mg L^{-1}), and log Q_e is the logarithm of the amount adsorbed (mg g^{-1}). Error bars represent the standard deviations of triplicate culture experiments.

cell wall. These results revealed the interactions between Hg and different functional groups such as amine, carboxyl, hydroxyl and silanol on the cell wall of *P. tricornutum.* Langmuir and Freundlich adsorption isotherm models were applied to understand the accumulation processes of Hg onto *P. tricornutum*, and the results obtained indicated that the accumulation of Hg by *P. tricornutum* was in accordance with the typical heterogeneous Freundlich adsorption process according to $\log Q_e = \log b + (1/n)\log C_e$, where Q_e is the amount adsorbed (mg g^{-1}), C_e is the equilibrium concentration of the Hg (mg L^{-1}), and b and n are the indicators of the adsorption capacity and adsorption intensity. Here, b = 14.42 and n = 1.52 (Figure 1(d)), and so the adsorption capacity and intensity are similar but somewhat weaker than typical Hg biosorbents such as *Spirulina platensis* (b = 81.43, n = 3.765) and *Aphanothece flocculosa* (b=141.61, n = 5.672) [22]. This result further confirmed a heterogeneous process of Hg accumulation by *P. tricornutum*, in which Hg interacted with the functional groups mentioned above and then entered the cell, resulting in both surface-bound and intracellular Hg distribution.

It is well known that ligands may change the chemical forms of heavy metals in the environment. Here Cys, Met, Trp and HA were chose as natural occurring ligands and

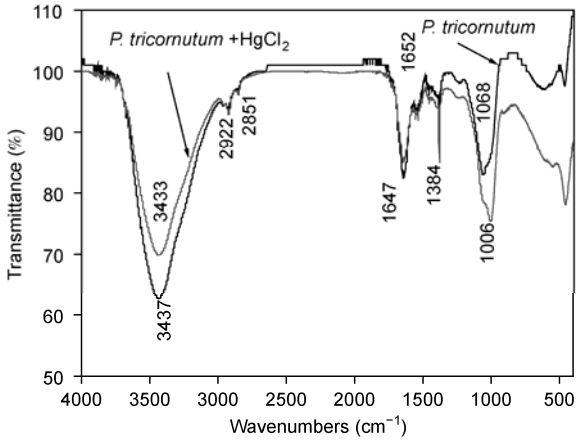

Figure 2 FT-IR spectra of *P. tricornutum* cell wall before and after cell-adsorption of Hg. The cells were stressed by 120 μg L^{-1} $HgCl_2$.

NTA and EDTA as anthropogenic ligands to mimic those in the seawater, and added respectively into the Hg-containing seawater culture solutions. $HgCl_2$ forms different chemical species accordingly with the ligands added, and these species formed, in turn, determine the toxicity of Hg and the sorption behavior as well as their intracellular transformation [23,24]. The results obtained indicated that the

growth status of *P. tricornutum* was inhibited remarkably in general in the culture solutions containing Hg and the ligands when compared with the control (Figure 3(a)). But compared with $HgCl_2$ (100 μg L^{-1}) alone in the culture solution, there was a 6.1% increase in the cell density when HA was added and 4.2% in the case of EDTA. These phenomena might be ascribed to the strong coordination abilities of HA (the stability constant log$K$ > 30 for Hg-HA) [25] and EDTA (log$K$ = 21.7) [26] to Hg, which greatly decreased the free Hg^{2+} concentration in the culture solution to 2.9×10^{-9} μg L^{-1} under the same mole concentration of HA (molecular weight was taken as 3373 D, 0.5 μmol L^{-1}) or EDTA (0.5 μmol L^{-1}). The corresponding total, surface-bound and intracellular Hg accumulated decreased 13.3%, 12.1% and 18.3% for HA, and 8.5%, 6.8% and 17.6% for EDTA. Inhibition of *P. tricornutum* growth was observed when Met (0.5 μmol L^{-1}), Trp (0.5 μmol L^{-1}) or NTA (0.5 μmol L^{-1}) were added into the culture medium, and the cell densities decreased 19.0%, 7.9% and 4.7% when compared with that of $HgCl_2$ (100 μg L^{-1}). However, the corresponding total, surface-bound and intracellular Hg increased by 31.1%, 30.0% and 36.1% for Met, 12.2%, 11.4% and 16.6% for Trp, and 8.3%, 7.8% and 10.9% for NTA (Figure 3(b)–(d)). Met and Trp improved the accumulation of Hg and chaperoned more Hg into the cell than did NTA. The Hg was complexed by all three, and easily taken into the cells, where it became more toxic than in the inorganic form $HgCl_2$ [27]. It is worth noting, however, that the addition of Cys (0.5 μmol L^{-1}) alleviated the toxicity of Hg and was favorable to the growth of *P. tricornutum*; the cell density increased 8.7% when compared with that of $HgCl_2$ (100 μg L^{-1}) although there were 18.5%, 17.8% and 22.6% increases for the total, surface-bound and intracellular Hg accumulated, implying that Cys might play a special role in detoxifying the toxicity of Hg towards *P. tricornutum*.

