Trace metal accumulation and toxicity in marine phytoplankton

By

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A Thesis submitted to

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in the Department of Biology

July 2006, Hong Kong
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By

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This is to certify that I have examined the above PhD thesis and have found that it is complete and satisfactory in all respects, and that any and all revisions required by the thesis examination committee have been made.

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ads-Cd/Cu  cell-surface-adsorbed Cd/Cu
ads-IC50  IC50 was calculated from ads-Cd/Cu
AL  actinic light
ANOVA  analysis of variance
AP  alkaline phosphatase
ASTM  American Society for Testing and Material
BLM  Biotic Ligand Model
CA  carbonic anhydrase
CCCP  carbonyl cyanide m-chlorophenylhydrazone
[Chl a]  chlorophyll a concentration
C₀  cell density at time zero
Cᵣ  cell density at time t
d  slope of the standard curve of fluorescence vs. chlorophyll a extracted from the commercially available algae *Anacystis nidulans*
D  diffusion coefficient
DFB  desferrioxamine B
DMS  dimethylsulphide
DNA  deoxyribonucleic acid
DOC  dissolved organic carbon
DTPA  diethylenetriaminepentaacetic acid
DTT  dithiothreitol
e  empirically determined constants related to the affinity or binding strength for metal
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>EDAX</td>
<td>energy-dispersive X-ray microanalysis</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EELS</td>
<td>electron energy loss spectroscopy</td>
</tr>
<tr>
<td>F</td>
<td>steady-state fluorescence</td>
</tr>
<tr>
<td>F_A</td>
<td>fluorescence after acidification</td>
</tr>
<tr>
<td>F_i</td>
<td>fluorescence before acidification</td>
</tr>
<tr>
<td>FIAM</td>
<td>Free-Ion Activity model</td>
</tr>
<tr>
<td>F_m</td>
<td>maximum fluorescence</td>
</tr>
<tr>
<td>F_m'</td>
<td>practical maximum fluorescence</td>
</tr>
<tr>
<td>F_o</td>
<td>initial/dead/constant fluorescence</td>
</tr>
<tr>
<td>F_o'</td>
<td>the baseline fluorescence after AL is turned off</td>
</tr>
<tr>
<td>GF/F</td>
<td>glass fiber/fine</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>HQS</td>
<td>8-hydroxyquinoline-5-sulphonate</td>
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<tr>
<td>IC50</td>
<td>median inhibition concentration</td>
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<tr>
<td>ICP-AES</td>
<td>inductively coupled plasma atomic emission spectrometry</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>insol-Cd</td>
<td>Cd concentration in the insoluble fraction</td>
</tr>
<tr>
<td>intra-Cd/Cu</td>
<td>intracellular Cd/Cu concentration</td>
</tr>
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<td>intra-IC50</td>
<td>IC50 was calculated from intra-Cd/Cu</td>
</tr>
<tr>
<td>k</td>
<td>empirically determined constants related to the maximum metal binding capacity</td>
</tr>
<tr>
<td>K_d</td>
<td>metal cell distribution coefficient</td>
</tr>
<tr>
<td>K_e</td>
<td>metal efflux rate constant</td>
</tr>
<tr>
<td>K_{cond}^{FeL,Fe}</td>
<td>Fe conditional stability constant of organic ligands</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>$K_m$</td>
<td>half-saturation constant</td>
</tr>
<tr>
<td>$K_u$</td>
<td>metal uptake rate constant</td>
</tr>
<tr>
<td>LD cycle</td>
<td>light-dark cycle</td>
</tr>
<tr>
<td>LMW</td>
<td>low molecular weight</td>
</tr>
<tr>
<td>$M$</td>
<td>cellular metal concentration</td>
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<tr>
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<td>$[M^{2+}]-IC50$</td>
<td>IC50 was calculated from $[Cd^{2+}]/[Cu^{2+}]$</td>
</tr>
<tr>
<td>mBrB</td>
<td>monobromobimane</td>
</tr>
<tr>
<td>ML</td>
<td>modulated light</td>
</tr>
<tr>
<td>$M_0$</td>
<td>cellular or intracellular Fe concentration at time zero</td>
</tr>
<tr>
<td>MSA</td>
<td>methanesulfonic acid</td>
</tr>
<tr>
<td>$M_t$</td>
<td>cellular or intracellular Fe concentration at time t</td>
</tr>
<tr>
<td>$M_w$</td>
<td>ambient metal concentration</td>
</tr>
<tr>
<td>$n$</td>
<td>acid correction factor for chlorophyll a measurement</td>
</tr>
<tr>
<td>-N</td>
<td>nitrogen-starved</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>reduced form of NADP</td>
</tr>
<tr>
<td>$+NH_4^+$</td>
<td>nutrient-enriched with $NH_4^+$ as the N source</td>
</tr>
<tr>
<td>$+NO_3^-$</td>
<td>nutrient-enriched with $NO_3^-$ as the N source</td>
</tr>
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<td>NOEC</td>
<td>no-observed effect concentration</td>
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<td>NTA</td>
<td>nitrilotriacetate</td>
</tr>
<tr>
<td>$p$</td>
<td>probability</td>
</tr>
<tr>
<td>-P</td>
<td>phosphorus-starved</td>
</tr>
<tr>
<td>$P_{680}$</td>
<td>the primary electron donor of PS II</td>
</tr>
<tr>
<td>PAM fluorometer</td>
<td>Pulse Amplitude Modulated fluorometer</td>
</tr>
</tbody>
</table>
Paraquat 1,1'-dimethyl-4,4'-bipyridinium
PCs phytochelatins
PS I photosynthetic system I
PS II photosynthetic system II
Q_A the first plastoquinone in photosynthetic system
R cell radius
RNA ribonucleic acid
sol-Cd/Cu Cd/Cu concentration in the soluble fraction
sol-IC50 IC50 was calculated from sol-Cd/Cu
SP saturation pulse light
UV ultraviolet
\( \nu \) volume of 90% acetone used for the chlorophyll extraction
\( V \) volume of the sample filtered for the chlorophyll measurement
\( \Phi_M \) maximum PS II quantum yield
\( \Phi_{M'} \) operational PS II quantum yield
\( \gamma \text{GC} \) \( \gamma \)-glu-cys
\( \mu \) cell-specific growth rate
\( \rho \) metal uptake rate
\( \rho_{\text{max}} \) maximum metal uptake rate
\( \Sigma \gamma \text{-glu-cys} \) \( \gamma \)-glu-cys group concentration in PC_2 and PC_3
Trace metal accumulation and toxicity in marine phytoplankton

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Abstract

In this study, trace metal bioaccumulation and toxicity in marine phytoplankton were examined. For the metal bioaccumulation, the hypothesis that there is a direct relationship between the algal specific growth rate (μ) and metal uptake rate (ρ) was tested. For this purpose, μ was adjusted by changing the environmental factors (temperature and light) and ρ under each condition was then quantified. A hyperbolic relationship between μ and ρ of Cd and Zn was observed. And the potential changes in cell size, biochemical composition, and cell cycle with temperature and light would not affect ρ. Although a positive relationship was also observed between Fe uptake and cell growth, this relationship might not be true as Fe uptake is closely related to temperature and light.

Since the Fe requirements are different under different light and temperature conditions, then how the marine phytoplankton fulfilled the different Fe requirements was examined based on the three variables (cell growth, Fe uptake, and efflux). Although more Fe is required under the low light intensity to synthesize more pigments for light interception, ρ of Fe decreased with the decreasing light intensity. However, the cellular Fe concentration was kept constant or slightly increased at low
light intensity as the cell growth was strongly inhibited. Contrastively, the high Fe requirement under the high temperature was fulfilled mainly through the Fe uptake induction. And $\mu$ was kept almost constant under different temperatures. Although there was an obvious Fe efflux out of the cell, it had no effects on the trend of intracellular Fe concentration under different light and temperature conditions.

As for the metal toxicity, the metal and algal species-dependent toxicity difference was examined. The cyanobacteria *Synechococcus* was found to be the most sensitive, as its cell growth and PAM parameters were the most dramatically inhibited at the same metal level and its NOEC (No-Observed Effect Concentration) was the lowest among the four species tested. The toxicity of the three metals was also different following the order: Cd > Cu > Zn from high to low toxicity according to their cellular concentration at NOEC. Meanwhile, the PAM technique had a similar sensitivity to $\mu$ as the metal toxicity endpoints for marine phytoplankton.

Metal toxicity to marine phytoplankton was thought to be dependent on the ambient free metal ion or the cell surface adsorbed metal concentration based on the Free Ion Activity Model and Biotic Ligand Model. However, these two models are based on several assumptions and a lot of exceptions have been reported. In this study, I quantified the metal concentrations in different subcellular compartments, which were then linked to their toxicity in algae. It was found that Cd concentration in the soluble fraction could account for most of its toxicity difference in different nutrient conditioned cells. However, it was not the case for Cu. Therefore, a more functionally based subcellular fractionation was required in the future.
Chapter 1

General introduction

1.1 Trace metal toxicity to phytoplankton

1.1.1 Historical review

Trace metal toxicity to all organisms including our human beings has been widely recognized since the outbreak of Minamata disaster (Hg poisoning caused by eating polluted fish and shellfish in Minamata Bay) and Itai-Itai disease (Cd poisoning caused by eating rice which was irrigated with water polluted by Cd from mining) at around 1950 in Japan. After that a lot of research work was conducted on the interactions between trace metals and organisms. As phytoplankton are the primary producers lying at the bottom of food chain, the metal accumulation and toxicity in them may influence other organisms at higher trophic levels. Therefore, a particularly intensive attention was paid to phytoplankton-metal interactions. However, most of these early (pre-1970) studies put emphasis mainly on the target organisms as well as several biological variables such as life stage, nutrition, and age [1]. Whether the ambient metal chemical speciation plays an important role in its accumulation were not quantitatively recognized until the development of computer programs designed to perform complex chemical equilibrium calculations [2, 3], the introduction of a chemically defined phytoplankton culture medium (Aquil) by Morel et al. [4], and also the newly developed trace metal clean technique [5, 6].

Sunda and Guillard [7] observed that algal growth inhibition and cellular Cu content are closely related to the free Cu ion activity while not to the total Cu concentration. The growth response to Cu exposure was the same under the same $[\text{Cu}^{2+}]$ (free Cu ion concentration) even though the total Cu concentration was different due to the concentration change in the organic ligand
(trishydroxymethylamino methane). FIAM (Free Ion Activity Model) was thus
developed [7, 8]. It was hypothesized that only the free metal ion can be directly
taken up by the organisms and metal toxicity was determined by the free metal ion
concentration in the environment. As a generalization of FIAM, the BLM (Biotic
Ligand Model) further linked metal toxicity to its adsorption on the cell surface and
these surface metal binding ligands were called as biotic ligands [9-11]. For fish,
the biotic ligand is either known or suspected to be the sodium or calcium channel
proteins in the gill surface. For other organisms, it is hypothesized that a biotic
ligand exists and that toxicity can be modeled in a similar way.

1.1.2 Future direction

The development of FIAM and BLM are of great benefit to our understanding
about the trace metal toxicity and they are considered as powerful tools in developing
water quality criteria and in performing aquatic risk assessment for metals [12].
However, both models are based on several assumptions and a lot of exceptions have
been reported [1, 13]. For example, some metal organic or inorganic ligands
complexes (e.g., Cd-diethylthiocarbamate and Ag-Cl complexes) are lipophilic and
can be directly taken up by organisms. Therefore, their toxic effects are more
related to these lipophilic complexes as their concentrations are much higher than
those of the free metal ion. Further efforts are thus warranted trying to better
predict the metal toxicity. Recently, intracellular metal concentration has been
proposed as a better predictor of metal toxicity than the free metal ion. De
Schamphelaere et al. [14] found that ads- (surface-bound) and intra- (intracellular)
Cu have better toxicity predictability than the free Cu ion when pH is varied for two
freshwater green algae Chlorella sp. and Pseudokirchneriella subcapitata.
Meanwhile, a great deal of metal subcellular fractionation work has been conducted in aquatic invertebrates [15-18]. A notable fraction of the accumulated metal was found to be sequestered in metallothioneins and metal rich granules, which play important roles in intracellular metal detoxification (e.g., 32-43% Zn was incorporated in the metal rich granules of two clam species: *Mactra veneriformis* and *Ruditapes philippinarum*). Namely, these metal fractions have little contribution to its toxic effects in the whole organisms. Therefore, quantification of metal distribution in those metal-sensitive subcellular fractions (e.g., organelles and heat-sensitive proteins) may explain the metal toxicity better. However, few work has yet been conducted to determine the relationship between metal subcellular fractionation and its toxicity.

### 1.2 Metal accumulation in phytoplankton

Trace metals can be transported into the cells through the following ways: 1) diffusion through the ion channel; 2) diffusion through the gated ion channel; 3) diffusion across lipid bilayer (e.g., HgCl₂); 4) facilitated diffusion; 5) active transport with ion pump; 6) Endocytosis (e.g., Fe-transferrin complex). And facilitated diffusion with carrier proteins is thought to be the main pathway for most trace metals to cross the membrane [19]. Therefore, the metal transport from the environment to the intracellular region can be separated by three steps: 1) diffusion from the bulk solution to the cell surface; 2) complexation with binding sites on the cell surface; 3) internalization into the cells [1].

In FIAM, it is assumed that trace metal internalization through cell membrane is the limiting step. Under this condition the metal uptake rate can be expressed with the Michaelis-Menten equation:
\[ \rho = \frac{\rho_{\text{max}} M_w}{K_m + M_w} \]  

(1-1)

where \( \rho \) represents the metal uptake rate, \( \rho_{\text{max}} \) is the maximum uptake rate when the metal binding ligands are saturated, \( M_w \) is the ambient metal concentration, and \( K_m \) is the half-saturation constant, defined as the metal concentration when \( \rho \) equals to \( 0.5\rho_{\text{max}} \). The expression of metal uptake rate with the Michaelis-Menten equation, which was originally developed to describe the rate of enzyme-mediated reactions, further proves that trace metals enter the cells mainly through facilitated diffusion.

However, the assumption that metal internalization through the cell membrane is the limiting step for the trace metal accumulation in cells is not always met. Some metals' concentrations (e.g., Fe) are so low in the ocean that their diffusion from the bulk solution to the cell surface becomes the limiting step. In this case, the relationship between the metal uptake rate and its concentration in the bulk solution conforms to the following equation [20]:

\[ \rho = 4\pi R D M_w \]  

(1-2)

where \( R \) and \( D \) represent the cell radius and the diffusion coefficient of the metal, respectively.

At the same time the change of cellular metal concentration with time can be expressed by the following differential equation:

\[ \frac{dM}{dt} = K_u M_w - (\mu + K_e) M \]  

(1-3)

where \( M \) represents the metal concentration in the organisms. \( K_u \) and \( K_e \) are equal to the uptake and efflux rate constant, and \( \mu \) is the growth rate of the organism. ‘\( K_u M_w \)’ in Eq. 1-3 is equivalent to the metal uptake rate (\( \rho \)) with an underlying assumption that there’s a linear relationship between \( \rho \) and \( M_w \), which is true only when \( M_w \) is quite low (\( \ll K_m \)) based on Eq. 1-1. Under steady-state condition the
cellular metal content will not change any more (i.e., \( \frac{dM}{dt} = 0 \)) and it can be quantified as:

\[
M = \frac{K_a M_w}{\mu + K_e}
\]  \hspace{1cm} (1-4)

The efflux rate constant \( K_e \) is thought to be negligible as compared with \( \mu \) in previous studies. However, this assumption may not be true for phytoplankton, as the metal efflux from phytoplankton was scarcely examined [21].

1.3 Light and temperature effects on metal accumulation

Both light and temperature are important variables in the ocean. However, few research work has been performed focusing on the influence of light and temperature on metal accumulation and further its global biogeochemical cycling. Metal uptake by phytoplankton may be affected by light and temperature in several ways. On the one hand, the biochemical composition, protein activity, and biovolume of the phytoplankton cells are greatly dependent on temperature and light [22-27]. Harrison et al. [22] found that the amount of N per cell an index of protein content was lower under low irradiances for a marine phytoplankton Chaetoceros calcitrans, which may affect the metal bioaccumulation in the cells. On the other hand, the free metal ion concentration in the ocean may also be affected by the light and temperature. Light can induce metal photo-reduction (e.g., Fe and Cu) and temperature can affect the thermal formation/dissociation of organic metal complexes as well as the oxidation of photo-produced metal [28]. When the light intensity is high, more Fe (III) was reduced to Fe (II) in the ocean. Although some of the Fe (II) was quickly re-oxidized to Fe (III) by \( O_2 \) or \( H_2O_2 \), high light intensity increased the steady-state concentration of biologically available Fe (II) and Fe (III)
and thus Fe uptake was induced. Furthermore, cellular requirements of those essential metals may also be different under different light and temperature conditions. Phytoplankton acclimated at lower light intensity tend to have a higher Fe requirement in the photosynthetic system for the additional pigment synthesis to intercept enough energy for cell growth [29]. Similar trend may also be observed for cells acclimated at higher temperature, as high temperature mimics low light for its effect on the photosynthetic system [30]. These factors are intertwined with each other, which makes it hard to directly estimate what roles the light and temperature have in the metal accumulation in phytoplankton.

1.4 Macronutrients and trace metal interactions

There’s a strong interaction between macronutrients and metals in phytoplankton. Exposure to high metal concentrations can suppress algal macronutrient assimilation either in a competitive (e.g., competition among nutrients and metals for the catalytic sites of the enzymes and carriers) or non-competitive (e.g., an alteration in the structure of the enzyme responsible for its uptake/transport) manner [31]. On the other hand, ambient nutrient concentration may also affect metal accumulation in phytoplankton. Cd uptake by -N (N-starved) algae (either laboratory culture or natural phytoplankton community) was strongly stimulated after the starvation was eliminated [32-34]. At the same time macronutrients play an important role in metal detoxification. P is known to sequester excessive metals in polyphosphate bodies of freshwater algae [35-37]. And the synthesis of PCs (phytochelatins), which are polypeptides for metal detoxification in cells [38], may also be regulated by the ambient nutrient concentration. It is thus hypothesized that
metal toxicity to phytoplankton may be dependent on the ambient nutrient concentrations, but no consistent results were obtained.

1.5 Research objectives

My PhD projects focus mainly on the two interrelated aspects: metal accumulation and its toxicity in marine phytoplankton. The steady-state intracellular metal accumulation (M) was determined by three variables: $\mu$, $\rho$ ($K_a M_w$) and $K_e$ (Eq. 1-4). In previous studies, $K_e$ was always thought to be negligible as compared with $\mu$. Therefore, M is equal to $\rho$ divided by $\mu$ under the steady-state condition. Sunda and Huntsman [39] summarized the feedback relationships between M and $\mu$, as well as the M and $\rho$ in marine phytoplankton. However, any interrelationship between $\rho$ and $\mu$ was less clear from such feedback regulation. In the previous study of our lab [32, 33], the effect of cell growth on metal uptake was examined by culturing the algae under different nutrient conditions (e.g., nutrient-enriched and starved conditions). Although the different nutrient condition could change the cell growth, it may also affect the metal uptake which obscured the observed positive correlation between $\rho$ and $\mu$. Therefore, a more direct examination of relationship between $\mu$ of a marine diatom *Thalassiosira pseudonana* and $\rho$ (Cd and Zn) was performed in chapter 2. $\mu$ was controlled by three environmental factors (light intensity, LD [light-dark] cycle, and temperature), which have no known direct effects on Cd and Zn uptake.

In chapter 3, the cellular Fe concentration, its $K_e$ and $\rho$, as well as $\mu$ under different light and temperature conditions were measured. As the cellular Fe requirement is different under different light and temperature conditions, it would be interesting to know how phytoplankton fulfill the different Fe requirement based on
the three variables \( (K_e, \rho, \text{ and } \mu) \). As light and temperature can affect Fe accumulation in several ways, the direct relationship between \( \mu \) and \( \rho \) cannot be shown as what we did for Cd and Zn. The Fe accumulation was quantified with both the long-term and short-term uptake experiments. These two techniques were widely used in metal accumulation research while no direct comparison has ever been performed. We also tried to measure Fe efflux under different light and temperature conditions and its contribution of cellular Fe concentration was examined.

At the same time metal toxicity to marine phytoplankton was studied. In chapter 4, we’d like to know the metal and algal species-dependent toxicity difference with a relatively new technique PAM (Pulse Amplitude Modulated) fluorometer and cellular metal concentration was used as a first trial to predict its toxicity in phytoplankton. Furthermore, the hypothesis that PAM technique is more sensitive than \( \mu \) as toxicity endpoints was also tested. Later in chapters 5 and 6, Cd and Cu toxicity under different nutrient conditions (e.g., \( +\text{NO}_3^- \) [nutrient-enriched with \( \text{NO}_3^- \) as the N source], \( +\text{NH}_4^+ \) [nutrient-enriched with \( \text{NH}_4^+ \) as the N source], -N and -P [P-starved] conditions) were examined. Their intracellular concentration as well as subcellular distribution was also quantified and was used to explain the potential toxicity difference under different nutrient conditions. The cellular contents of LMW (low molecular weight) thiols and their influence in Cu detoxification were also examined in chapter 6.

1.6 Trace metals used

The metals used in my study include Cd, Cu, Fe, and Zn. They are all essential elements except Cd, although it can substitute Zn as a cofactor of CA
(carbonic anhydrase) when the cells were depleted of Zn [40-42]. As a result of excessive urbanization, a lot of terrestrial and aquatic systems are getting highly polluted by Cd, Cu, and Zn. A mass of research work has been performed to examine their potential influence in the whole ecosystem including our human beings, which is instructive to the establishment of water control criteria and risk assessment of the trace metal pollution. Contrastively, Fe is a potentially limiting nutrient for the growth of marine phytoplankton due to its extremely low concentration in some oceanic and upwelling coastal areas [43-45]. Fe limitation of phytoplankton growth and further the drawdown of greenhouse gas CO₂ from the atmosphere are among the ‘hottest’ issues in recent oceanography. The physicochemical characters and biological functions of these metals were shown as follows.

Cd is a non-essential element with only one oxidation state (II). Up to 70% of the dissolved Cd in the ocean was complexed with some unknown organic ligands [46]. As for the inorganic Cd species remained, 95% was held in strong Cd-Cl complexes (i.e. CdCl⁺, CdCl₂⁰, and CdCl₃⁻) [47]. Cd is among the earliest trace metals whose toxicity have been recognized since the breaking out of Itai-Itai disease (ouch-ouch joint pain) at around 1950. As a very common environmental pollutant, it is one of the most frequently used trace metals for the toxicity research. Its toxic effects on the photosynthetic system will be shown later in Chapter 4.

Cu has three oxidation states (I, II, III) in biological system. Cu (I) and (II) are the most effective monovalent and divalent cations to bind with organic ligands, respectively [48]. Cu exists mainly as particulates (e.g. CuS) in the ancient ocean and the dissolved Cu concentrations were very low. Thus only trace amounts are required by organisms. As the O₂ concentration in the atmosphere and seawater strongly increased with more and more photosynthetic organisms appearing on the
earth, the binding ligands were oxidized and Cu was released to the dissolved phase. Thus Cu became a potentially toxic element to the organisms. Its toxic effect to the phytoplankton will be discussed later in Chapter 4. As a micronutrient, Cu also has a major beneficial role in photosynthetic system as a structural and electron exchange component of plastocyanin. As the Fe concentration in the ocean is very low, it is often found that the phytoplankton can use plastocyanin as a substitute for Fe containing enzyme cytochrome C₆. Cu is also used in the reduction of NADP (nicotinamide adenine dinucleotide phosphate) to NADPH (reduced form of NADP) and acts as a cofactor of cytochrome C oxidase in the respiration system [48-50].

Fe has three oxidation states within the cell (II, III, IV) [49] and is particle reactive. As the ocean turned from anoxic to oxygenic condition, the concentrations of total dissolved Fe have fallen by about 12 orders of magnitude [48]. Thus different strategies have been used by the phytoplankton to alleviate the effects brought about by Fe depletion in the environment, such as the substitution of cytochrome C₆ by plastocyanin and the release of DMS (dimethylsulphide) to seize Fe containing particles in the atmosphere [51]. Another very important way is the excretion of the Fe specific complexing ligands (siderophores), which can bind with Fe and prevent it from precipitation [45]. Later the siderophore-Fe complexes can be transported into the cells directly. As a nutrient, Fe is fundamental to the physiology of prokaryotic and eukaryotic cells. It is used in photosynthesis and respiratory electron transport, nitrate or nitrite reductase, nitrogenase for N₂ fixation, and some catalase, peroxidase etc. [52].

Zn has a high electron affinity as a Lewis acid. It can bind to the same centers as iron but it has no redox chemistry which enables it to function as a catalyst center. Zn is more commonly used in the eukaryotes than in the prokaryotes as the dissolved
Zn concentration increased by about four orders of magnitude as a result of the increase of the O₂ concentration in the atmosphere. Zn is an important cofactor of DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) polymerases, CA, and AP (alkaline phosphatase). Zn can also exert some toxic effects when its concentration gets to a very high level, which will be shown later in Chapter 4.

1.7 PAM method for the toxicity monitoring

The PAM fluorometer, as developed by Schreiber and manufactured by Walz (Germany), has been applied to the toxicity research for nearly twenty years [53-55]. It was reported that this non-intrusive technique is more sensitive than other parameters (e.g. growth rate) as toxicity endpoints [56].

Generally the light energy adsorbed by the chlorophyll can be dissipated through four different ways: (a) conversion into chemically bound energy in the photosynthesis products; (b) conversion into energy used in the metabolic processes which has not been stored; (c) reemission as fluorescence; (d) conversion into heat in the photosynthetic system and increase the temperature [57]. Although the amount of energy dissipated as fluorescence is very small (ca. 3-4 %), it can act as an indicator of the light energy distribution in the cells and further the physiological condition of the algae.

A typical fluorescence induction curve with PAM method was shown in Fig. 1-1. The cells have been put in darkness for about 15 min to completely oxidize/open the PS II (photosynthetic system II) reaction center. The cooperation of PS I (photosynthetic system I) and PS II to drive the photosynthetic electron transport are temporally impaired after being in darkness for 15 min. At the beginning of the PAM fluorescence measurement only the ML (modulated light) is
on and it is so weak that it will not affect the complete open state of the reaction center. Thus the fluorescence observed represents the fluorescence yield when all PS II reaction centers are open. And the fluorescence, which is also called as constant, ground, or dead fluorescence ($F_0$), derives from energy migration processes in the pigment antenna and is independent of photochemical events as well as the light intensity. $F_m$ (maximum fluorescence) is the fluorescence measured with the SP (saturating pulse light) on. Under this condition the PS II reaction centers have been completely reduced / closed and no light energy can be transferred through the photosynthesis transport chain. Thus the maximal fluorescence yield is obtained. After the AL (actinic light) is turned on, the baseline fluorescence level increases first due to the gradual reduction of the PS II reaction centers. It then decreases to a steady level as PS I reoxidizes the PS II reaction centers and there’s also an obvious non-photochemical quenching at this time. The change of fluorescence baseline after the cells are exposed to AL from darkness is often mentioned as Kautsky effect [58, 59]. The practical maximal fluorescence ($F'_m$) in Fig 1-1 represents the fluorescence yield with the PS II reaction centers closed but with an obvious non-photochemical quenching as compared with $F_m$. $F$ (steady-state fluorescence) represents the baseline fluorescence yield when the cells are fully acclimated to the AL. After the AL is off, the baseline fluorescence level, which is indicated as $F'_0$, decreases as more PS II reaction centers are reoxidized.

Generally two PAM parameters ($\Phi_m$ and $\Phi'_m$) were frequently used in the toxicity research work, which represent the maximum and operational PS II quantum yield, respectively. Their calculations are shown as follows [55, 56, 60-62]:

$$\Phi_m = \frac{F_m - F_0}{F_m}$$  \hspace{1cm} (1-5)
\[ \Phi_m' = \frac{F_m' - F}{F_m'} \]
Figure 1-1 Typical fluorescence induction curve with PAM method. The fluorescence yields for the calculation of PAM parameters were labeled ($F_o =$ initial fluorescence; $F_m =$ maximum fluorescence; $F_m' =$ practical maximum fluorescence; $F =$ steady-state fluorescence; $F_o' =$ final fluorescence with AL off). The turning on/off (+/-) of different types of light sources was also indicated in the figure (ML = modulated light; SP = saturating pulse; AL = actinic light).
Chapter 2

Relationships between cell specific growth rate and uptake rate of

Cd and Zn by a coastal diatom

2.1 Abstract

Despite that the influences of metal chemistry on its uptake in marine phytoplankton have been well studied, the effects of physiological processes such as the cellular growth remain less well-known. In this study, $\mu$ of a coastal diatom Thalassiosira pseudonana (clone-3H) was modified by three environmental factors, including the temperature, irradiance, and LD cycle. The uptake of Cd and Zn was subsequently quantified using short-term exposure. These environmental factors had notable effects on the metal uptake. $\rho$ increased with increasing irradiance, temperature, and illumination period. We demonstrated that $\rho$ initially increased in proportion with an increase in $\mu$, and then remained constant. The uptake of Cd and Zn by the diatoms was not inhibited by two photosynthetic inhibitors, suggesting that the photosynthetic activity had little contribution to their uptake. There was no obvious diel trend for the metal uptake and the cell size was kept relatively constant under the different environmental conditions. Thus in this study, cell size, photosynthetic activity, and cell cycle were unlikely to account for the variations in $\rho$ at different temperatures, irradiiances, and LD cycles. The dependence of metal uptake on $\mu$ may have important implications for the prediction of metal accumulation by marine phytoplankton in different ecosystems or during phytoplankton blooming period. Further studies are needed to examine the underlying mechanisms for such relationship at the cellular and sub-cellular levels.
2.2 Introduction

There has been a longstanding interest in trace metal uptake by marine phytoplankton [39, 48, 63]. Many aspects about trace metal uptake have been considered, including their transport mechanisms, physiological and biochemical functions etc. [1, 40, 42, 52, 64, 65]. There are also many studies on the influences of metal speciation and concentration in their uptake by marine phytoplankton. The relationship between $M_w$ and $p$ has been well established using trace metal buffer solutions [39, 66]. Recent studies also considered the metal uptake by phytoplankton under different nutrient regimes [32, 67], or metal and phosphate uptake under different iron concentrations [68]. However, the effects of temperature, irradiance and LD cycle, which are all important environmental variables in the ocean, on metal uptake by phytoplankton have been scarcely examined. Most metal uptake experiments by marine phytoplankton have been conducted at the optimal temperature and light conditions for cell growth. However, several studies showed that the biochemical composition, protein activity, and biovolume of phytoplankton are greatly dependent on temperature and light [22-27]. Whether or not these biological changes can affect the algal metal uptake remains mostly unknown.

According to a simple kinetic equation (Eq. 1-4), $M$ under steady-state conditions can be predicted by $p$ divided by $\mu$ of the cells (assuming that the metal efflux is negligible as compared to the cell growth) [39]. However, the inter-relationship between $p$ and $\mu$ remains to be tested for further refinement of the kinetic equation [32]. Previous limited studies testing the inter-relationship between $\mu$ and $p$ were all performed by varying one environmental parameter (e.g., metal speciation, metal concentration, nutrient condition). Whether these factors
can affect both $\mu$ and $\rho$ simultaneously, or if there is an interaction between $\mu$ and $\rho$ is rather speculative. Understanding such an interaction has great implications on the prediction of metal dynamics in marine phytoplankton due to a change in their growth rate (e.g., bloom conditions) and thus their potential transfer to higher trophic levels. Nevertheless, this relationship has been seldom considered in previous studies.

In this study, three environmental factors (temperature, irradiance and LD cycle) were used to modify $\mu$ of a coastal diatom *Thalassiosira pseudonana*. $\rho$ of Cd and Zn was subsequently quantified and the inter-relationship between $\rho$ and $\mu$ of the diatom cells was tested. Zn is an important micronutrient to the phytoplankton, involved in the hydrolysis of phosphate esters, the replication and transcription of nucleic acids, and the hydration and dehydration of $\text{CO}_2$ (e.g., CA) [52]. Cd can substitute for Zn when Zn is depleted and has similar physiological functions for the diatoms [40-42]. To examine whether $\rho$ of Cd and Zn was dependent on the photosynthetic system, we employed two photosynthetic inhibitors to suppress the photosynthetic activity of the cells. Recent studies have used these inhibitors to diagnose the uptake mechanisms [21, 69]. CCCP (carbonyl cyanide m-chlorophenylhydrazone) can disconnect electron transport from ATP synthesis, thereby inhibiting photophosphorylation and partly disrupting the energy production of the cells. The other inhibitor, the herbicide paraquat (1,1’-dimethyl-4,4’-bipyridinium) used in this study can accept electrons from PS I and inhibit the reduction of ferredoxin. We also tested the metal uptake as influenced by diel cycles. Recent works have demonstrated the diel periodicity of some biochemical variables such as protein activity, cellular C and N contents of the phytoplankton [70-72]. In addition, changes in $\rho$ with $M_w$ were examined to ensure
that the Cd and Zn uptake was below saturation at the metal concentration levels used in this study.

2.3 Materials and methods

Axenic culture of *Thalassiosira pseudonana* (clone 3H), which is a common coastal diatom, was obtained from the Provasoli-Guillard Center for the Culture of Marine Phytoplankton, Bigelow Laboratory, Maine, USA. The cells were maintained in f/2 medium [73] with sterile techniques, in an incubator at 19°C with a light illumination of 170 μmol photons m⁻² s⁻¹ on a 14:10 LD cycle. All seawater was collected 10 km off East Hong Kong to minimize the influences of anthropogenic activities, and was filtered through 0.22 μm Poretics membrane before being used in the experiments. The background dissolved Cd and Zn concentrations in the seawater were 0.49 nmol l⁻¹ and 12.3 nmol l⁻¹, respectively.

2.3.1 General experimental protocols

Two radionuclides, ¹⁰⁹Cd (in 0.1 mol l⁻¹ HCl) and ⁶⁵Zn (in 0.1 mol l⁻¹ HCl), were used to trace the uptake of stable Cd and Zn by the diatoms. They were obtained from New England Nuclear, Boston, USA. The ¹⁰⁹Cd (2.53 nmol l⁻¹) and ⁶⁵Zn (0.02 nmol l⁻¹) radionuclides, and stable Cd (nominal concentration of 17.8 nmol l⁻¹) and Zn (nominal concentration of 76.9 nmol l⁻¹) were spiked into the 0.22 μm filtered seawater without any other nutrient addition, and the pH was adjusted to 8.2 ± 0.1 using Suprapure NaOH. This uptake medium was then equilibrated for 12 h under the same conditions as the uptake period before the uptake experiments. The total concentration of Cd and Zn in the uptake medium was 20.8 nmol l⁻¹ and 89.2 nmol l⁻¹, respectively, including the background, radionuclides, and stable metals. The radioactivity of ¹⁰⁹Cd (at 88 keV) and ⁶⁵Zn (at 1115 keV) was determined by a
Wallac gamma detector. Counting time was adjusted to result in propagated counting errors of <5%.

The diatoms were acclimated at different temperatures and light conditions before the uptake measurements. A 14:10 LD cycle was used throughout this study except the LD cycle experiment. During the acclimation period, the diatoms were grown under different temperatures and light conditions for 4-5 days until they reached the mid-exponential growth phase. Cells were enumerated every 12 h by a Coulter Counter. Cell size was also measured concurrently. $\mu$ was quantified as the slope of linear regression between the natural log of the cell density and the growth time. When the cells reached the mid-exponential phase, they were harvested by filtering gently (<100 mm Hg) onto the 3 $\mu$m polycarbonate membrane and then rinsed with 0.22 $\mu$m filtered seawater three times. They were then resuspended into the uptake medium at an approximate cell concentration of $3\times10^5$ cells ml$^{-1}$. The light and temperature conditions in the uptake period were the same as those during the acclimation period. Each experimental treatment had three replicates. The short-term uptake lasted 1 or 4 h. During the uptake period, a 10 ml aliquot was filtered through the 1 $\mu$m polycarbonate membrane for measurements of the radioactivity in the cells at each time point (1, 2, 3, and 4 h for the 4 h uptake or 0.25, 0.5, 0.75, and 1 h for the 1 h uptake). The metal adsorbed onto the cell wall was removed by Ti-technique wash [74, 75]. A 2 ml aliquot was also removed for measurement of total radioactivity in the uptake medium. The accumulated content of Cd and Zn was calculated as follows:

Accumulated content ($\mu$g g$^{-1}$) = 

\[
\frac{\text{radioactivity g}^{-1} \text{ dry weight cells} \times \text{total metal concentration } \mu\text{g l}^{-1}\text{)}}{\text{the radioactivity of medium l}^{-1}\text{)}}
\]  

(2-1)
Thus, only the intracellular accumulation of Cd and Zn was quantified in our study. The cell number was counted both at the beginning and the end of the uptake experiments. The dry weight of the cells was measured as described by Wang and Dei [33]. Briefly, the cells were first filtered through a pre-combusted GF/F (glass fiber/fine) membrane, rinsed with ammonium formate, and dried at 80°C for 1d.