2.2 Transformation of Hg inside *P. tricornutum*

In order to understand the transformation of Hg species inside the *P. tricornutum* cell and the possible corresponding detoxifying mechanisms, we analyzed the intracellular extract of *P. tricornutum* (with NH_4HCO_3, pH 7.8) using SEC/UV/ICP-MS. The results obtained are shown in Figure 4(a). Hg and S were simultaneously detected in two main fractions (retention time from 8.0 to 10.5 and 20.3 to 22.6 min on the SEC). According to the calibration curve on the SEC using the standard compounds of known molecular weight under the same chromatographic conditions, we could estimate the Hg associated with high molecular weight (HMW) biomolecules (MW > 20 kD) in the first fraction (accounting for 68% of the intracellular Hg) and low molecular weight biomolecules (MW < 1500 D) in the second fraction (24%). It should be noted that the first fraction had a strong absorption at 280 nm but the second fraction had almost no

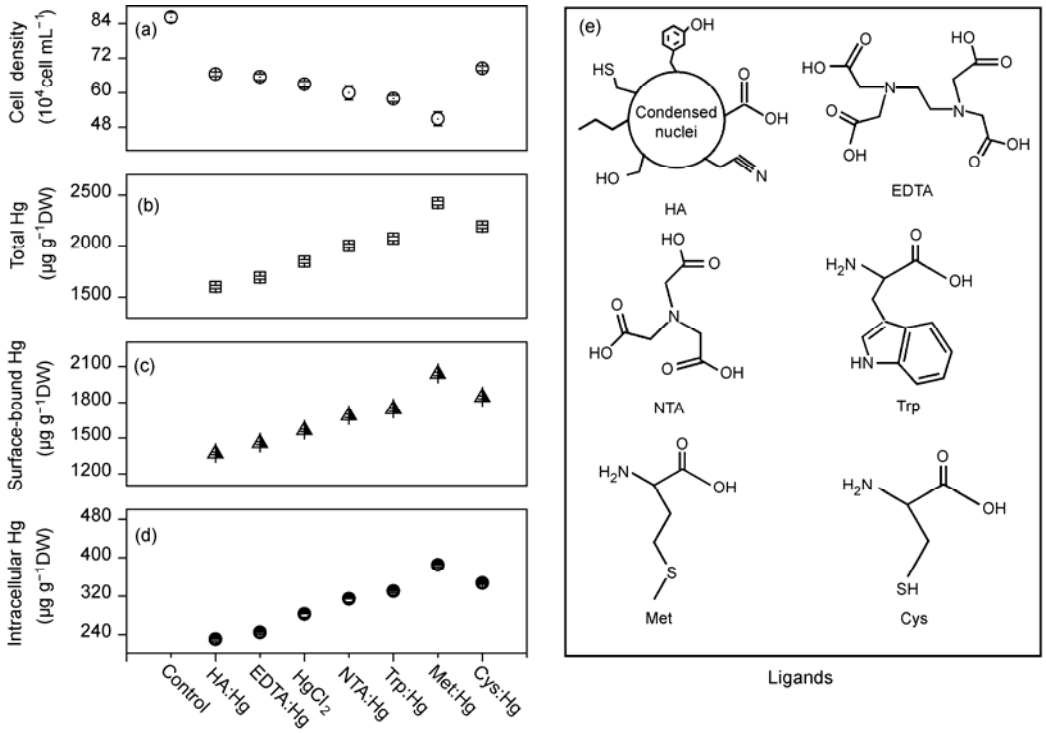

Figure 3 The cell density of *P. tricornutum* under exposure to 100 μg L^{-1} $HgCl_2$ alone and together with different ligands after 6 d (a). The total (b), surface-bound (c) and intracellular (d) Hg accumulated by *P. tricornutum*. The molar concentration ratio of Hg and the ligand was 1:1. The structure of the ligands (e). Error bars represent the standard deviations of triplicate cultures.