2.3.2 Uptake kinetics at different ambient Cd and Zn concentrations

During the acclimation period, the cells were grown at 15 °C with the irradiance 340 μmol photons m⁻² s⁻¹. When the cells reached the mid-exponential phase, they were collected and the metal uptake experiment was conducted. The experimental 0.22 μm filtered seawater used for the uptake medium was first passed through the Chelex 100 resin column and the dissolved organic matters were destroyed by UV (Ultraviolet) irradiation (24 h, with removal efficiency close to 100%). There were 6 concentration treatments with two replicates for each treatment. The total metal concentrations (including the stable metals and radioisotopes) used were 1.28, 3.55, 35.5, 150, 350, 1000 nmol l⁻¹ for Cd, and 1.2, 5.0, 10, 21, 100, 200 nmol l⁻¹ for Zn. Trace metal clean technique was used and all uptake experiments were conducted in a Class 100 clean bench. The relationship between ρ and Mₜ follows the Michaelis-Menten equation (Eq. 1-1).

2.3.3 Irradiance and temperature experiments

During the acclimation period, the cells were grown under 3 different irradiances (40, 170, and 340 μmol photons m⁻² s⁻¹) and 3 temperatures (15 °C, 19 °C, and 24 °C). Thus, there were a total of 9 different temperature × irradiance treatments. These light intensities were chosen to obtain a relatively low, intermediate, and a quite high cell growth, respectively. And the cell growth was not photoinhibited yet under the highest irradiance. However, temperature had
much less effects on the cell growth based on our preliminary experiments. The
cells were grown under these conditions for 4-5 days and the cell concentration was
monitored every 12 h. Different irradiances were achieved using different numbers
of white fluorescent tubes and adjusting the distance between the cultures and light
sources. When the cells reached the mid-exponential phase, the metal uptake by the
cells was then performed over a 4-h period. In all experiments, the uptake was
conducted simultaneously for each temperature treatment.

2.3.4 LD cycle experiment

Before the metal uptake quantification, the cells were cultured for 6 days with a
light intensity of 170 μmol photons m⁻² s⁻¹ and at two temperatures (19 °C or 24 °C).
Four different LD cycles were used: 4:20, 10:14, 16:8 and 24:0 h. No complete
dark treatment was included in this experiment because the cells did not grow under
the full-dark condition over the 6-day period. In some treatments (i.e., fast cell
growth during the acclimation period), the cells were transferred to the new f/2
medium to avoid nutrient depletion.

2.3.5 Photosynthetic inhibitors

The concentrations of the inhibitors used in our experiments were 5 and 25
μmol l⁻¹ for CCCP and 10, 50, and 150 μmol l⁻¹ for paraquat [69]. There was a
control treatment without the addition of inhibitors for CCCP and paraquat
treatments, respectively. The cells were acclimated for 3-4 days under the
illumination of 170 μmol photons m⁻² s⁻¹ and 19 °C. They were then exposed to
different concentrations of the inhibitors for 2 h. Afterwards, the cells were filtered
and resuspended in the uptake medium.

2.3.6 Diel variation
The cells were grown for 3-4 days under the irradiance of 170 μmol photons m$^{-2}$ s$^{-1}$ and 19 °C in a 14:10 LD cycle. The uptake experiments were then conducted over a 24 h period at every 2-3 h interval, under the same LD cycle. When the cells were under the dark cycle, all the bottles for the uptake experiments were covered with aluminum foil to minimize any possibility for the cells to be exposed to light. All the cells used in the experiments were from the same culture. Each uptake experiment lasted 1 h, and the measurement of metal accumulation was conducted at 15 min intervals.

2.4 Results

2.4.1 Metal uptake at different metal concentrations, irradiiances, and temperatures

As expected, both the Cd and Zn uptake followed the Michaelis-Menten uptake kinetics (Fig. 2-1). The $p_{\text{max}}$ and $K_m$ were 4.4 μg g$^{-1}$ h$^{-1}$ and 38.8 nmol l$^{-1}$ for Cd, and 26.3 μg g$^{-1}$ h$^{-1}$ and 93.4 nmol l$^{-1}$ for Zn, respectively. These experiments were conducted using DOC (dissolved organic carbon) -free seawater. In our subsequent experiments, the metal uptake was quantified at concentrations below the $K_m$ (20.8 nmol l$^{-1}$ for Cd, and 89.2 nmol l$^{-1}$ for Zn), but without removal of DOC from seawater. Since the DOC was not removed in the subsequent experiments, the resulting free metal concentrations were expected to be much lower than the $K_m$ -based free metal ion concentration.

During the 4-h uptake period of the temperature and irradiance experiment, the total metal concentrations in the medium decreased by <15% for Zn and <5% for Cd. Measurable but not much increase of cell number (<1.2× increase) was found during this period. There was an approximately linear relationship between the
accumulated metal content and the exposure time (between 1 and 4 h) (Fig. 2-2). Previous study indicated that Ti-washing removed 81% and 90% of extracellular Cd and Zn from the diatom cells [75]. The y-intercept of the linear regression between the accumulated metal content and the exposure time represented the amount of metal adsorbed onto the cell surface [76] or the transporter-bound metals after rinsing, and the slope of this regression represented $\rho$ of the metals. Our preliminary experiments also demonstrated that the y-intercept represented the initial surface sorption of the cells, since the metal sorption by the heat-killed cells was essentially comparable to the calculated y-intercept of the linear regression (data not shown). In general, the accumulated Cd and Zn contents at each time point of exposure increased with increasing irradiance levels at each temperature. Additionally, the accumulated contents of Cd and Zn were also positively related to the temperature. This experiment was repeated and the trends of metal uptake at different temperatures and irradiances were consistent between these two separate experiments.

The calculated $\rho$ of Cd and Zn as a function of irradiance levels were shown in Fig. 2-3. At each temperature, $\rho$ generally increased with increasing irradiance at the two lower light levels, but remained rather constant when the irradiance was further increased to 340 $\mu$mol photons m$^{-2}$ s$^{-1}$. With the irradiance increasing from 40 to 340 $\mu$mol photons m$^{-2}$ s$^{-1}$, $\rho$ of both metals increased by 2-4x, at 15°C, 19°C, and 24 °C, respectively. Two-way ANOVA (two-way analysis of variance for temperature and irradiance) showed that there was a strong interaction between these two factors ($p < 0.001$, Table 2-1). And $\rho$ of both Cd and Zn increased significantly with increasing temperature or irradiance ($p < 0.001$). The most remarkable influence of irradiance on the metal uptake was observed at the two lower irradiance
levels. $\mu$ measured in the two replicated experiments were highly comparable at
different temperatures and irradiances (Fig. 2-3). The cell size of the diatoms was
also concurrently measured for treatments under different temperatures and
irradiances. In general, the cell sizes were comparable and not statistically
significantly different among the different treatments ($p > 0.05$). The average cell
diameter was 5.0 $\mu$m (i.e., cell volume was about 524 $\mu$m$^3$).

We further examined the relationship between $\mu$ and $\rho$ quantified in the
temperature and irradiance experiments (Fig. 2-4). In general, $\rho$ of Cd and Zn
increased significantly with increasing $\mu$ ($p < 0.05$) and then leveled off at a high $\mu$.

2.4.2 Metal uptake at different LD cycles

The accumulated contents of Cd and Zn also increased linearly over time in
experiments with different LD cycles (data not shown). After 4 h of exposure, there
was a 2.7× and 1.8× increase in the accumulated Cd content and 1.9× and 1.4×
increase in the intracellular accumulated Zn content when the cells were maintained
under 24 h light illumination as compared to cells maintained under 4 h light
illumination each day at 19°C and 24°C, respectively. The accumulated
intracellular contents of Cd and Zn were conspicuously low at the lowest light
illumination period (4 h). The relationship between $\rho$ and the light exposure time
was shown in Fig. 2-5. At 24 °C, $\rho$ of Cd and Zn increased by 1.8× and 1.5×,
respectively, when increasing the light illumination period from 4 h to 24 h each day.
Similarly, at 19 °C, $\rho$ increased by 2.7× and 1.7× for Cd and Zn, respectively.
One-way ANOVA (Table 2-2) indicated that LD cycle significantly affected the
uptake of Cd and Zn by the diatoms ($p < 0.05$). $\mu$ of the diatoms also increased
with increasing light illumination period (Fig. 2-5). Similarly, $\rho$ of Cd and Zn
increased with increasing $\mu$ (Fig. 2-6).
2.4.3 Metal uptake with additions of inhibitors

The photosynthetic activity of the diatom cells was inhibited by the two inhibitors (CCCP and paraquat at different concentrations), as indicated by the lower $\mu$ for the inhibitor-treated treatments as compared to the control treatment during the uptake period (1.25× increase for the control treatment vs. 1.15× increase for the 150 $\mu$mol l$^{-1}$ paraquat treatment). The accumulated intracellular metal contents also proceeded linearly over 1-4 h exposure (data not shown). There was no consistent trend for the variation in the accumulated cellular metal content with different concentrations of inhibitors. The calculated $\rho$ at different concentrations of inhibitors are shown in Fig. 2-7. Statistical analysis (Table 2-2) indicated that there was no significant difference in the Cd uptake for treatments with the addition of CCCP or Paraquat ($p > 0.05$). In contrast, the Zn uptake rate increased with the addition of paraquat and CCCP and then decreased at higher concentrations (150 $\mu$mol l$^{-1}$ for paraquat and 25 $\mu$mol l$^{-1}$ for CCCP).

2.4.4 Diel variations of metal uptake

The diatom cells were maintained at a 14:10 h LD cycle and the metal accumulation was quantified at different periods of the day. The accumulated intracellular contents of Cd and Zn proceeded linearly with exposure time for diatoms harvested at different diel periods (data not shown). The calculated $\rho$ of Cd and Zn over the 24 h period were shown in Fig. 2-8. In general, $\rho$ was in the range of 0.53-1.20 $\mu$g g$^{-1}$ h$^{-1}$ for Cd, with most in the range of 0.60-0.90 $\mu$g g$^{-1}$ h$^{-1}$, and it was in the range of 3.90-4.90 $\mu$g g$^{-1}$ h$^{-1}$ for Zn, with one exception of 5.35 $\mu$g g$^{-1}$ h$^{-1}$ measured at 8:00 am. There was no consistent trend for $\rho$ of Cd as influenced by different diel periods, although statistical analysis indicated that diel period did significantly affect the Cd uptake ($p < 0.05$, one-way ANOVA) (Table 2-2).
Conversely, there was no diel variation for Zn uptake at different diel period (p > 0.05, one-way ANOVA).

2.5 Discussion

Both ρ of Cd and Zn quantified in this study were comparable to previous studies on the same diatom species under similar conditions [67]. In addition, the calculated ρmax was also comparable to those measured in previous studies [77]. Since it was difficult to accurately calculate the [Cd^{2+}] (free Cd ion concentration) and [Zn^{2+}] (free Zn ion concentration) with MINEQL+ (a chemical equilibrium modeling system, version 4.5 for windows) without using the buffer solution for the uptake medium, direct comparison of the K_m with previous studies was impossible. In our short-term uptake experiments (4 h), there was a possibility of interference by metal efflux from the cells, thus the quantified metal uptake may only represent the net uptake rate. However, within such a short exposure period (<4 h), the metal efflux may be considered negligible as compared to the much higher metal influx rate (e.g., Thalassiosira weissflogii, [78]; Chlorella kessleri, [21]).

In this study, we demonstrated that temperature and irradiance had notable influences on ρ of Cd and Zn by the diatom Thalassiosira pseudonana. Increasing the temperature and irradiance both enhanced the uptake of Cd and Zn. Furthermore, temperature and irradiance interacted in their influences on ρ. With the irradiance and temperature constant, changes in the LD cycle also impacted ρ. Light and temperature both control the photosynthesis of the cells. Previous studies have shown that the biochemical composition and the cell size of phytoplankton were different for cells acclimated under different light and temperature conditions [23-27]. For example, cellular N, chlorophyll a and C:N ratio increased with
increasing temperature [25]. Furthermore, there was also a diel variation in the cellular composition and cell size [70, 72, 79] as well as a diel pattern in the cell cycle [71]. For example, in the marine diatom *Skeletonema costatum*, the cellular organic N increased from 2.4 pg cell\(^{-1}\) (at the beginning of the photophase) to 3.3 pg cell\(^{-1}\) (at the end of the photophase) and then decreased again to 2.5 pg cell\(^{-1}\) (end of the scotophase) [72]. All these biochemical, biovolume, and cell cycle changes may potentially lead to a change in \(\rho\) as influenced by temperature, irradiance, and LD cycle.

To address whether \(\rho\) of Cd and Zn was dependent on the phytoplankton's photosynthetic activity, which can be affected by temperature and light, we used two inhibitors (CCCP and the herbicide paraquat) to block the photosynthesis of the cells [21, 69]. CCCP can disconnect electron transport from ATP synthesis, thereby inhibiting the photophosphorylation and preventing the formation of ATP required in the energy supply. Paraquat acts at the reducing end of PS I by inhibiting ferredoxin reduction and ATP production. In our experiments, \(\rho\) of Zn increased rather unexpectedly with the addition of two inhibitors at different concentrations as compared to the control treatment. The reason for such an increase remains unknown, although Moye et al. [69] also found that paraquat enhanced the accumulation of methylmercury by the green algae (*Selenastrum capricornutum*). However, Moye et al. [69] showed that additions of CCCP at the same concentrations used in our study (5 and 25 \(\mu\)mol l\(^{-1}\)) abruptly decreased the methylmercury uptake by 85%. Hassler and Wilkinson [21] also demonstrated that the addition of the inhibitors CCCP and vanadate (BDH) reduced (by up to 95%) the uptake of Zn by *Chlorella kessleri*. Our study suggested that \(\rho\) of Cd and Zn by the
diatoms was not affected by photosynthetic activity which was closely related to the
temperature and light conditions.

Previous studies have demonstrated that the cell size [79], the cell-cycle [71],
enzyme activity (e.g. nitrate reductase) [70], and the cellular N and C [72] exhibited
considerable diel patterns. Our experiments generally found that there was no
consistent or notable pattern of diel variations in Cd and Zn uptake by the diatoms.
Furthermore, there was no significant difference among most ρ quantified over 24 h
for Cd (p > 0.05). However, metal accumulation increased significantly with
increasing light illumination period (ρ < 0.05), e.g., a 2.7× and 1.7× increase in ρ of
Cd and Zn, respectively, at 19°C with increasing light illumination period from 4 h to
24 h each day. Consequently, the diel cycle in the morphology, physiology or
biochemistry of the diatoms was not responsible for the correlation between ρ and
the LD cycle, despite that the cells under different LD cycles may show different cell
cycles.

The cell size of the diatoms was reported to increase with irradiance
(Thalassiosira pseudonana, [22]) and temperature (Thalassiosira weissflogii, [80]) or
remain constant (Thalassiosira pseudonana, [25]; Skeletonema costatum, [81]).
And the cell size also tended to increase when the diatom Thalassiosira pseudonana
was cultured under a LD cycle compared with the continuous light [25]. In our
study, we did not find any obvious change in the cell size of T. pseudonana, which
may substantially affect ρ due to a change in the surface area to volume ratio. The
cell size can thus be ruled out as controlling ρ at different temperatures and light
conditions. Additionally, the N content of the diatoms (as an index of the protein
content) was almost constant regardless of the changes in temperature and light [22,
25]. Thus, biochemical composition, cell size and changes in metabolism or
photosynthetic activity were not responsible for the relationship between $\rho$ and irradiance or LD cycle observed in this study. Furthermore, the metal binding sites were not saturated according to our uptake kinetic experiments, thus the level-off of $\rho$ under higher irradiances was unlikely due to the saturation of surface metal binding sites.

In our study, we found that $\rho$ of both Cd and Zn increased with increasing $\mu$ in both replicated experiments with different combinations of temperature and irradiances. It appears that there was a hyperbolic relationship between $\rho$ and $\mu$. At the lowest temperature (15°C), the maximum $\mu$ was only 1.2 d$^{-1}$, and the hyperbolic relationship between $\rho$ and $\mu$ became less apparent. At this temperature, $\rho$ of Zn increased linearly with increasing $\mu$ in each replicated experiment. Similar hyperbolic correlation was also evident in the LD cycle experiment. In earlier studies, Wang and Dei [32] described a positive linear or exponential correlation between $\mu$ and $\rho$ of Cd, Se, and Zn in different species of marine phytoplankton. In their study, $\mu$ was regulated by different additions of macronutrients, while we used light and temperature in this study. Because both $\mu$ and $\rho$ can be dependent on the macronutrients, such correlation as found by Wang and Dei [32] cannot provide a definite conclusion on the direct dependence of $\rho$ on $\mu$. For example, algal cells maintained under N-enriched conditions may be able to synthesize more ligands for metal transport (i.e., high $\rho$), while the growth of the cells was also stimulated. In this study, we have shown that the cellular metabolism and photosynthetic activity brought about by changes in temperature, irradiance and LD cycle had little effect on metal uptake. Consequently, $\rho$ may be potentially dependent on $\mu$ of the diatoms.

Under the steady-state conditions, $M$ of phytoplankton can be calculated by $\rho$ divided by $\mu$ [39, 52]. Sunda and Huntsman [39] summarized the feedback
relationships between M and \( \mu \), as well as M and \( \rho \) in marine phytoplankton. However, it remains unknown whether there's any interrelationship between \( \rho \) and \( \mu \). Our study indicated that when the cell growth increased under a certain condition, \( \rho \) would initially increase in proportion with \( \mu \), maintaining the same M. Zn is a cofactor of some enzymes such as CA, DNA and RNA polymerases, and AP [40, 41]. Thus, the physiological requirement for Zn may increase with the growth of the phytoplankton, leading to a higher Zn uptake. This can be considered as positive feedback between \( \rho \) and \( \mu \). When \( \mu \) reached a higher level, \( \rho \) was unable to keep up resulting in a decrease in M and the cell growth may thus be limited. In this regard, \( \mu \) can equally well be described by a negative feedback regulation from \( \rho \).

The relationship between \( \mu \) and \( \rho \) has an important implication for understanding the metal dynamics during phytoplankton bloom seasons (e.g., eutrophication or spring blooms). Luoma et al. [82] found that particulate Cd and Zn concentrations (nmol l\(^{-1}\)) in San Francisco Bay increased by 6\( \times \) and 3\( \times \), respectively, during the spring bloom period. Our study implies that such an abrupt increase in particulate Cd and Zn concentrations in natural phytoplankton may be due to the proportionally higher \( \rho \) of Cd and Zn as compared to higher \( \mu \) during the bloom period. Such a nearly proportional increase for \( \rho \) and \( \mu \) also explains why there was a much smaller increase for Cd and Zn content (nmol g\(^{-1}\)) in the phytoplankton (2\( \times \) and 1.1\( \times \) for Cd and Zn, respectively). However, the inter-relationship between these two parameters still needs to be further tested under field conditions. In addition, there was a notable difference between the Cd and Zn uptake at different temperatures but with similar \( \mu \). Temperature may thus affect \( \rho \) of Cd and Zn by other metabolic processes in additional to the controls by \( \mu \). The mechanisms contributing to such marked difference remains unknown.
Our study demonstrated that the metal uptake by the diatoms was dependent on their $\mu$, which should be taken into consideration when examining the interrelationship among $M$, $\rho$, and $\mu$. Such an interrelationship can be potentially responsible for the observed increase in particulate metal concentration during phytoplankton bloom periods and the survival of phytoplankton under sublethal metal concentration conditions. It should be noted that the interaction between $\rho$ and $\mu$ was not indirectly exerted through change in $M$. However, the cellular mechanisms underlying the interrelationship between $\rho$ and $\mu$ need to be further studied. Whether or not this relationship exists for other metals and other phytoplankton species also needs to be examined.
Table 2-1. Two-way ANOVA statistical analysis of Cd and Zn uptake rates under different irradiances (I) and temperatures (T) in the two separate experiments.

<table>
<thead>
<tr>
<th>Source</th>
<th>Exp. 1</th>
<th></th>
<th>Exp. 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>MS</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td>Cd</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>T</td>
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<td>284.28</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>I</td>
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<td>0.43</td>
<td>156.32</td>
<td>&lt;0.001</td>
</tr>
<tr>
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<td>0.14</td>
<td>51.06</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Error</td>
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<td>0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>T</td>
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<td>368.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>I</td>
<td>2</td>
<td>70.80</td>
<td>103.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T × I</td>
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<td>23.60</td>
<td>34.37</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Error</td>
<td>17</td>
<td>0.69</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2-2. One-way ANOVA statistical analysis of the Cd and Zn uptake rates in the LD cycle, photosynthetic inhibitor and diel variation experiments. BG represents between groups and WG means within groups.

| Exp. | Source | Cadmium (Cd) | | | Zinc (Zn) | | |
|------|--------|-------------|---|---|---|---|
|      |        | df | MS | F | p | df | MS | F | p |
| 19°C |        |    |    |   |   |    |    |   |   |
| LD cycle | BG | 3 | 0.117 | 53.382 | <0.001 | 3 | 3.358 |
|         | WG | 8 | 0.002 | 8 | 0.359 | 9.344 | 0.005 |
| 24°C |        |    |    |   |   |    |    |   |   |
|       | BG | 3 | 0.252 | 21.801 | <0.001 | 3 | 32.158 |
|       | WG | 8 | 0.012 | 8 | 2.396 | 13.42 | 0.002 |
| CCCP |        |    |    |   |   |    |    |   |   |
|       | BG | 2 | 0.010 | 3.57 | 0.07 | 2 | 10.33 |
|       | WG | 9 | 0.003 | 9 | 0.90 | 11.51 | 0.003 |
| Inhibitor |        |    |    |   |   |    |    |   |   |
| Paraquat | BG | 3 | 0.009 | 3.01 | 0.09 | 3 | 9.12 |
|         | WG | 8 | 0.029 | 8 | 1.13 | 8.08 | 0.008 |
| Diel |        |    |    |   |   |    |    |   |   |
|       | BG | 7 | 0.087 | 12.88 | <0.001 | 7 | 0.79 |
|       | WG | 13 | 0.007 | 15 | 0.62 | 1.27 | 0.3301 |
Fig. 2-1. *Thalassiosira pseudonana*. Metal uptake rates as a function of total ambient metal concentration. Data are mean ± SD (n = 2).
Fig. 2-2. *Thalassiosira pseudonana*. Accumulated Cd (left panel) and Zn (right panel) content in the diatoms acclimated under different temperatures and irradiances (μmol photons m⁻² s⁻¹) during the short-term uptake period in Expt. 2. Data are mean ± SD (n = 3).
Fig. 2-3. *Thalassiosira pseudonana*. The relationship between the diatom specific growth rate (μ) or the uptake rates (ρ) of Cd and Zn and the irradiance level. Open symbols: Exp. 1; Closed symbols: Exp. 2. Data are mean ± SD (n = 3).
Fig. 2-4. *Thalassiosira pseudonana*. The relationship between the diatom specific growth rate ($\mu$) and the uptake rates ($p$) of Cd and Zn measured at different combinations of temperature and irradiance. Data are mean ± SD (n = 3).
Fig. 2-5. *Thalassiosira pseudonana*. The relationship between the diatom specific growth rate ($\mu$) or the uptake rates ($\rho$) of Cd and Zn and the light exposure time at two different temperatures. Data are mean ± SD (n = 3).
Fig. 2-6. *Thalassiosira pseudonana*. The relationship between the uptake rates ($\rho$) of Cd and Zn and the diatom specific growth rates ($\mu$) at two temperatures with different LD cycles. Data are mean ± SD (n = 3).
Fig. 2-7. *Thalassiosira pseudonana*. Uptake rates ($\rho$) of Cd and Zn by the diatoms with the addition of different concentrations of inhibitors. Data are mean ± SD ($n = 3$).
Fig. 2-8. *Thalassiosira pseudonana*. Diel variations in the uptake rates of Cd and Zn by the diatoms during a 24 h diel period. White bar means the metal uptake rate during the light period and the gray bar means the metal uptake during the dark period. Data are mean ± SD (n = 3).
Chapter 3

Fulfilling iron requirements by a coastal diatom under different temperatures and irradiances

3.1 Abstract

The strategies used by a coastal diatom, *Thalassiosira pseudonana*, for potentially different Fe requirements under different temperatures and irradiances were examined based on three parameters: $\rho$, $\mu$, and $K_e$. These three variables determined the cellular Fe concentration and they were all quantified under different temperatures and irradiances during long-term (days) and short-term (hours) $^{59}$Fe exposures. Results obtained from both exposures were consistent. Although more Fe was required under the lower irradiance, $\rho$ decreased by 1.78× and 2.20× as the irradiance decreased from 340 to 40 $\mu$mol photons m$^{-2}$ s$^{-1}$ when measured by short and long term exposures, respectively. Under this condition, $\mu$ decreased from 1.30 - 1.50 to 0.51 - 0.63 d$^{-1}$ in order to keep a relatively high intracellular Fe concentration under lower irradiance. The opposite trend was observed for temperature. The higher Fe requirement at higher temperature was fulfilled mainly through an increase of $\rho$ with increasing temperature. For example, $\rho$ increased by 1.21× and 2.55× as the temperature increased from 15 to 24 °C in the short and long term exposures, respectively. In contrast, $\mu$ was relatively constant (0.92 – 1.06 d$^{-1}$) under these three temperatures. There was a remarkable Fe efflux from the diatoms, with $K_e$ ranging from 0.008 to 0.017 h$^{-1}$. Such a high Fe efflux suggested that it should not be neglected in the calculation of intracellular Fe concentration. Furthermore, Fe efflux may be an important process for Fe regeneration in surface seawater. However, Fe efflux had a negligible effect on the different Fe requirements under different temperatures and irradiances.
3.2 Introduction

Fe is an essential trace metal to marine phytoplankton. It is involved in many biochemical processes, such as photosynthetic and respiratory electron transport, pigment synthesis, nitrate and nitrite reduction, and other numerous biochemical reactions [83]. Its bioaccumulation mechanism by phytoplankton has been examined [64, 84, 85]. To quantify the Fe accumulation in marine phytoplankton, both long-term and short-term exposures have been widely used [84, 86, 87]. In the long-term exposure experiments, Fe accumulation is generally quantified at the end of the exposure with a steady-state assumption. The ambient metal concentration was kept constant during this period with the help of high concentration of buffer reagents (e.g., EDTA [Ethylenediaminetetraacetic acid]). This method is relatively simple with only one time point, but the metal uptake kinetics cannot be obtained nor can the cell surface adsorption which is completed within a few minutes [88]. The cells may also excrete some metabolites during the long-term exposure period and thus the water chemistry and metal speciation may be affected, which are critical in metal accumulation [89-91]. In the short-term exposure experiments, cellular metal accumulation is quantified at different time points lasting a few minutes to hours. The uptake kinetics and cell surface adsorption can be measured using this method, and the potential effect of organisms on the ambient environment can be minimized. Although these two methods have been both frequently applied in metal accumulation studies, no direct comparison of both approaches has been made in previous studies.

Both temperature and light are important environmental variables in the ocean. Raven [29] calculated that more Fe was needed in the phytoplankton at lower irradiance for the much higher pigment synthesis in order to intercept more light
energy. It has also been found that phytoplankton growth at high temperature mimics the low light acclimation [30] and thus requires more Fe [92]. Under steady state condition the cellular Fe concentration can be expressed with Eq. 1-4. According to this equation the cellular Fe concentration is determined by three variables (i.e., \( \rho \), \( \mu \), and \( K_e \)). It is thus interesting to examine how phytoplankton fulfill their different cellular Fe requirements under different light and temperature conditions based on these three variables.

The Fe uptake (i.e., \( \rho \)) under the influence of environmental factors, such as light and temperature, has not been well studied. Both light and temperature can directly affect Fe chemistry in seawater as well as phytoplankton growth and cellular composition. Their net effects on Fe accumulation in marine phytoplankton and further Fe biogeochemical cycling are rather speculative. In several studies, no consistent results were found about the influences of temperature and light on Fe uptake. For example, \( \rho \) can either increase, remain constant, or even decrease with the increase of irradiance [93-95]. Meanwhile, direct quantification of Fe efflux in marine phytoplankton has not yet been performed. It remains unknown whether temperature and light intensity have any effects on Fe efflux. The potential existence of Fe efflux can not only affect the intracellular Fe concentration (as indicated in Eq. 1-4), but also provide an important Fe source for the growth of marine phytoplankton because dissolved Fe concentration is exceptionally low in surface waters (e.g., high nitrate low chlorophyll area). The input of regenerated Fe is estimated to be orders of magnitude greater than the external supply rate of iron [96]. Hutchins et al. [97] found evidence of the potential biological recycling of Fe between cyanobacteria and diatoms and it remains to be determined whether Fe efflux plays a role in such potential biological recycling.
In order to know how phytoplankton fulfill their different Fe requirements under different temperatures and irradiances, we conducted both long-term and short-term uptake experiments to examine their influences on Fe uptake by a coastal diatom, *Thalassiosira pseudonana*. μ under the different light and temperature conditions were also quantified. We further measured the intracellular Fe efflux from diatoms as well as its dissociation from the cell surface under each condition. The diel cycle of Fe uptake by the diatoms was also examined since the Fe requirement by phytoplankton may vary diurnally [98].

3.3 Materials and methods

An axenic culture of *Thalassiosira pseudonana* (CCMP 1335, clone 3H) was obtained from the Provasoli-Guillard Center for the Culture of Marine Phytoplankton, Bigelow Laboratory, Maine, USA. The cells were maintained in f/2 medium [73] under sterile conditions, in an incubator at 19°C with a light illumination of 170 μmol photons m⁻² s⁻¹ on a 14:10 h LD cycle. All seawater was collected 10 km off East Hong Kong to minimize the influences of anthropogenic activities. It was filtered through a 0.22 μm Poretic membrane before being used in the experiments.

3.3.1 Short-term uptake experiments

3.3.1.1 General experimental protocols. All the incubation and sampling experiments were performed in a Class 100 laminar flow cabinet to avoid inadvertent trace metal contamination. The polycarbonate bottles for the diatom culture and uptake experiments were first soaked in acid and rinsed with Milli-Q water (18.0 MΩ) at least six times following trace metal clean techniques. The 0.22 μm filtered seawater used for the uptake media was first passed through Chelex 100 ion-exchange resin to remove any background Fe with a removal efficiency higher
than 95% (data not shown). The $^{59}\text{Fe}$ radioisotope (in 0.1 mol l$^{-1}$ HCl, from New England Nuclear, Boston, USA) was used to trace the Fe uptake by the diatoms. Equal molar amounts of Fe ($^{59}\text{Fe}$ in 0.1 mol l$^{-1}$ HCl and stable Fe in 0.5 mol l$^{-1}$ HCl) and Na$_2$EDTA were mixed for 5 min. Afterwards, the mixture was spiked into the uptake medium with a nominal Fe concentration of 10 nmol l$^{-1}$ (0.5 nmol l$^{-1}$ $^{59}\text{Fe}$ and 9.5 nmol l$^{-1}$ stable Fe) as well as 10 nmol l$^{-1}$ Na$_2$EDTA. The HCl added with the mixture was neutralized with Suprapure NaOH (1 mol l$^{-1}$). These uptake media were equilibrated at the targeted temperature and irradiances overnight. The 1:1 ratio of Fe to Na$_2$EDTA was used for a detectable $^{59}\text{Fe}$ count during the short-term uptake period. Although most of the Fe added should have precipitated under this concentration based on the simple equilibrium calculations, the premix of Fe with EDTA repressed its precipitation due to the slow dissociation rate of Fe-EDTA complex [99-101]. Our preliminary experiments showed that most of the Fe added (> 98%) was in the dissolved phase after equilibration. The radioactivity of $^{59}\text{Fe}$ was quantified at 1072 keV with a Wallac gamma detector. The counting time was adjusted to result in propagated counting errors < 5%.

The diatoms were first semi-continuously cultured under different environmental conditions (e.g., different temperatures or light intensities) depending on the different requirements of the experiment, in f/2 medium, except that the trace metal concentrations used were at the f/10 levels. The cell density was counted with a Coulter Counter every 12 h. After being grown for several generations, the cells were collected, rinsed with the Fe-free 0.22 $\mu$m seawater, and then resuspended into the uptake medium. The short-term uptake experiments lasted for 1, 2, or 8 h, during which period there were several time points (e.g., 4 time points for the short-term uptake under different irradiances). At each time point, a 10-20 ml
(depending on the exposure time and cell density used) sample was filtered through the 1 \( \mu \text{m} \) polycarbonate membrane. The cell surface adsorbed Fe was removed with the Ti solution [74]. A 1 ml aliquot was also collected for measurement of the total radioactivity in the uptake medium. The intracellular accumulated Fe content was calculated as follows:

\[
\text{Accumulated Fe content (nmol cell}^{-1}) = \frac{\text{radioactivity cell}^{-1} \times \text{total metal concentration nmol l}^{-1}}{\text{the radioactivity of medium l}^{-1}} \tag{3-1}
\]

\( \rho \) was then obtained as the slope of the regression line between the intracellular accumulated Fe and the exposure time, with an assumption that the potential cell growth and Fe efflux had a negligible effect on the linear relationship during the short exposure period. The \( y \)-intercept of the linear regression represents the transporter bound Fe [76]. Each uptake treatment had two replicates, as the standard deviation for the algal uptake was small. With the assumption that the algae were under steady-state condition, the intracellular Fe concentration could then be calculated using Eq. 1-4 with \( K_e \) neglected. Later the intracellular Fe concentration with and without the consideration of efflux will be compared after \( K_e \) was determined.

The algal cell sizes under different temperatures and irradiances were also quantified with a Coulter Counter. The cell sizes (diameters) were about 5 \( \mu \text{m} \), and there was no significant difference in the size among different treatments \( (p > 0.05) \).

3.3.1.2 Uptake with refreshed medium. The diatom cells were first cultured at 19 °C and 170 \( \mu \text{mol photons m}^{-2} \text{ s}^{-1} \) with a LD cycle of 14:10 h. After they arrived at the mid-exponential phase, an eight-hour short-term uptake experiment with 13 time points (0.25, 0.5, 0.75, 1, 2, 3, 3.25, 3.5, 3.75, 4, 5, 6, and 8 h) was performed. During this eight-hour experimental period, the uptake medium was
refreshed after three hours, as >70% (data not shown) of Fe was scavenged by the cells after eight hours without medium refreshing.

3.3.1.3 Uptake at different irradiances or temperatures. The algal cells were first acclimated at different irradiances or temperatures (40, 85, 170, and 340 μmol photons m\(^{-2}\) s\(^{-1}\) or 15°C, 19°C, and 24°C) for at least eight generations. Different irradiances were obtained by adjusting the distance from the light sources to the diatom culture. A one-hour short-term uptake with several time points (0.25, 0.5, 0.75, 1 h for the irradiance experiment and 0.125, 0.25, 0.5, 0.75, 1 h for the temperature experiment) was then conducted after the diatoms reached the mid-exponential phase under different light intensities or temperatures. The intracellular accumulated Fe and ρ were then calculated.

3.3.1.4 Diel variation of Fe uptake. The cells were first acclimated at 19°C and 170 μmol photons m\(^{-2}\) s\(^{-1}\) with a LD cycle of 14:10 h for at least eight generations. After they arrived at the mid-exponential phase, a total of ten one-hour short-term Fe uptake experiments (five during the light period and five during the dark period) were performed continuously over about 30 h period (i.e., from 11:00 h on the first day to 15:30 h on the second day) with two to three-hour intervals between each experiment. The cells for all ten short-term uptake measurements were taken from the same bottle. When the uptake was performed during the dark period, all the bottles were covered with aluminum foil to minimize the potential effects of light.

3.3.2 Long-term uptake experiments

The long-term uptake experiments were performed in the same culture/uptake medium as those used during the acclimation period of the short-term uptake experiments. Different temperature or light intensity treatments were also the same
as in the short-term uptake experiments. $^{59}$Fe (0.92 nmol l$^{-1}$) was used to trace the Fe accumulation along with 2.33 μmol l$^{-1}$ stable Fe (f/10 level). EDTA (100 μmol l$^{-1}$) was added to keep the free metal ion concentration constant during the experimental period. The pH of the medium was adjusted to 8.2 ± 0.1 with Suprapure NaOH (1 mol l$^{-1}$). The cell density was counted every day. At the same time, a 10-20 ml sample was filtered through the 1.0 μm polycarbonate membrane, soaked in Ti-solution for 1 min [74], rinsed with the 0.22 μm filtered seawater three times, and then the radioactivity was counted with the Wallac gamma detector. Another 5 ml sample without Ti-wash was also collected to quantify the total cellular Fe accumulation. A 1 ml water sample was collected simultaneously for the measurement of total Fe radioactivity in the medium. The intracellular and total cellular Fe contents at each time point were calculated by Eq. 3-1. The difference between these two parameters indicated the loosely cell surface adsorbed Fe. A was then calculated without considering $K_c$ first, similar to the short term uptake experiment. The whole experiment lasted until the cells had acclimated for at least eight generations. During this period, the algal cells were sub-cultured once to avoid nutrient depletion. In the irradiance experiments, the subculture was performed on the third and fourth days for the two lower and higher irradiance treatments, respectively. In the temperature experiment, the subculture was conducted on the fifth day for all treatments.