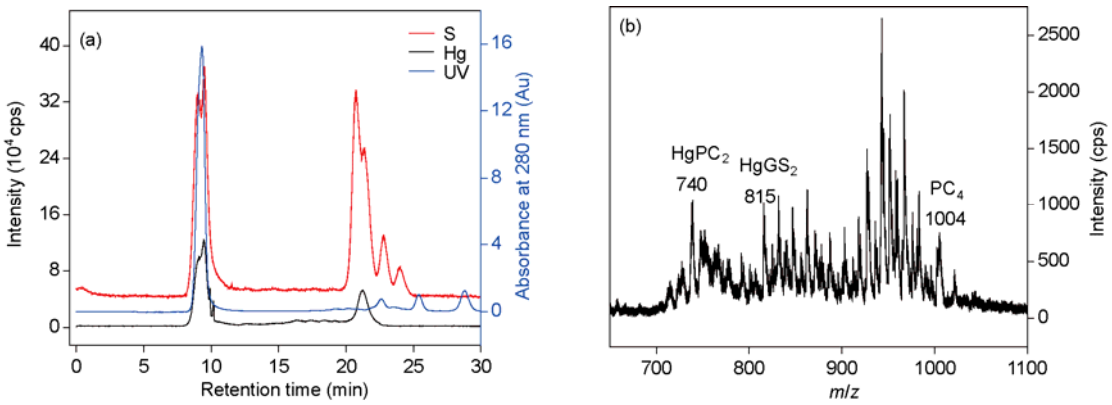

Figure 4 SEC/UV/ICP-MS showing the different Hg fractions in an NH_4HCO_3-soluble intracellular extract of *P. tricornutum* exposed to 120 μg L^{-1} $HgCl_2$ for 6 d (a). The red line represents the S signals, the black line the Hg signals and the blue line the UV absorbance at 280 nm, MALDI-TOF-MS spectrum (in positive ion mode) of the second fraction (b).

absorption at 280 nm. In this regard, we can speculate that Hg interacted with large proteins in the first fraction while in the second fraction with small peptides not containing phenylalanine, Trp and tyrosine such as GSH and PCs. This was confirmed by the fact that $HgGS_2$ and $HgPC_2$, as well as PC_4, were observed when the fraction was determined using MALDI-TOF-MS (Figure 4(b)). Previous studies also showed that some water plants (*Hydrilla verticillata* and *Vallisneria spiralis* L. [17]), a microalga (*Thalassiosira weissflogii* [28]) as well as some terrestrial plants (*Brassica napus* [29] and *Brassica chinensis* L. [30]) can produce PCs under the exposure to Hg.

In order to further investigate the intracellular metabolic behavior or defense mechanisms of *P. tricornutum* when exposed to Hg, the second intracellular fraction of *P. tricornutum* was acidified to pH 1 then infused into ESI-IT-MS. GSH and PC_2 to PC_5 were detected ($[GSH+H]^+$, *m/z* 308; $[PC_2+H]^+$, *m/z* 540; $[PC_3+H]^+$, *m/z* 772; $[PC_4+H]^+$, *m/z* 1004; $[PC_5+H]^+$, *m/z* 1236), confirming that Hg indeed induced the production of PCs inside *P. tricornutum* cells. The amounts of PCs induced were determined using DTNB-derivatization spectrophotometry at 410 nm (Figure 5 and Table 1). The results obtained under the exposure of different amounts of Hg indicated that not only the amount but also the type of PCs increased along with the increase in Hg concentration in the culture solution. When Hg concentration was 120 μg L^{-1}, 65 ± 13 nmol g^{-1} PC_2, 36 ± 11 nmol g^{-1} PC_3, 37 ± 10 nmol g^{-1} PC_4 and 16 ± 8 nmol g^{-1} PC_5 were induced. These results suggested that *P. tricornutum* was able to synthesize PCs to defend against the toxicity of Hg, in which Cys is an important precursor [30]. This helps us to explain the phenomenon whereby Cys in the culture solution can enhance the uptake of not only the total Hg but also the surface-bound and intracellular Hg while alleviating the toxicity of Hg when compared with the other ligands added. We thus speculated that the intrinsic defence mechanism of *P. tricornutum* was triggered to start synthesizing PCs when exposed to Hg, which in turn alleviated cellular toxicities through their superior mutual interactions.