3.3.3 Fe efflux from diatoms

The Fe efflux experiment was performed using $^{59}$Fe labeled diatoms resulting from the long-term Fe uptake experiments described above. The Fe efflux for both living and dead cells was quantified. The algal cells were first collected, rinsed with Chelex resin passed 0.22 μm filtered seawater three times, and then resuspended
into the efflux medium. The dead cells were obtained after the diatoms were heated in a 50 °C water bath for 10 min [102] and were kept intact as observed under the microscope. The efflux medium contained the Chelex resin passed 0.22 μm filtered seawater with the addition of 5 μmol l\(^{-1}\) desferrioxamine B (DFB). The medium was equilibrated in the dark for a few hours before the start of the Fe efflux experiment. DFB is a trihydroxamate siderophore that specifically complexes inorganic Fe (III) with an extremely high conditional stability constant (\(K_{\text{Fe,Fe}}^{\text{cond}} = 10^{16.5} \text{ L mol}^{-1}\)) ([103] and references therein). A preliminary study conducted in our laboratory also demonstrated that Fe uptake by \(T.\) \(pseudonana\) was inhibited with the addition of DFB (Chen and Wang unpubl.). Thus, DFB can prevent or at least minimize the reassimilation of eliminated Fe.

The efflux experiment lasted for eight hours with several time points of sampling. At each time point 10 or 20 ml aliquots were removed, soaked in Ti-solution for 1 min, and rinsed with 0.22 μm seawater three times. The radioactivity of the cells was then counted and the percentage of Fe retained in the cells was calculated. The total cellular Fe retained was also quantified with the 0.22 μm filtered seawater rinse only. The Fe retained in both the intracellular and total cellular pools were modeled with the following equation:

\[ M_t = M_0 e^{-K_{\text{Fe}} t} \]  

(3-2)

where \(M_0\) and \(M_t\) represent the cellular or intracellular Fe concentration at the beginning and at the incubation/depuration time \(t\), respectively.

3.4 Results

3.4.1 Short-term Fe uptake
Our preliminary experiment employed an eight-hour exposure period to quantify Fe uptake. A hyperbolic increase in intracellular accumulated Fe with the increase of exposure time was observed, as shown by the dashed line in Fig. 3-1a. More than 70% of Fe in the uptake medium was scavenged by the algal cells at the end of eight hours. Thus, the Fe uptake experiment was then performed by renewing the medium after three hours of exposure to examine whether the observed hyperbolic pattern was due to the marked decline of the ambient Fe concentration. In this experiment, the intracellular accumulated Fe concentration first increased linearly and then started to level off after three hours. When the medium was refreshed, there was a sudden increase and then a linear increase until the end of the eight-hour uptake period. The intracellular accumulated Fe increased by about 10 times (i.e., from 2.75×10^{10} to 2.60×10^{9} nmol cell^{1}) from 0.25 hour to eight hours of exposure. A good linear regression was obtained when data at the three- and eight-hour time points were excluded. A similar slope of the linear increase before and after the medium renewal is shown in Fig. 3-1b. The intracellular Fe accumulation before the medium renewal was subtracted from that after the renewal. Thus, the intracellular Fe accumulation at each time point before and after the medium renewal was comparable, suggesting that the decrease in the ambient Fe concentration was responsible for the observed hyperbolic increase in the intracellular accumulated Fe concentration over the eight hours of exposure. The calculated transporter bound Fe (i.e., the y-intercept of the linear regression between cellular accumulated Fe and exposure time) before and after the medium renewal was also comparable. In all subsequent experiments, the uptake was performed within one to two hours of exposure, and there was <20% decrease in the ambient Fe concentration during this relatively short period.
In the short-term uptake experiments, the diatoms were first acclimated under different irradiances and temperatures for at least eight generations before the uptake experiments. There was a very quick response in \( \mu \) (quantified during the acclimation period), which increased with an increase in light intensity or temperature (e.g., 0.51 to 1.3 d\(^{-1}\) when the light intensity increased from 40 to 340 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) (Fig. 3-2a and b). The effect of temperature on the cell growth was much less obvious than was the effect of irradiance, with \( \mu \) equal to 0.98, 0.98, and 1.0 d\(^{-1}\) at 15, 19, and 24 °C, respectively. The Fe accumulations quantified at different temperatures and light intensities are shown in Fig. 3-3. As expected, the intracellular accumulated Fe concentration increased linearly with an increase in exposure time during the one hour period (Fig. 3-3a and b), which further validated our assumption that the effects of cell growth and Fe efflux on cellular Fe accumulation could be neglected during a short period. For example, the intracellular accumulated Fe concentration increased from 5.53×10\(^{-10}\) to 1.05×10\(^{-9}\) nmol cell\(^{-1}\) at the lowest irradiance level. Similarly, it increased linearly from 4.1×10\(^{-10}\) to 1.29×10\(^{-9}\) nmol cell\(^{-1}\) at the lowest temperature. At the same time the cellular accumulated Fe concentration increased with an increase in light intensity at each of the four time points, respectively. For example, the cellular accumulated Fe concentration increased from 1.05×10\(^{-9}\) to 1.89×10\(^{-9}\) nmol cell\(^{-1}\) after one hour as the light intensity increased from 40 to 340 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\). There was also a slight increase in the intracellular accumulated Fe concentration at the highest temperature treatment. Such a positive relationship was further evidenced by the calculated transporter bound Fe and \( \rho \), both of which increased with an increase of irradiance and temperature (Fig. 3-3c-f). For example, the transporter bound Fe and \( \rho \) were 1.83× and 1.78× higher for the highest irradiance treatment as compared with
the lowest one. As \( \mu \) was higher under higher irradiance, there was a 1.42x decrease in M as the irradiance increased from 40 to 340 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) (Fig. 3-3g). An opposite trend was found for the different temperature treatments due to the similar \( \mu \) under different temperatures. Accordingly, M increased slightly (1.18x) with an increase of temperature (Fig. 3-3h).

In the diel variation experiment, the diatom cells were maintained at a 14:10 h LD cycle and the Fe accumulation was quantified at different periods of the day. The intracellular accumulated Fe concentration proceeded linearly with the exposure time for diatoms harvested at different diel periods (data not shown). The calculated \( \rho \) (over 30 h) are shown in Fig. 3-4. They ranged from 1.16x10\(^{-9}\) to 1.58x10\(^{-9}\) nmol cell\(^{-1}\) h\(^{-1}\), and were consistent with the results under different temperatures and light intensities in Fig. 3-3. Statistical analysis showed that there was no diel variation in \( \rho \) at different diel periods (\( p > 0.05 \), one-way ANOVA).

### 3.4.2 Long-term Fe uptake

\( \mu \) of *T. pseudonana* under different temperatures and irradiances in the long term experiment are shown in Fig. 3-2c and d. They were comparable to those observed in the short-term uptake experiments under the same conditions. For example, \( \mu \) increased from 0.63 (0.51) to 1.5 (1.30) d\(^{-1}\) when the ambient light intensity increased from 40 to 340 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) in the long- (short-) term Fe uptake experiments. However, the temperature had a much smaller effect on \( \mu \). It increased from 0.92 to 1.06 d\(^{-1}\) when the temperature increased from 15°C to 24°C.

Because 100 \( \mu \)mol L\(^{-1}\) of EDTA was added to the long-term uptake medium, most of the Fe (77-81%) was present in the ambient water throughout the whole experiment, whereas 13-16% of Fe was adsorbed onto the cell surface, and 6-7% was taken up into the cell after five and six days (Fig. 3-5a and b). The cell surface Fe
adsorption, which accounted for 13-16% of the total Fe added, was completed within a few minutes [88]. Thus, the bioavailable Fe concentrations were considered to be relatively constant during this experiment and the steady-state condition was reached after several generations of growth.

The calculated Fe accumulation under different conditions is shown in Fig. 3-6. The cell surface adsorbed Fe concentration increased when the cells were first inoculated into a new $^{59}$Fe labeled medium and then decreased continuously until the next subculture or medium renewal. Following a sudden increase after the renewal, the cell surface adsorbed Fe decreased again similar to pattern observed before the subculture (Fig. 3-6a and b). For example, the cell surface adsorbed Fe concentration at the highest irradiance decreased from $1.88 \times 10^{-6}$ to $1.04 \times 10^{-6}$ nmol cell$^{-1}$ before the subculture. Afterwards, it increased to $1.95 \times 10^{-6}$ and decreased finally to $1.17 \times 10^{-6}$ nmol cell$^{-1}$. Similar results were also observed in the other irradiance treatments and temperature experiments. This trend with time was however not observed for M and $\rho$. For most of the treatments in the irradiance and temperature experiments, both parameters remained almost constant and did not change with time (Fig. 3-6c-f). The only exception was the highest temperature treatment, in which M increased from $3.31 \times 10^{-7}$ to $6.64 \times 10^{-7}$ nmol cell$^{-1}$ and $\rho$ from $1.47 \times 10^{-8}$ to $2.93 \times 10^{-8}$ nmol cell$^{-1}$ h$^{-1}$ as the exposure time increased from 2 to 6 days (Fig. 3-6d and f).

The final loosely cell surface adsorbed Fe concentrations, M and $\rho$ are compared for different irradiances and temperature treatments. The cell surface adsorbed Fe concentration was higher under higher irradiance or temperature (Fig. 3-6a and b), e.g., $4.28 \times 10^{-7}$ and $1.17 \times 10^{-6}$ nmol cell$^{-1}$ under 40 and 340 $\mu$mol photons m$^{-2}$ s$^{-1}$, respectively, and $8.29 \times 10^{-7}$ and $1.26 \times 10^{-6}$ nmol cell$^{-1}$ at 15°C and
24°C. M remained constant at all four different irradiance levels, but was much higher at 24 °C as compared with the other two lower temperature treatments (Fig. 3-6c and d). Due to the higher μ at a higher light intensity, the calculated ρ increased with an increase in irradiance (Fig. 3-6e). It increased by 2.2× as the irradiance increased from 40 to 340 μmol photons m⁻² s⁻¹. Similarly, ρ increased with an increase in temperature (Fig. 3-6f). When the temperature increased from 15 °C to 24 °C, ρ increased from 1.15×10⁸ to 2.93×10⁸ nmol cell⁻¹ h⁻¹.

3.4.3 Fe efflux from the diatoms

Figure 3-7 shows the relative Fe concentration decreases in both living and heat-killed cells under different irradiances and temperatures. For the dead cells, there was a remarkable decrease in both the total (by 57-72%) and intracellular Fe concentration (by 40%) during the eight-hour efflux period (Fig. 3-7a-c). Similarly, there was a notable decrease in total cellular Fe concentration during the experimental period with the living cells. The relative Fe retained at the end of eight hours ranged from 45.7% to 62% and 48% to 52.5% under different irradiances and temperatures, respectively (Fig. 3-7d, e). The Fe elimination from the intracellular pools of living cells was smaller as compared with the dead cells. The relative decrease ranged from 5.8 to 11% and 10 to 11% for different irradiances and temperatures (Fig. 3-7f, g).

The Fe elimination of the two different pools (i.e., intracellular and total cellular pools) followed an exponential pattern for both dead and living cells, as described by Eq. 3-2. The calculated Kₑ under different light and temperature conditions are shown in Table 3-1. The Fe elimination of both pools was faster for dead cells as compared with living cells under the same irradiance and temperature. The calculated Kₑ for the total cellular Fe of dead cells was about 1.6× higher than
that of living cells under the highest irradiance of 340 μmol photons m\(^{-2}\) s\(^{-1}\) (i.e., 0.149 vs. 0.095 h\(^{-1}\)). Furthermore, \(K_e\) for intracellular Fe of dead cells was about 5× higher than that for living cells at 24 °C (i.e., 0.081 vs. 0.016 h\(^{-1}\)). On the other hand, Fe elimination from both pools of the dead and living cells was higher under the higher irradiance and temperature conditions except for the intracellular pools of living cells under different temperatures. For example, in temperature experiment with dead cells, 41.2% total cellular Fe was retained after eight hours at 15°C, whereas only 28.4% was retained at 24°C (Fig. 3-7c). Such a trend of Fe elimination was also demonstrated by the increase of \(K_e\) with an increase in temperature and irradiance (Table 3-1). However, the intracellular Fe elimination of living cells was relatively constant under different temperatures with \(K_e\) ranging from 0.015 to 0.017 h\(^{-1}\).

3.5 Discussion

3.5.1 Fe accumulation under different temperatures and irradiances

Previous studies employed either long-term or short-term experiments to quantify \(\rho\), but comparison of these two methods in calculating Fe uptake has not yet been directly conducted. Our quantified Fe accumulations in short-term and long-term uptake experiments were generally comparable to the results of the previous studies employing similar bioavailable Fe concentrations [84, 104]. In our study, the trends obtained from both methods were mostly consistent. Since different total Fe and EDTA concentrations were used in the short-term and long-term uptake experiments (i.e., Fe: 10 nmol l\(^{-1}\) vs. 2.33 μmol l\(^{-1}\); EDTA: 10 nmol l\(^{-1}\) vs. 100 μmol l\(^{-1}\)), the bioavailable Fe concentrations were completely different.
Consequently, the absolute values of $\rho$, $M$, and other parameters varied by orders of magnitude between these two experiments.

Light and temperature are both important environmental variables and play important roles in Fe-phytoplankton interactions by affecting the ambient bioavailable Fe concentration and the physiology of phytoplankton. Light can induce Fe photo-reduction and temperature can affect the thermal formation/dissociation of organic Fe complex as well as the oxidation of photo-produced Fe (II) [28]. Both parameters can also affect the biochemical composition and cell volume of the phytoplankton. For example, cellular N, chlorophyll a, and C:N ratio increase with increasing temperature [25]. Meanwhile, phytoplankton acclimated at lower light intensity tend to have a higher Fe requirement in the photosynthetic system, similar to cells acclimated at higher temperature. Furthermore, the effects of temperature and irradiance on Fe accumulation and cell surface adsorption may be dependent on cellular Fe quota. Therefore, Fe accumulation in phytoplankton at different light levels and temperatures is complicated and contrasting conclusions have been drawn in previous studies.

Both short-term and long-term uptake experiments showed that $\rho$ was higher under higher irradiance while $M$ was slightly decreased (short-term experiment) or kept relatively constant (long-term experiment) with an increase in the light intensity. The effects of irradiance on $\rho$ and $M$ have been examined in a few previous laboratory and field studies [93-95], but there was no generally consistent result. Both parameters either increased, remained constant, or even decreased with an increase of irradiance. Raven [29] calculated that more Fe was required at a lower photon flux density (i.e., irradiance) due to the much higher pigment content to
intercept more light energy. Our results suggested that M under the four different irradiances was not as different as what we thought before.

Several mechanisms may explain our results. First, diatoms may supply enough Fe for extra pigment synthesis under lower irradiance by transporting Fe from other organelles to the thylakoid where the photosynthetic system is located. For example, 50% of the cellular Fe occurred within photosynthetic units at high light whereas 90% occurred at low light [93]. Second, the coastal diatom *T. pseudonana* may take up luxury Fe. M can be 20–30 times higher than required for maximum growth [84]. Thus, abundant Fe storage in the cells was sufficient for pigment synthesis even under low light intensity. Third, the light intensity used in our study may be not low enough to result in an increase in M. It is possible for a threshold irradiance above which the phytoplankton can complete extra pigment synthesis with Fe redistribution or utilization of Fe storage. Below this threshold level, the diatoms may have to increase ρ or further decrease µ to obtain a high M for normal physiological function. Strzepek and Price [95] found that a very high M occurred only at an extremely low light intensity. Finally, there may be more bioavailable Fe at the higher light intensity due to photoreduction [105, 106], which may further complicate the results.

Recently, Rijkenberg et al. [107] reported a diel cycle of bioavailable Fe concentrations with the highest Fe concentration at noon as a result of photoreduction, which subsequently induced Fe uptake in the day time. Concurrently, the Fe requirement was higher during the daytime as the cellular chlorophyll levels reached their minimum around the middle of the dark period and began to increase prior to the photoperiod [98]. However, diel Fe uptake was rather
constant during the 30 h period in our study. Consequently, the biological demand for Fe by the diatoms is probably decoupled from Fe uptake.

Temperature effects have been much less examined. In both short-term and long-term Fe uptake experiments, $\rho$ and M were higher at 24 °C, although such a trend was less obvious in the short-term experiment. Strzepek and Price [95] found that $\rho$ and $\mu$ for *Thalassiosira weissflogii* increased proportionally with an increase in temperature, resulting in a similar M under different temperatures. However, phytoplankton growth at high temperature mimics low light acclimation [30] and there are more PS II reaction centers, leading to a greater Fe requirement [92]. This may explain the higher M and $\rho$ at higher temperatures observed in our study. Furthermore, Sunda and Huntsman [28] found that more Fe was bioavailable under the lower temperature conditions due to its different effects on photochemical and thermal reaction rates. Thus, the increase in intracellular Fe concentration and uptake with the increase in temperature may have even been underestimated in our study.

### 3.5.2 Fe efflux under different temperatures and irradiances

A remarkable Fe efflux from intracellular pools was observed in our study with $K_e$ ranging from 0.008 to 0.017 h$^{-1}$. In our study, any Fe excreted was trapped by DFB [103] to minimize the re-assimilation of Fe by the diatoms. $K_e$ observed in our study may be underestimated if there was a notable Fe redistribution/internalization from the cell surface to the intracellular compartments during the depuration period [64]. The cell surface adsorbed Fe was not removed before the start of the efflux experiment because the Ti reagent may damage the living cells and further affect Fe elimination [84]. Fe release from the whole cells was also considerable, with a $K_e$ estimate of 0.048-0.095 h$^{-1}$ for the living cells.
Theoretically, Fe release from the whole cell included both Fe efflux from the intracellular compartments and Fe dissociation from the cell surface. Since most accumulated Fe was distributed on the cell surfaces, the Fe efflux from intracellular pools can be neglected and $K_e$ for the Fe elimination from the whole cell can be considered as the Fe dissociation from the cell membrane. This hypothesis was supported by the increase of $K_e$ with the increase in irradiance or temperature because the dissociation is energy dependent [28, 86].

Measurements of the loss of Fe from the dead cells were designed to serve as a control for monitoring the release of Fe from the cells only as a result of Fe dissociation. The gross Fe elimination from the intracellular compartments could be calculated as the difference between the loss from the dead cells (i.e., Fe dissociation from cell surface only) and the Fe efflux from the whole living cell. However, the Fe elimination from heat killed cells was even faster than that from the living cells, indicating the characteristic change of cell surface metal binding ligands after heating (i.e., 0.092 – 0.151 h$^{-1}$ vs. 0.048 – 0.095 h$^{-1}$). Moreover, there was also a notable decrease in intracellular Fe concentration in heat killed cells with $K_e$ ranging from 0.059 to 0.081 h$^{-1}$. Thus the membrane permeability of diatoms may have changed, although the heat killed cells were kept intact (observed under the microscope). In addition, the Fe dissociation from the cell surface may be overestimated due to DFB competition binding [108]. However, the Fe release from the intracellular compartments of living cells was not affected because DFB-Fe complex was not directly bioavailable [86, 103] and neither was DFB, at least for this algal species.

The potential growth dilution on the decrease of the intracellular Fe concentration was subtracted from the results given the notable cell growth during the efflux experiment (ca. 1.3× increase of cell density). Without calibration for
growth dilution, the decrease in M during the eight-hour period would be more obvious. Thus the relative Fe decrease from intracellular compartments can be considered as the true Fe efflux out of the cells, which may even be underestimated in our study. \( K_e \) was often neglected as compared with \( \mu \) when calculating the cellular metal concentration for the phytoplankton [109]. Our results implied that \( K_e \) was significant (i.e., \( K_e: 0.008 - 0.017 \, \text{h}^{-1} \) vs. \( \mu: 0.026 - 0.063 \, \text{h}^{-1} \)) and cannot be neglected, at least for the case of Fe. M or \( \rho \) before and after the consideration of \( K_e \) were compared in Table 3-2 for the short or long term experiment. A notable difference was found with and without \( K_e \), although the trends for different temperatures and irradiances were similar.

Our study demonstrated a remarkable Fe efflux in marine diatoms. Since no Fe efflux data was available for the marine phytoplankton from the literature, our results were compared with the Fe efflux from macrophage, arcard myocytes, and Xenopus oocytes [110-112]. Because different time scales were used and only the relative percentage of Fe decrease was available in these previous studies, \( K_e \) was calculated with the assumption that Fe release from the cell follows first-order kinetics. For macrophage, \( K_e \) values ranged from 0.013 to 0.038 \, \text{h}^{-1} \) [112, 113], and ranged from 0.006 to 0.009 \, \text{h}^{-1} \) and from 0.001 to 0.013 \, \text{h}^{-1} \) for cardiac myocytes [111] and Xenopus oocytes [110], respectively. Thus, Fe efflux measured in our study was in agreement with these different cell types. Fe dissociation from the cell surface was also compared with the other studies. The rate constant for Fe dissociation from the Fe transporter on the cell surface was about 0.72 \, \text{h}^{-1} \) for the coccolithophorid \( P. \text{carterae} \) [64], which was much higher than what was observed in our study for the whole binding ligands of \( T. \text{pseudonana} \). The rate constants of total Cd export from the marine diatom \( T. \text{weissflogii} \) and Zn export from \( \text{Chlorella} \)
*kessleri*i were 0.11-0.16 h⁻¹ and 0.084-0.222 h⁻¹ for Cd and Zn, respectively [21, 78], which were somewhat higher than the Fe dissociation (i.e., 0.048 – 0.095 h⁻¹) obtained in our study. Thus, metal dissociation from the cell surface may be algae, metal species, and binding ligands specific.

Two possibilities may explain the remarkable Fe efflux from the diatoms. On the one hand, the diatom used in our study is a coastal species, which was rarely limited by Fe, and thus highly efficient usage of intracellular Fe was probably not well developed. On the other hand, the high Fe efflux may also have some advantages with respect to other organisms. Since there is a luxury Fe uptake for marine phytoplankton [84], the cells may first take up much more Fe than their own biological requirements when the Fe concentration in the environment is high (e.g., episodic aeolian deposition). Subsequently, Fe may be excreted out of the cells in the form of more dissolved and bioavailable species, which can be taken up again by the phytoplankton as well as for the other organisms [97]. Thus, the phytoplankton can act as a buffer system for Fe. Gordon et al. [114] calculated that only 4-20% of total primary productivity was supplied by the new iron and that each individual iron atom may be cycled in the surface water 169 times before being removed with sinking particles. Our results further prove the importance of Fe regeneration in the surface water, but the forms of Fe that are excreted need to be further examined.

Although more Fe may be required under the low irradiance for the synthesis of more pigments to intercept enough light energy, ρ was much lower at low irradiance based on the results of both short and long term uptake experiments. Under this condition, the diatoms had to decrease μ in order to keep a relatively high M. Consequently, ρ was decoupled with the different Fe requirements under different irradiances. This was further evidenced by the relatively constant ρ in the
diel variation experiment. Several other mechanisms (e.g., intracellular Fe re-distribution and luxury uptake) may be involved to fulfill the higher Fe requirement under low irradiance. An opposite trend was found for temperature. The higher Fe requirement at high temperature was mainly fulfilled by increased $\rho$, while $\mu$ was kept relatively constant. Although $K_e$ plays an important role in the calculation of $M$ and Fe regeneration in the ocean, it has a negligible effect for different Fe requirements under different temperatures and irradiances.
Table 3-1. Fe efflux rate constant ($K_e$) for total and intracellular pools of heat-killed and living diatoms *Thalassiosira pseudonana*. Mean ± SD (n=2).

<table>
<thead>
<tr>
<th>Cells</th>
<th>$K_e$ (h$^{-1}$)</th>
<th>$r^2$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>0.092±0.016</td>
<td>0.875</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>85 Dead (total)</td>
<td>0.106±0.009</td>
<td>0.968</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>170</td>
<td>0.120±0.018</td>
<td>0.883</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>340</td>
<td>0.149±0.009</td>
<td>0.980</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>40 Irradiance (μmol photons m$^{-2}$ s$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>85 living (total)</td>
<td>0.061±0.008</td>
<td>0.910</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>170</td>
<td>0.082±0.011</td>
<td>0.912</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>340</td>
<td>0.095±0.010</td>
<td>0.948</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>40 living (intra)</td>
<td>0.008±0.003</td>
<td>0.567</td>
<td>0.051</td>
</tr>
<tr>
<td>85</td>
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<td>0.629</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>170</td>
<td>0.013±0.003</td>
<td>0.823</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>340</td>
<td>0.017±0.002</td>
<td>0.964</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>15 Temperature (°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19 dead (total)</td>
<td>0.103±0.010</td>
<td>0.947</td>
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<td>24</td>
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<td>0.916</td>
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<td>24</td>
<td>0.151±0.010</td>
<td>0.974</td>
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<tr>
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<td>0.943</td>
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<td>19</td>
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<td>0.016±0.004</td>
<td>0.792</td>
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Table 3-2. Comparison of intracellular Fe concentration (M) and Fe uptake rate (ρ) with and without considering Fe efflux.

<table>
<thead>
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<th>Parameters</th>
<th>Environmental variables</th>
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<th>With efflux</th>
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<td>Irradiance</td>
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<td>2.75×10⁻⁶₈</td>
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<tr>
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<td></td>
<td></td>
<td>24</td>
<td>2.86×10⁻⁶₈</td>
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<tr>
<td>ρ in long term uptake</td>
<td>Irradiance</td>
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<td>1.15×10⁻⁶₈</td>
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<tr>
<td>(nmol cell⁻¹ h⁻¹)</td>
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<td>85</td>
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<td>24</td>
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Fig. 3-1. (a) Increase in cellular accumulated Fe concentration with exposure time during an 8 h period. Medium was refreshed after 3 h and the dashed line represents Fe accumulation without medium refreshing. (b) Increase of cellular accumulated Fe concentration during 3 h period pre- and post- the medium refreshing. The cellular accumulated Fe concentration before the medium refreshing was subtracted from that after the medium refreshing. Data are mean ± SD (n = 2).
Fig. 3-2. Increase of $\ln (\text{cell density at time } t \times \text{initial cell density}^{-1})$ with the increase of growth time under (a, c) different irradiances ($\mu$mol photons m$^{-2}$ s$^{-1}$) and (b, d) temperatures ($^\circ$C) for both (a, b) the short-term and (c, d) long-term uptake experiments. The growth rate was quantified during the acclimation period for the short-term uptake experiments.
Fig. 3-3. (a, b) Increase of cellular accumulated Fe concentration with exposure time under different irradiances (μmol photons m⁻² s⁻¹) and temperatures (°C), respectively. (c, d) The transporter bound and (g, h) intracellular Fe (M) concentrations as well as (e, f) Fe uptake rates (ρ) for cells under different irradiances and temperatures. Data are mean ± SD (n = 2).
Fig. 3-4. Fe uptake rates ($\rho$) at different time during a 30 h period with 14:10 h LD cycle. Data are mean ± SD ($n = 2$).
Fig. 3-5. Relative distribution of Fe in the cells (intra), on the cell surface (extra), and in the aquatic system (aquatic) for the long term uptake experiment with (a) 170μmol photons m$^{-2}$ s$^{-1}$ and (b) 19°C treatments as examples for the different irradiances and temperatures effects experiments. Data are mean ± SD ($n = 2$).
Fig. 3-6. (a, b) Cell surface adsorbed (extra) and (c, d) intracellular Fe (intra) concentration as well as (e, f) the calculated Fe uptake rate ($\rho$) at different time points for the different irradiances ($\mu$mol photons m$^{-2}$ s$^{-1}$, panels a, c, e) and temperatures (°C, panels b, d, f) effects experiments, respectively, in the long-term uptake experiment. Data are mean ± SD ($n = 2$).
Fig. 3-7. Relative retention of intracellular (intra) and total cellular (total) Fe in (a-c) heat killed and (d-g) living cells for different irradiances (μmol photons m⁻² s⁻¹, panels b, d, f) and temperatures (°C, panels a, c, e, and g) during the 8 h depuration period. Data are mean ± SD (n = 2).
Chapter 4

Comparison of Cd, Cu, and Zn toxic effects on four marine phytoplankton by PAM fluorometry

4.1 Abstract

The toxic effects of Cd, Cu, and Zn on four different marine phytoplankton, Dunaliella tertiolecta, Prorocentrum minimum, Synechococcus sp., and Thalassiosira weissflogii were examined by comparing $\mu$, PAM parameters ($\Phi_M$ and $\Phi_M'$), chlorophyll a content, and M over a 96 h period. The calculated no-observed effect concentration (NOEC) based on both $\mu$ and two PAM parameters were mostly identical. Thus, these PAM parameters and $\mu$ were comparable in their sensitivities as the endpoints for trace metal toxicity to marine phytoplankton. The cyanobacteria Synechococcus sp. was the most sensitive species among the four algal species tested due to its higher cell surface area to volume ratio. The toxicity of the three tested metals followed the order of Cd > Cu > Zn based on M of the four algae at NOEC. The cellular metal bioaccumulation followed the same Freundlich isotherm for each metal regardless of the algal species, indicating that the metal accumulation was a non-metabolic process under high ambient metal concentrations and the cell surface metal binding were comparable among the different species. For all the algae examined in our study, the bioaccumulation potentials of Cu and Zn were similar to each other, while the Cd bioaccumulation was much lower under environmentally realistic metal concentration.
4.2 Introduction

Over the last decade, the PAM fluorescence method has been developed to study photosynthetic processes in plants [53, 54]. This technique has since been widely used to study the effects of environmental stressors (e.g. herbicides, trace metals, nutrient depletion, and viral infection) on higher plants and phytoplankton [115-119]. By using this method, the maximum PS II quantum yield $\Phi_M$ and operational PS II quantum yield $\Phi_M'$ can be quantified [53, 62]. Thus, any direct or indirect photosynthetic activity impairments caused by environmental stressors would be observed by the changes of these parameters. It has been proposed that this non-intrusive fluorescence technique offers a rather rapid and sensitive approach to evaluate pollutant effects on plants and freshwater algae as compared to the frequently used endpoints such as $\mu$, CO$_2$ fixation, O$_2$ evolution, respiration, and ATP levels [56, 120]. However, the sensitivity of these parameters to trace metal toxic effects on marine phytoplankton remains unknown.

Many studies have examined the Cu toxic effects on photosynthesis of plants and phytoplankton. Cu can affect photosynthetic electron transport on the reducing side of PS I at the level of the ferredoxine [121-123]. In addition, Cu can alter the PS II on the oxidizing side by inhibiting the electron transport at P$_{680}$ (the primary electron donor of PS II) as well as by inactivating some PS II reaction centers [124, 125]. A few studies have indicated that Cu may also impair the PS II electron transport on its reducing side by affecting the rate of oxydoreduction [126-129]. For Cd and Zn, much less is known about their toxic effects on the photosynthetic apparatus. Zn may affect the oxidizing side of PS II by non-competitively inhibiting Ca$^{2+}$ and Mn$^{2+}$ binding at their native sites on the water oxidizing complex [130]. A high Cd concentration can modify the PS II activity of bean plants [131]
and disassemble the PS II proteins of pea [132]. Consequently, these three trace metals can affect photosynthetic electron transport chain to different degrees, which may be reflected by the changes of the PAM parameters.

A few decades have elapsed since the formulation of FIAM [1, 7, 8], but there were fewer trace metal toxicity studies based on the ambient free metal ion concentration [63, 133-135] as compared to those based on the total metal concentration. To our knowledge, few studies also attempt to link metal cellular bioaccumulation with metal toxicity in marine phytoplankton [136]. In this study, we compared the toxic effects of three trace metals Cd, Cu, and Zn on four different species of marine phytoplankton, using different physiological endpoints such as $\mu$ and the PAM parameters. These physiological endpoints were then correlated with the actual metal accumulation in the phytoplankton cells. Our objectives were to (1) examine whether the PAM parameters were more sensitive than $\mu$ as physiological endpoints to trace metal toxicity on different marine phytoplankton, (2) examine the sensitivity of different algal species to three trace metals, the potential mechanisms involved in these toxic effects as well as the toxicity order of these metals, and (3) establish the relationship between metal toxicity and cellular metal bioaccumulation in different species of marine phytoplankton. Relationships between metal cellular bioaccumulation and metal toxicity in marine phytoplankton can provide critical information for the development of BLM for this particular group of marine organisms.

4.3 Materials and methods

4.3.1 Phytoplankton and culture conditions
Axenic cultures of *Dunaliella tertiolecta* (CCMP 1320, green alga), *Prorocentrum minimum* (CCMP696, dinoflagellate), *Synechococcus sp.* (CCMP 1333, cyanobacteria), and *Thalassiosira weissflogii* (CCMP 1048, diatom) were all obtained from the Provasoli-Guillard Center for the Culture of Marine Phytoplankton, Bigelow Laboratory, West Boothby Harbor, Maine, USA. The cells were maintained in f/2 medium (including trace metals Fe, Cu, Mo, Zn, Co, Mn, and EDTA) [73] with sterile techniques, in an incubator at 19 °C with a light illumination of 170 μmol photons m⁻² s⁻¹ on a 14:10 LD cycle. *Dunaliella tertiolecta* and *Thalassiosira sp.* are the recommended model phytoplankton for the toxicity tests by ASTM (American Society for Testing and Materials). Furthermore, *Synechococcus* and *Prorocentrum minimum* were reported to be quite sensitive and tolerant to trace metals, respectively [133]. The pH value of the medium was 8.2 ± 0.1. Seawater was collected 10 km off East Hong Kong to minimize the influences of anthropogenic activities, and was filtered through 0.22 μm Poretic membrane before being used in the experiments.

**4.3.2 Medium for metal toxicity test**

The 0.22 μm filtered seawater and all macronutrients (N, P, and Si) used throughout the whole toxicity study were passed through a Chelex 100 resin column (analytical grade from BIO-RAD, Hercules, CA, USA) to remove background trace metals. The dissolved organic matters in the water were then destroyed by irradiating the media with UV for at least 24 h (with removal efficiency close to 100%). Trace metal clean technique was used throughout the whole experiment period. All the beakers and bottles (polycarbonate from Nalgene, Rochester, NY, USA) used were soaked in 10% HNO₃ until the day before the toxicity tests and then rinsed with Milli-Q water (18.0 MΩ) seven times. Nutrients and metals addition were
performed in the class-100 clean bench to minimize the potential metal contamination. The N, P, Si, and vitamin concentrations enriched in the seawater were the same as the f/2 medium, while the trace metals’ (i.e., Fe, Cu, Mo, Zn, Co, and Mn) concentrations were at the f/10 levels. The relatively high nutrient concentrations used as compared with the natural environment were to ensure the algal growth not limited by the nutrient depletion. It would not affect the difference or trend observed between different treatments as the same concentrations were applied, although the nutrient concentration can influence the metal bioaccumulation [32, 33]. Nitrilotriacetate (NTA, 0.1 mmol l⁻¹) was used to keep the free metal ion concentrations constant in the media during the toxicity experiment. A total of six concentration treatments with three replicates each were performed for the different metals and different algal species. The total nominal metal concentrations (Cd, Cu, or Zn) used for each treatment were listed in Table 4-1. Treatment A served as a control with the nominal Cd concentration 10% of the background (i.e. 90% were removed by the chelex resin) and the concentrations of Cu and Zn the same as those in the f/10 medium. The pH values of the media were adjusted to 8.2 ± 0.1 with 1 mol l⁻¹ Suprapure NaOH (Merck, Darmstadt, GERMANY). The free metal ion concentrations ([M²⁺]) listed in Table 4-1 were calculated by using the software MINEQL+ (Version 4.5 from Environmental Research Software, Hallowell, ME, USA) with the calibration of the ionic strength. Such a wide range of metal concentrations was used to ensure the appearance of toxic effects and to examine the change of the parameters with increasing metal concentration. The media were equilibrated for at least 24 h under the same conditions before starting the toxicity tests.

4.3.3 Toxicity tests by PAM-fluorometry
The algal cells were first inoculated into the f/2 medium except that the trace metals' concentrations for this new culture were the same as the f/10 medium. The temperature, light intensity, and LD cycle were the same as those used for the algal stock culture. During this acclimation period, the cell density of the algae *D. tertiolecta*, *P. minimum*, and *T. weissflogii* were enumerated by a Coulter Counter every 12 h, whereas the growth of *Synechococcus sp.* was quantified with a TD-700 fluorometer. After the cells arrived at the mid-exponential phase, they were collected by centrifugation (4000 g, 15 min) and then resuspended into the toxicity test media. The initial cell density of different metal concentration treatments for each alga was maintained the same (1.6×10⁵ cells ml⁻¹ for *D. tertiolecta*; 10⁴ cells ml⁻¹ for *P. minimum*; 3.8×10⁶ cells ml⁻¹ for *Synechococcus sp.*; 8×10³ cells ml⁻¹ for *T. weissflogii*), and the PAM fluorescence measurements were performed after 5 h, 24 h, 48 h, and 96 h. At the same time the cell number was quantified either with the Coulter Counter or a TD-700 fluorometer (for *Synechococcus sp.* only). Because the cell specific growth rates $\mu$ at the higher metal addition treatments became smaller during the 96 h exposure period, only the overall cell specific growth rate was calculated by the following equation:

$$\mu = \frac{\ln(C_f/C_0)}{t}$$  \hspace{1cm} (4-1)

where $C_0$ and $C_f$ represent, respectively, the initial and final cell density or fluorescence, and $t$ is the duration of the whole toxicity experiment (96 h).