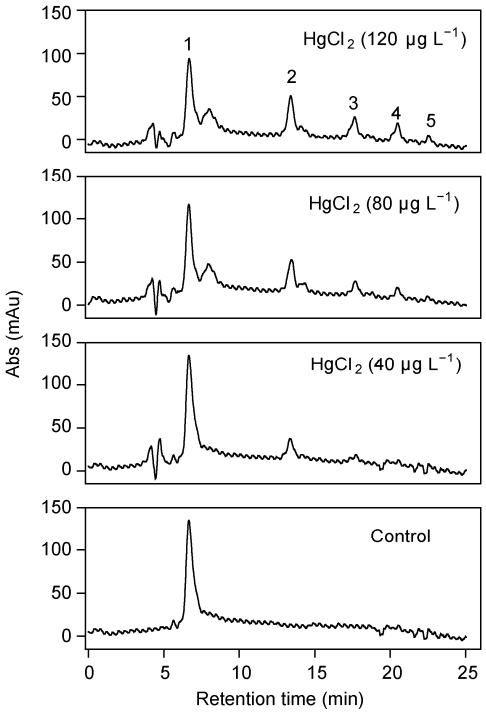

Figure 5 Chromatogram of PCs produced by *P. tricornutum* under the exposure of different concentrations of $HgCl_2$. The identified peaks using ESI-IT- MS are GSH (1), PC_2 (2), PC_3 (3), PC_4 (4), and PC_5 (5).

More surprisingly, MeHg was observed both inside the cells and in the culture solution when *P. tricornutum* was exposed by $HgCl_2$ (Figure 6). As we know, MeHg is a more toxic form of Hg and the major risk to the health of humans via consumption of contaminated fish and rice in some Hg-contaminated areas [6,31]. It is well documented that MeHg can be transformed from inorganic Hg in natural waters via biological ways (for example, periphyton [32], plankton produced iodomethane [33], macroalgae [34,35] and especially anoxic bacteria such as sulfate-reducing bacteria [36] in deep and littoral sediments are found to produce methylated

Table 1 Amount of GSH and PCs induced in *P. tricornutum* after exposure to different concentrations of Hg [a)]

Exposure conditions	GSH and PCs (nmol g^{-1} DW)					ΣPC (μmol g^{-1} DW)
	GSH	PC_2	PC_3	PC_4	PC_5	
Control	103 ± 15	n.d.	n.d.	n.d.	n.d	0
Hg [b)] (40)	109 ± 13	39 ± 8.3	12 ± 6.8	n.d.	n.d.	0.11 ± 0.1
Hg [b)] (80)	97 ± 11	46 ± 9.6	19 ± 6.5	20 ± 8.9	n.d	0.23 ± 0.1
Hg [b)] (120)	88 ± 12	65 ± 13	36 ± 11	37 ± 10	16 ± 8	0.47 ± 0.2

a) All values were expressed as mean ± SD of three repetitions from triplicate cultivations; DW, dry weight; ΣPC, molar concentrations of the sum of γ-Glu-Cys units of the detected PC variants. n.d., not detected. b) μg L^{-1}.

mercury) and non-biological ways [37] (such as methyltin, methylcobalamin and humic matter in sediments). In order to verify the production of MeHg when *P. tricornutum* was exposed to $HgCl_2$, we detected and identified MeHg in both the intracellular extract of *P. tricornutum* and the corresponding culture solution using HPLC-AFS and GC-MS. MeHg was detected in the *P. tricornutum* culture solutions using HPLC-AFS (Figure 6(b)) strictly following the MeHg extraction protocols [21], but no MeHg was detected in the control culture solutions containing no *P. tricornutum*. This was further confirmed by the GC-MS results which showed the Hg species of MeHgPh (*m*/*z* = 294), HgPh (279), MeHg (217) and Hg (202) as well as Ph (77), when using $NaBPh_4$ derivatization and electron impact as the ion source (Figure 6(c) and (d)). After one day of $HgCl_2$ exposure (at 20, 80 and 120 μg L^{-1}, respectively), the amounts of MeHg produced were found to be 2.1, 6.2 and 9.8 μg g^{-1} DW inside *P. tricornutum*; and 27, 42 and 63 ng L^{-1} in the culture solutions. Although the amount of MeHg to total Hg is much small, these results suggested that *P. tricornutum* could not only produce MeHg inside its cells but also release it into the culture solution. The relative lower MeHg concentration in the culture solution might be ascribed to the slow release rate of MeHg from *P. tricornutum* and the escape into the atmosphere from the culture solution due to its relatively higher vapor pressure at high ionic strengths (such as in the sea water used in this study) [38,39]. Although these observations were made, little is known at this moment concerning the methylating mechanism of *P. tricornutum*. More intensive research should be performed in the near future.