Fluorescence induction measurements were performed with a PAM 101/103 fluorometer equipped with an ED-101PM emitter-detector-cuvette unit (Heinz Walz GmbH, Effeltrich, GERMANY) after dark-acclimation of algae for 15 min [118]. $F_o$ of dark-adapted algae was measured by using ML which has a very low light intensity to avoid the reduction of the PS II primary electron acceptor $Q_A$. $F_m$ was
induced by the SP (2000 µmol photons m\(^{-2}\) s\(^{-1}\)), which completely reduced all \(Q_A\). Our preliminary experiment found that the light intensity used for SP was high enough for the complete reduction of \(Q_A\), indicating that the PS II reaction centers were completely closed. Such a high light intensity did not cause any obvious photoinhibition effect. The electron transport during the toxicity test was triggered by turning on a continuous AL with the irradiance similar to the growth light intensity (170 µmol photons m\(^{-2}\) s\(^{-1}\)). During the fluorescence induction period, SP were triggered every 1 min. \(\Phi_M\) and \(\Phi_M'\) were calculated from the fluorescence induction curve based on Eqs. 1-5 and 1-6 as described by Juneau et al. [56]. Both parameters were obtained from the same PAM fluorescence induction curve.

The NOEC was calculated as the highest \([M^{2+}]\) at which no statistically significant toxic effect was observed based on the toxicity endpoints (i.e., \(\mu\), \(\Phi_M\), and \(\Phi_M'\)) as compared to the control treatment. The NOEC was calculated in our study for all the four different algal species and three metals based on the statistical analysis results of one-way ANOVA with post hoc multiple comparisons (software used: SPSS 11.0 by SPSS, Chicago, USA). Significant difference was accepted at \(p < 0.05\).

4.3.4 Cellular metal concentration measurement

\(M\) was quantified after the measurement of PAM fluorescence and cell number/fluorescence at 96 h. One hundred ml aliquots were taken from each replicate of the six treatments and placed into two 50 ml plastic centrifuge tubes which had been soaked in 10% HNO\(_3\) for several days to minimize potential trace metal contamination. After centrifugation at 4000 g for 15 min, the supernatant was decanted and the pellets were resuspended into 10 ml (5 ml for each tube) UV-Chelex 0.22 µm filtered seawater to remove the labile metals associated with
cell surface. The concentrated algae were transferred to a 15 ml centrifuge tube and spun for 15 min, afterwards the cells were resuspended again into 10 ml of UV-Chex 0.22 μm seawater and centrifuged. This procedure was repeated a few more times depending on the metal concentration of the treatment. Finally, after the supernatant was decanted, a 1 ml Suprapure 70% HNO₃ (BDH from Merck, Darmstadt, GERMANY) was added into the cell pellets, and cells were digested for 2 to 3 days at room temperature. The digested samples were diluted and the metal concentrations were measured by inductively coupled plasma – mass spectrometry (ICP-MS). Standard curve using a series of dilution of metal standards (Perkin-Elmer, Boston, MA, USA) was run each time when the samples were analyzed. Metal concentrations in the blanks were also quantified and they were below the detection limit. M was expressed with three different units: ng cell⁻¹, μmol per cell volume (μmol l⁻¹), and μmol per cell surface area (μmol m⁻²) in order to explore the potential mechanisms of the cellular metal accumulation for different algal species. Trace metal clean technique was used throughout the whole experiments. All sample preparations were conducted in a trace metal class-100 clean bench.

4.3.5 Quantification of the chlorophyll a content

The cellular chlorophyll a contents were measured at both 24 h and 96 h. The culture (10-20 ml) was gently (< 50 mm Hg) filtered through the polycarbonate membrane (1 μm) and rinsed three times with 0.22 μm seawater. The membrane was soaked in 10 ml 90% acetone in darkness at 4 °C. After 24 h, the fluorescence of the acetone solution before and after the acidification with 10% HCl was measured with the TD-700 fluorometer. The chlorophyll a concentration was calculated according to Parsons et al. [137]:
\[ [\text{chl a}] = \frac{(F_i - F_A) \times V}{V} \times n \times d \quad (4-2) \]

where \([\text{chl a}]\) is the concentration of chlorophyll a, \(F_i\) and \(F_A\) represent the fluorescence before and after the HCl acidification, respectively, \(V\) is the volume of 90% acetone used for the chlorophyll extraction, \(V\) is the volume of the sample filtered for the measurement, \(n\) is the acid correction factor (ca. 2), and \(d\) is the slope of the standard curve of fluorescence vs. chlorophyll a extracted from the commercially available algae \textit{Anacystis nidulans}.

4.4 Results

4.4.1 PAM fluorescence parameters (\(\Phi_M\) and \(\Phi_M'\))

Remarkable toxic effects were observed for most of the algae exposed to high concentrations of Cd, Cu, and Zn with PAM fluorometry. Typical changes of the fluorescence induction curve with the increase of \([M^{2+}]\) in the medium are shown in Fig. 4-1, using \textit{D. tertiolecta} as an example. For all these metals, both the initial \(F_0\) and \(F_m\) levels decreased when the metal concentration increased. For \textit{D. tertiolecta}, there was a 80% and 85% decrease of the initial \(F_0\) and \(F_m\), respectively, as \([\text{Cd}^{2+}]\) increased from \(10^{-13}\) to \(10^{-6.9}\) mol \(l^{-1}\). When \([\text{Cu}^{2+}]\) increased from \(10^{-13}\) to \(10^{-8.7}\) mol \(l^{-1}\), the \(F_0\) and \(F_m\) decreased by 50% and 55%. Similar trend was also found for Zn, i.e., the \(F_0\) and \(F_m\) decreased by 30% and 55% respectively, as \([\text{Zn}^{2+}]\) concentration increased from \(10^{-10.2}\) to \(10^{-5.3}\) mol \(l^{-1}\). The changes of the fluorescence induction curve with the increase of \([M^{2+}]\) in the media were similar for the other three algal species tested (data not shown).

Based on these induction curves, \(\Phi_M\) and \(\Phi_M'\) were calculated. The relative changes of these two PAM parameters (the real value of the metal treatment divided by that of the control) for each tested algal species under different \([M^{2+}]\) and
exposure time are shown in Figs. 4-2 to 4-4. \( \Phi_M \) and \( \Phi_M' \) decreased at higher [M\(^{2+}\)] in most cases, although these two parameters were kept almost constant for all the treatments with different [M\(^{2+}\)] in some toxicity tests, e.g., Cd - *P. minimum*, Cu - *D. tertiolecta*, and Zn - *P. mimimum*. Both parameters were inhibited to a greater degree when the exposure time was increased from 5 h to 96 h. Among the four algae tested in our study, *Synechococcus sp.* had the most dramatic changes in the quantum yields (\( \Phi_M \) and \( \Phi_M' \)). For example, there was about 80%, 60%, and 90% decrease for both \( \Phi_M \) and \( \Phi_M' \) when *Synechococcus sp.* was grown in a medium containing high [Cd\(^{2+}\)] (10\(^{-6.9}\) mol l\(^{-1}\)), [Cu\(^{2+}\)] (10\(^{-8.7}\) mol l\(^{-1}\)), or [Zn\(^{2+}\)] (10\(^{-5.3}\) mol l\(^{-1}\)) respectively, as compared with the control treatments (Figs. 4-2 to 4-4). The toxic effects of Cd and Zn were also observed much rapider on *Synechococcus sp.* as compared with the other three species (Figs. 4-2 and 4-4). After 5 h of exposure, the \( \Phi_M \) and \( \Phi_M' \) had already decreased by 50%. On the other hand, the PS II quantum yields for *P. minimum* were kept almost constant when exposed to different [Cd\(^{2+}\)] and [Zn\(^{2+}\)] (Figs. 4-2 and 4-4). The highest [Cd\(^{2+}\)], [Cu\(^{2+}\)], and [Zn\(^{2+}\)] used for this alga were 10\(^{-6.5}\), 10\(^{-5.6}\), and 10\(^{-4.3}\) mol l\(^{-1}\), respectively, as compared to the highest [Cd\(^{2+}\)], [Cu\(^{2+}\)], and [Zn\(^{2+}\)] of 10\(^{-6.9}\), 10\(^{-8.7}\), and 10\(^{-5.3}\) mol l\(^{-1}\) used in experiments with *Synechococcus sp.*

### 4.4.2 Cell specific growth rate

The cell specific growth rates \( \mu \) under different [M\(^{2+}\)] for different species are shown in Fig. 4-5. Similar to \( \Phi_M \) and \( \Phi_M' \), *Synechococcus sp.* showed the most dramatic decrease in \( \mu \) among the four algal species tested (i.e., there was 100% decrease at the highest [M\(^{2+}\)] as compared with the control treatment at 96 h) with the increase of [M\(^{2+}\)]. Comparing to the initial cell density, the final cell density remained unchanged or even decreased when the [M\(^{2+}\)] was increased to 10\(^{-6.9}\), 10\(^{-8.7}\),
and $10^{-7.2}$ mol l$^{-1}$ for Cd, Cu, and Zn, respectively. For the other species, only $\mu$ of \textit{D. tertiolecta} and \textit{P. minimum} reached 0 at the highest [Cd$^{2+}$] and [Cu$^{2+}$]. At the same time $\mu$ either decreased continuously with increasing [M$^{2+}$] (e.g. \textit{T. weissflogii}), or started to decrease significantly when the [M$^{2+}$] reached a threshold level ($p < 0.05$).

### 4.4.3 Cellular metal concentration

The cellular metal concentration M, including both the cell surface strongly adsorbed metals and the intracellular metals, of the different algae exposed to Cd, Cu and Zn for 96 h is shown in Fig. 4-6. For all the algal species/metal combination, M increased with the increase of [M$^{2+}$] in the medium and reached almost saturation for some species under the highest [M$^{2+}$] (e.g. the cellular Cd concentration for \textit{Synechococcus sp.}). With the increase of [Cd$^{2+}$] from $10^{-13}$ to $10^{-6.5}$ mol l$^{-1}$, there was about 3 to 5 orders of magnitude increase in the cellular Cd concentration for all the four algal species tested. On the other hand, there was less than 3 orders of magnitude increase for the cellular Cu concentration over the different ranges of [Cu$^{2+}$] used for different algae ($10^{-13} - 10^{-8.7}$ mol l$^{-1}$ for \textit{D. tertiolecta} and \textit{Synechococcus sp.}; $10^{-13} - 10^{-7.1}$ M for \textit{T. weissflogii}; $10^{-13} - 10^{-5.6}$ M for \textit{P. minimum}).

As seen for Cu, the cellular Zn increased by $< 3$ orders of magnitude over the range of [Zn$^{2+}$] used in our study ($10^{10.2} - 10^{5.3}$ mol l$^{-1}$ for \textit{D. tertiolecta}, \textit{Synechococcus sp.}, and \textit{T. weissflogii}; $10^{10.2} - 10^{4.3}$ M for \textit{P. minimum}). For all these four algal species, M (ng cell$^{-1}$) of \textit{Synechococcus sp.} was significantly lower than the other three algae, mainly due to the much smaller cell size ($p < 0.05$). Thus after being calibrated to the cell volume (µmol l$^{-1}$), M of \textit{Synechococcus sp.} were even higher than that measured for the other three species.
However, both units (ng cell\(^{-1}\) and μmol l\(^{-1}\)) could not indicate the metal body burden accurately due to the difference in cell size and the existence of large vacuole for some algae [135]. \(M\) was then expressed as \(\mu\text{mol m}^{-2}\) (cell surface area basis calculated from the cell diameter with the spherical assumption), and it was found that the difference in \(M\) of different algal species under the same \([M^{2+}]\) disappeared (Fig. 4-6). There was a linear relationship between the log \([M^{2+}]\) and log \(M\), which could be described by the Freundlich isotherm [138]:

\[
\log M = \frac{1}{e} \log [M^{2+}] + k
\]

where \([M^{2+}]\) is the free metal ion (Cd\(^{2+}\), Cu\(^{2+}\), or Zn\(^{2+}\)) concentration in the medium, \(M\) is the cellular metal concentration, \(k\) and \(e\) are the empirically determined constants, with \(k\) being related to the maximum binding capacity, and \(e\) is related to the affinity or binding strength. By performing the linear regression, the two empirical constants, \(k\) and \(e\), can be both calculated (Fig. 4-6). Comparing the two empirical constants of different trace metals, it was found that the bioaccumulation ability of Cu and Zn was comparable, while it was much weaker for Cd at low \([M^{2+}]\).

4.4.4 NOECs of different algal species for different metals

NOECs based on \(\mu\) and PAM parameters (\(\Phi_M\) and \(\Phi_M'\)) are compared in Table 4-2. For most of the toxicity tests NOECs calculated for these two different endpoints were identical. NOEC for \(\mu\) was lower than those for PAM parameters in the toxicity tests of Cu - *T. weissflogii*, but the reverse was true in the toxicity tests of Cd - *Synechococcus sp.* and Zn - *T. weissflogii*. Fig. 4-7 compares the geometric mean of NOECs obtained from \(\mu\) and the two PAM parameter for the four different algal species. NOECs of the cyanobacteria *Synechococcus sp.* and the diatom *T. weissflogii* were lower than the green alga *D. tertiolecta* and the dinoflagellate *P. minimum*, indicating that they were more sensitive to metal exposure. However,
when comparing M ($\mu$mol l$^{-1}$) for different algal species at NOEC, the difference between different species became orders of magnitude less obvious, although the M leading to NOEC was the lowest for the diatom (Fig. 4-7). Among the three metals, Zn was the least toxic, as its NOEC was orders of magnitude higher than those for Cd and Cu. In contrast, there was no consistent trend for Cd and Cu, whose toxicity depended on the different algae. When comparing M at NOEC, the toxicity order of these three metals became clearer: Cd > Cu > Zn from high to low toxicity.

4.4.5 Cellular chlorophyll a content

[Chl a] of three algal species at both 24 and 96 h is shown in Fig. 4-8. [Chl a] of Synechococcus sp. was not presented because of the incomplete chlorophyll extraction from the GF/F membrane. The polycarbonate membranes were later used for the extraction of the other algal species. The diatom T. weissflogii had the highest [chl a] ($4.78 - 7.18 \times 10^{-12}$ g cell$^{-1}$) while [chl a] of D. tertiolecta was the lowest ($6.04 - 9.81 \times 10^{-13}$ g cell$^{-1}$). For D. tertiolecta, [chl a] was kept almost constant (e.g., 8.17 - 8.82 x $10^{-13}$ g cell$^{-1}$ at 96 h) regardless of the [M$^{2+}$] used, even though obvious toxic effects were found at high [Cd$^{2+}$] and [Zn$^{2+}$]. For T. weissflogii, the addition of Cd significantly increased [chl a] at the highest concentrations (e.g., from 5.15 to 6.75 x $10^{-12}$ g cell$^{-1}$ at 96 h), while Zn and Cu significantly reduced [chl a] in the diatoms ($p < 0.05$). For P. minimum, [chl a] decreased with increasing [Cu$^{2+}$] (e.g., from 2.28 x $10^{-12}$ to 5.19 x $10^{-13}$ g cell$^{-1}$ at 96 h), but remained comparable for Cd and Zn. For both T. weissflogii and P. minimum, [chl a] had a bigger change after 96 h exposure as compared to 24 h.

4.5 Discussion

4.5.1 Comparison of $\mu$ and PAM fluorometry and algal species sensitivity
PAM fluorometry is known to be a non-intrusive method having some advantages for ecotoxicological research, such as the high speed and the supply of information about the underlying toxicity mechanisms for higher plants and freshwater algae [56, 120]. In our study, the sensitivity of two endpoints ($\mu$ and PAM parameters: $\Phi_M$ and $\Phi_{M'}$) was compared by studying the toxic effects of Cd, Cu, and Zn on four different marine algal species. The NOECs calculated based on these two endpoints were very similar regardless of which endpoint was used for the calculation, suggesting that they had similar sensitivity in quantifying the metal toxicity to marine phytoplankton. Juneau et al. [56] calculated that the copper 5 h IC50 (median inhibition concentration) values of $\Phi_{M'}$ parameter for the freshwater algae *Chlamydomonas reinhardtii* and *Pseudokirchneriella capricornutum* were 13 and 30 $\mu$g l$^{-1}$, respectively, which were much lower than the 24 h IC50 values found in other studies with growth inhibition as endpoint (46 to 227 $\mu$g l$^{-1}$) [139, 140], indicating that $\Phi_{M'}$ is more sensitive than $\mu$. However, in our study, the IC50 values were lower than the previous studies (data not shown) probably due to the fact that the IC50 values calculated in the previous studies were based on the total dissolved metal concentration in the medium instead of the free metal ion concentrations.

In our study, the PAM parameters have similar sensitivities with $\mu$ as endpoints for the metal toxicity to marine phytoplankton. However, they offer the advantage as these parameters reveal the mechanisms involved in metal toxicity. For example, the Cu toxic effects on the photosynthetic system (PS I and II biochemistry) had been extensively examined. The inhibition of both $\Phi_M$ and $\Phi_{M'}$ found in our study indicated that Cu affects the whole electron transport chain. Zn can reduce the PS II activity by non-competitively inhibiting Ca$^{2+}$ and Mn$^{2+}$ binding at native sites on
water oxidase [130]. Thus a similar reduction of $\Phi_M$ and $\Phi_M'$ was also observed. Such an inhibition of the electron transport by Zn was partially due to the change in the ultrastructure of the thylakoid membrane as well as the destruction of the antennae pigments [141]. Contrary to other metals, the direct effect of Cd on the photosynthetic apparatus of algae was less documented. A high Cd concentration can modify the PS II activity of bean plants [131] and disassemble the PS II proteins of pea [132], which result in the decrease of $\Phi_M$. Similar inhibitive effects may also take place in phytoplankton, as both $\Phi_M$ and $\Phi_M'$ were lowered in the higher Cd concentration treatments of our study. In addition, the PAM fluorescence method is related to its higher efficiency and rapidity compared to the growth inhibition measurements especially for the field ecotoxicological work [120, 142, 143].

Based on the calculated NOECs, *Synechococcus* sp. and *T. weissflogii* were more sensitive than the other two algal species: *D. tertiolecta* and *P. minimum*. This result was partly consistent with previous study [133] showing that the prokaryotic cyanobacteria were most sensitive to both Cu and Cd, whereas the diatoms were the least sensitive, and coccolithophores and dinoflagellates were intermediate. Our study showed that the diatom *T. weissflogii* was even more sensitive to Cd and Zn than the cyanobacteria *Synechococcus* sp.. The type of toxicity response by *T. weissflogii* was different from the other three algal species. With increasing ambient $[\text{M}^{2+}]$, $\mu$ or the PAM parameters of *T. weissflogii* decreased continuously, even when the ambient metal concentrations were low and nontoxic to the other three algal species. Thus, the NOEC for *T. weissflogii* was much lower than the other three algal species. However, *Synechococcus* sp. was still the most sensitive species by taking into account its most abrupt changes of the endpoints. Differences between M of these four algal species at NOEC were orders of
magnitude smaller than that of the NOEC itself, especially for *Synechococcus* sp.. Since the cell size of *Synechococcus* sp. was very small (leading to a higher cell surface area to volume ratio), this species can accumulate the metals more efficiently than the other algae. Thus, the higher sensitivity of *Synechococcus* to the metals (especially to Cd and Cu) was not due to its lower ability to regulate the metal uptake or eliminate the cellular metal, but to its higher cell surface area to volume ratio and thus higher metal bioaccumulation potentials.