In conclusion, *P. tricornutum* can accumulate Hg and its bioaccumulation factor is at 10^4 levels. After biouptake, Hg inside *P. tricornutum* undergoes species transformation through interactions with the induced HMW sulfur-containing

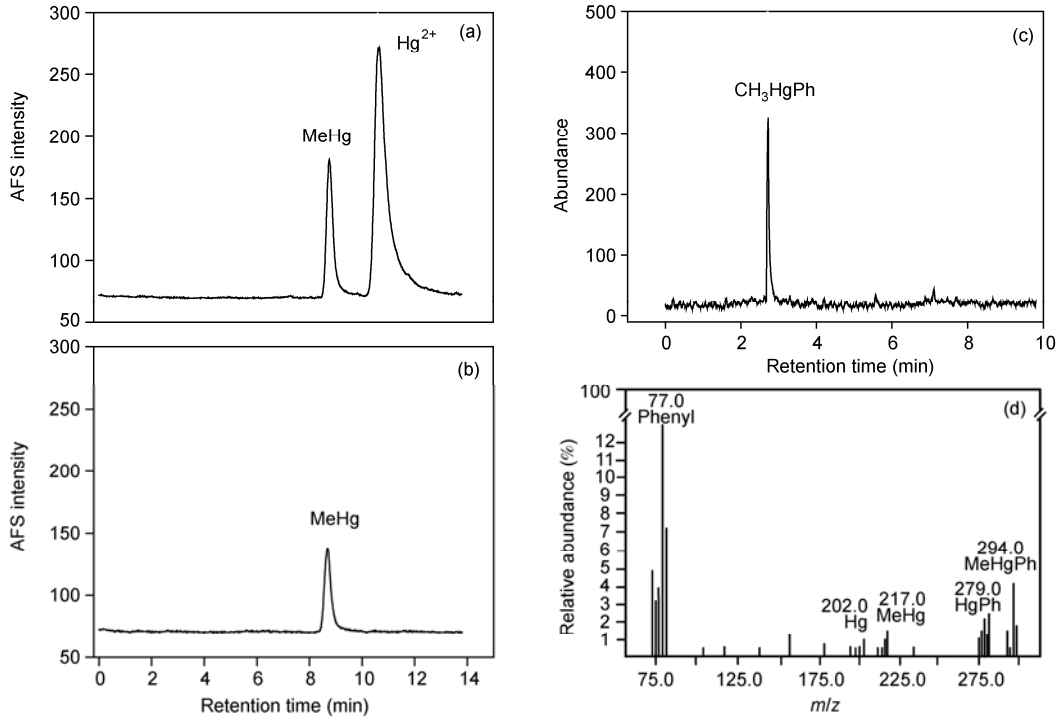

Figure 6 (a) The typical HPLC-AFS chromatogram of $MeHg^+$ and Hg^{2+} standards, 0.5 mg L^{-1}, sample injection 10 μL; (b) the typical HPLC-AFS chromatogram of the intracellular extract of *P. tricornutum* under the exposure of 120 μg L^{-1} $HgCl_2$; (c), (d) the GC-MS spectrum of the intracellular extract of *P. tricornutum* under the exposure of 120 μg L^{-1} $HgCl_2$ with $NaBPh_4$ derivatization.

proteins and PCs alleviating the toxicity of Hg; although the mechanism needs further and intensive investigation, on the other hand, *P. tricornutum* has the ability to convert $HgCl_2$ into MeHg, and to release the MeHg out of the cells, thus behaving as another defense mechanism. Even though all these characteristics of *P. tricornutum* were observed under the exposure of extremely high Hg concentration hopefully understanding the possible mechanisms of acute Hg stress response at this moment, these knowledge learned was helpful to understand the self-cleaning ability of aquatic ecosystems and the biogeochemical cycling of Hg as well as the bioaccumulation and biomagnification of Hg along the food-chain. Clearly, further researches are expected to perform under the exposure of Hg at the nature levels to find the chronic exposure response of *P. tricornutum*, so that a more comprehensive and accurate understanding of the role of marine algae in the self-cleaning ability of the oceans and the biogeochemical cycling of Hg can be achieved.

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