In our study, *D. tertiolecta* was very tolerant of Cu while *P. minimum* was very tolerant of Cd and Zn, e.g., no obvious toxic effects were observed even under the highest [M$^{2+}$] we can make with the solubility limitation. Their NOECs were operationally defined as the highest [M$^{2+}$] used for these two species. At the same time the cellular Cd and Cu concentrations for these two algal species under the highest [M$^{2+}$] treatments were comparable to other phytoplankton at NOEC, which could not be completely accounted for by their differences in cell size. Thus, the high tolerance of these algal species may result from the regulation of metal uptake or elimination, which leads to a lower cellular metal concentration at the same [M$^{2+}$] [144-146].

4.5.2 Cellular metal concentration

Methods for the measurement of cellular metal concentration have been available [134, 136, 147]. However, it is difficult to compare M from different studies, because most of these previous works were performed based on the total ambient dissolved metal concentration. Direct measurements of the cellular metal concentration based on the free metal ion concentration were rather scarce. According to FIAM, the cellular metal bioaccumulation depends mainly on the free metal ion concentration/activity in the ambient environment. Thus, the cellular
metal concentration may be completely different under the similar total ambient metal concentration due to the potential difference in free metal ion concentration of the environment. Few toxicity studies have been performed based on the free metal ion concentration [134, 135].

When the cellular metal concentrations were expressed with the unit μmol per cell surface area (i.e., μmol m⁻²), the relationship between M and [M²⁺] followed the same Freundlich isotherm for each metal, respectively, regardless of the algal species. Thus it appeared that metal bioaccumulation at high metal concentrations was a non-metabolic process. Similarly, Fisher et al. [136] reported that the cellular accumulated metal concentration for heat-killed cells was comparable to that of the living cells. Brand et al. [133] found that the prokaryotic cyanobacteria (e.g. *Synechococcus* sp.) were the most sensitive algal species to Cu and Cd as compared to the eukaryotic species. It was advanced that Cu and Cd are bound more strongly to the mucopolysaccharide cell walls of the cyanobacteria than to the fundamentally different types of cell walls of eukaryotic algae (predominantly cellulose). Our results however suggested that *Synechococcus* sp. had similar metal bioaccumulation ability as the eukaryotic algal species, although their cell surface characteristics are different.

Based on the Freundlich isotherm obtained from M (μmol m⁻²) with the increase of [M²⁺], it was evident that Cu and Zn had similar bioaccumulation ability. For Cd, its accumulation slope (i.e., 1/e in Eq. 4-3) was higher than that of Cu and Zn (i.e., 0.66 vs. 0.33/0.36 in Fig. 4-6). However, its bioaccumulation was still lower than Cu and Zn at low environmental metal concentrations. The metal bioaccumulation by an alga was known to involve three processes: (1) diffusion of the metal from the bulk solution to the biological surface; (2) binding site complexation at the cell
surface; and (3) uptake or internalization of the metal [1]. The surface binding can reach saturation over a very short period (e.g. less than 10 min). It is known that the surface metal binding sites were mainly composed of carboxylic, sulfhudryl, and phosphatic groups [148, 149]. Cu (as Cu$^{2+}$) and Zn could bind strongly to oxygen and nitrogen-containing ligands, while Cd was known to bind more strongly with sulfur-containing ligands [150], which may explain why the bioaccumulation of Cu and Zn was higher than Cd under the same [M$^{2+}$]. Franklin et al. [147] found that the metal cell distribution coefficient (Kd) of Cu > Cd > Zn. These calculations were based on the total ambient metal concentration, which may not indicate the real particle reactivity of these metals based on [M$^{2+}$]. In addition, the toxicity test they performed was in freshwater under which the metal speciation may be completely different from the marine waters. For example, Cd$^{2+}$ was the dominant Cd species in the freshwater while Cd-Cl complexes were the main inorganic Cd species in seawater which may not be directly bioavailable [1]. Thus the Kd value of Cd in the freshwater should be higher than that in seawater under the same total dissolved metal concentration due to more bioavailable Cd, which could even be higher than that of Zn [147].

There was a trend that Cu and Zn decreased the chlorophyll a content of T. weissflogii and P. minimum, presumably because these metals inhibited the reductive steps in the biosynthetic pathway of photosynthetic pigments [151]. In addition, Zn can inhibit the enzymatic activity of protochlorophyllide reductase, which was involved in the reduction of protochlorophyll to chlorophyll [152]. The chlorophyll a increase for one of the treatments in Cu toxicity test to T. weissflogii (i.e. F at 24h and D at 96 h) may be due to hormesis effect. This was evidenced by the shift of the chlorophyll a increase treatment from F to D when the exposure time increased
from 24 h to 96 h. In contrast to Cu and Zn, the chlorophyll a content of *T. weissflogii* increased at high Cd concentrations. The reason underlying such increase remains unknown, and needs further investigation.

Our study suggests that the PAM parameters ($\Phi_M$ and $\Phi_{M'}$) can be used as endpoints to quantify the metal toxicity to marine algal species. This technique may potentially be used to study the toxicity under the field conditions, especially since it can give clues on the mechanisms of toxicity. Our study also shows considerable variations in metal sensitivity among different species of marine phytoplankton. Such difference needs to be considered in the future development of BLM to predict metal toxicity in marine phytoplankton. In our study, we used the trace metal buffer systems to control $[M^{2+}]$ during the 96 h incubation period. Whether $[M^{2+}]$ was constant throughout the exposure period remains to be further verified. In addition, we used high macronutrient concentrations in the toxicity testing to avoid any potential interference of macronutrients on metal toxicity. It would be interesting to further examine the interaction of macronutrients and metals in their toxicities to marine phytoplankton.
Table 4-1. Total dissolved ($M_w$) and free metal ion concentrations ([M$^{2+}$], mol l$^{-1}$) used for the four different algal species: *Dunaliella tertiolecta* (DT), *Prorocentrum minimum* (PM), *Synechococcus* sp. (Syn), and *Thalassiosira weissflogii* (TW).

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Table 4-2. Comparison of no-observed-effect concentration (NOEC, mol l\(^{-1}\)) values derived from the changes of cell specific growth rate (\(\mu\)) and PAM parameters \(\Phi_M\) and \(\Phi_{M'}\) for the four algal species, *Dunaliella tertiolecta* (DT), *Prorocentrum minimum* (PM), *Synechococcus* sp. (Syn), and *Thalassiosira weissflogii* (TW).

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N.D.: not determined.

*: NOEC from \(\mu\) was significantly different from those from both \(\Phi_M\) and \(\Phi_{M'}\).
Fig. 4-1. *Dunaliella tertiolecta.* PAM fluorescence induction curves at different $[\text{Cd}^{2+}]$ ($10^{-13}$, $10^{-8.1}$, and $10^{-6.9}$ mol l$^{-1}$ from left to right), $[\text{Cu}^{2+}]$ ($10^{-13}$, $10^{-10.1}$, and $10^{-8.7}$ mol l$^{-1}$ from left to right), and $[\text{Zn}^{2+}]$ ($10^{-10.2}$, $10^{-7.2}$, and $10^{-5.3}$ mol l$^{-1}$ from left to right) after 96 h of exposure.
Fig. 4-2. *Dunaliella tertiolecta*, *Prorocentrum minimum*, *Synechococcus* sp., and *Thalassiosira weissflogii*. Relative changes of the maximum quantum yield ($\Phi_M$) and operational quantum yield ($\Phi_M'$) as a function of $[\text{Cd}^{2+}]$ at different times of exposure. Data are mean $\pm$ SD ($n = 3$).
Fig. 4-3. *Dunaliella tertiolecta*, *Prorocentrum minimum*, *Synechococcus sp.*, and *Thalassiosira weissflogii*. Relative changes of the maximum quantum yield ($\Phi_M$) and operational quantum yield ($\Phi_M'$) as a function of $[Cu^{2+}]$ at different times of exposure. Data are mean ± SD ($n = 3$).
Fig. 4-4. *Dunaliella tertiolecta*, *Prorocentrum minimum*, *Synechococcus* sp., and *Thalassiosira weissflogii*. Relative changes of the maximum quantum yield ($\Phi_M$) and operational quantum yield ($\Phi'_M$) as a function of [Zn$^{2+}$] at different times of exposure. Data are mean ± SD (n = 3).
Fig. 4-5. Dunaliella tertiolecta, Prorocentrum minimum, Synechococcus sp., and Thalassiosira weissflogii. Changes of the cell specific growth rate at different ambient free metal ion concentrations ([M^{2+}]). Data are mean ± SD (n = 3).
Fig. 4-6. *Dunaliella tertiolecta* (○), *Prorocentrum minimum* (●), *Synechococcus sp.* (▼), and *Thalassiosira weissflogii* (▽). Cellular metal concentrations expressed with three different units (ng cell⁻¹, μmol l⁻¹, and μmol m⁻²) at different ambient free metal ion concentrations ([M²⁺]). Data are mean ± SD (n = 3).
Fig. 4-7. *Dunaliella tertiolecta* (DT), *Prorocentrum minimum* (PM), *Synechococcus sp.* (Syn), and *Thalassiosira weissflogii* (TW). Comparison of NOEC for different algal species (left panel) and the calculated cellular metal concentration at the NOEC for each algal species (right panel). Data are mean ± SD (n = 3).
Fig. 4-8. *Dunaliella tertiolecta* (DT), *Prorocentrum minimum* (PM), and *Thalassiosira weissflogii* (TW). Chlorophyll a content ([Chl a]) of different algal species at 24 h and 96 h of exposure at different metal concentrations. A, D, E, and F represent different metal concentration treatments shown in Table 4-1. Significant difference from the control treatment is indicated by * (p < 0.05, one-way ANOVA). Samples for *D. tertiolecta* at 96 h were accidentally lost. Data are mean ± SD (n = 3).
Chapter 5

Cd toxicity to two marine phytoplankton under different nutrient conditions

5.1 Abstract

Cd accumulation and toxicity in two marine phytoplankton (diatom *Thalassiosira weissflogii* and dinoflagellate *Prorocentrum minimum*) under different nutrient conditions (+NO$_3^-$, -N and -P conditions) were examined in this study. Strong interactions between the nutrients and Cd uptake by the two algal species were found. Cd accumulation as well as N and P starvation themselves inhibited the assimilation of N, P, and Si by the phytoplankton. Conversely, N starvation strongly inhibited Cd accumulation but no influence was observed under P starvation. However, the Cd accumulation difference between +NO$_3^-$ and -N cells was smaller when [Cd$^{2+}$] was increased in the medium, indicating that net Cd accumulation was less dependent on the N-containing ligands at high Cd levels. As for the subcellular distribution of the accumulated Cd, most was distributed in the insoluble fraction of *T. weissflogii* while it was evenly distributed in the soluble and insoluble fractions of *P. minimum* at low Cd levels. A small percentage of cellular Cd (< 15%) was adsorbed on the cell surface for both algae at the lowest [Cd$^{2+}$], which increased when the [Cd$^{2+}$] increased. Cd toxicity in phytoplankton was quantified as depression of growth and $\Phi_m$, and was correlated with the [Cd$^{2+}$], intracellular Cd concentration (intra-Cd), and Cd concentrations in the cell-surface-adsorbed (ads-Cd), soluble (sol-Cd), and insoluble (insol-Cd) fractions. According to the estimated IC50 based on the different types of Cd concentration, the toxicity difference among the different nutrient-conditioned cells was the smallest when sol-Cd was used,
suggesting that it may be the best predictor of Cd toxicity under different nutrient conditions.
5.2 Introduction

Interactions between trace metals and phytoplankton have been extensively examined [39]. Low levels of trace metals such as Cu and Zn are biologically required in several indispensable metabolic processes. They may compete with each other for binding sites and cause toxic effects when accumulated at high cellular concentrations (i.e., the highly accumulated metals may complex nonspecifically with some important coordination sites which are normally occupied by some other essential metals). In the FIAM, metal accumulation and its toxicity are thought to be dependent on the free metal ion concentration in the environment [7, 8]. As the derivative of FIAM, the BLM links metal toxicity to cell-surface-adsorbed metal concentration and the metal binding sites on the cell surface are called the ‘biotic ligands’ [10]. Both models assume that metal internalization through the cell membrane is the limiting step when the metal moves from the bulk solution into the cell and only the free metal ions can directly enter cells [13]. Exceptions to both models have however been reported [1, 13].

Recently, intracellular metal concentration has been proposed as a better predictor of metal toxicity than the free metal ion. De Schamphelaere et al. [14] found that surface-bound and internal copper are better predictors of toxicity than the free copper ion when pH is varied for two freshwater green algae *Chlorella* sp. and *Pseudokirchneriella subcapitata*. Meanwhile, a great deal of metal subcellular fractionation work has been conducted in aquatic invertebrates [15-18]. In these earlier studies, metal concentrations in different subcellular compartments were quantified and their relationship with ambient metal concentration as well as their bioavailability to predators was examined. To the best of our knowledge, no work has yet been conducted to determine the relationship between the subcellular
fractionation of metals and their potential toxicity in phytoplankton. Therefore, in this study we examined whether metal toxicity to phytoplankton can be better predicted by the intracellular metal concentration or metal concentrations in the subcellular pools.

Several recent studies have considered the interactions between macronutrients and metals in phytoplankton. Exposure to high metal concentrations can suppress algal macronutrient assimilation [31, 153, 154]. On the other hand, N addition stimulated Cd uptake while P addition had no influence on both laboratory algal cultures (Chlorella autotrophica, Phaeodactylum tricornutum, Prorocentrum minimum, Tetratselmis levis, Thalassiosira pseudonana) and field phytoplankton community [32-34]. Payne and Price [135] found that cellular N and S contents were higher at higher metal levels as both elements are involved in metal detoxification. P is known to sequester excessive metals in polyphosphate bodies of freshwater algae [35-37]. A few studies have thus suggested that metal toxicity to phytoplankton may be dependent on the ambient nutrient concentrations, but no consistent results were obtained. Rijstenbil et al. [155] found that the diatom Thalassiosira pseudonana was more sensitive to Cu under P limitation, as metal exclusion/elimination systems may be impaired. Chen and Lin [156] also found that metal tolerance of Selenastrum capricornutum decreased under nutrient-limited conditions. However, Riedel et al. [157] showed that Cu had greater inhibition in the nutrient-enriched mesocosms.

In this study, we examined Cd toxicity to a marine diatom Thalassiosira weissflogii and a dinoflagellate Prorocentrum minimum under different macronutrient conditions. Intracellular Cd bioaccumulation as well as its subcellular distribution was quantified. The main objectives of our study were to
examine: 1) interactions between the uptake of macronutrients and Cd by phytoplankton, 2) the effects of macronutrients on Cd toxicity to algal cells, and 3) whether the intracellular or subcellular Cd concentration can better predict its toxicity to phytoplankton. Our previous work showed that *P. minimum* is much more tolerant of trace metals than is *T. weissflogii* [158], allowing us to explore the mechanisms underlying species-specific tolerance through this study.

5.3 Materials and methods

5.3.1 Phytoplankton and culture conditions

Axenic cultures of the dinoflagellate *Prorocentrum minimum* (CCMP696) and the diatom *Thalassiosira weissflogii* (CCMP 1048) were obtained from the Provasoli-Guillard Center for the Culture of Marine Phytoplankton, Bigelow Laboratory, West Boothbay Harbor, Maine, USA. The cells were maintained in f/2 medium [73] with sterile techniques, in an incubator at 19 °C with a light illumination of 170 μmol photons m⁻² s⁻¹ on a 14:10 LD cycle. Si was not used in the culture of the dinoflagellate *P. minimum*. The pH value of the medium was 8.2 ± 0.1. Seawater for the culture was collected 10 km off East Hong Kong to minimize any anthropogenic influences and was filtered through a 0.22 μm Poretic membrane before use.

5.3.2 Medium for metal toxicity test

Artificial seawater was used throughout the toxicity test. It was made by adding several salts (i.e., CaCl₂, H₃BO₃, KBr, KCl, MgCl₂, NaCl, NaF, NaHCO₃, Na₂SO₄, SrCl₂) to Milli-Q water (18.0 MΩ) following Aquil medium recipes [4, 159]. The 0.22 μm filtered artificial seawater and all nutrients except the trace metal stocks (i.e., N, P, Si, and vitamins) used during the toxicity study were passed through
Chelex 100 ion-exchange resin columns to remove background trace metals. The Chelex 100 resin was purified following the procedure by Price et al. [159] in case of organic substances leakage from the resin. In our preliminary experiments, there was no difference in μ between the algae cultured in natural and artificial seawater.

A trace metal clean technique was used throughout the whole experiment. All the beakers and bottles (polycarbonate) were soaked in 10% HCl until the day before the toxicity tests when they were rinsed with Milli-Q water seven times. Nutrient and Cd additions were performed in a class-100 clean bench to minimize potential metal contamination. Cd toxicity was examined for algae acclimated under different nutrient conditions (i.e., +NO₃, -N and -P conditions). The N, P, Si (T. weissflogii only), and vitamin concentrations in the artificial seawater were the same as in the f/2 medium except that N (or P) was not added in the N (or P) starvation toxicity tests. Trace metal concentrations were at the f/10 levels. A total of six Cd concentration treatments (treatments A-F) with four or five replicates each were used in every toxicity test. NTA (0.1 mmol l⁻¹) was used to keep the free metal ion concentrations constant in the media. The total dissolved cadmium concentrations (nominal) used for treatments A to F were 2×10⁻⁹, 1×10⁻⁷, 1×10⁻⁶, 1×10⁻⁵, 1×10⁻⁴ and 5×10⁻⁴ mol l⁻¹. The pH values of the media were adjusted to 8.2 ± 0.1 with 1 mol l⁻¹ Suprapure NaOH (Merck, Darmstadt, Germany). [Cd²⁺] was calculated by using the MINEQL+ software package (Version 4.5 from Environmental Research Software, Hallowell, ME, USA) with the updated thermodynamic constants and the calibration set on ionic strength. They were 3.40×10⁻¹¹, 1.70×10⁻⁹, 1.70×10⁻⁸, 1.74×10⁻⁷, 1.95×10⁻⁶, 1.20×10⁻⁵ mol l⁻¹ for treatments A to F, respectively. The high [Cd²⁺], although less environmentally realistic, had to be used in order to observe a
complete dose response from 0 to 100% inhibition. The media were equilibrated for at least 24 h under the same conditions as the following toxicity tests.

5.3.3 Toxicity tests by PAM-fluorometry

Algal cells were first inoculated from the stock into the f/2 medium except that the trace metal concentrations were at the f/10 levels. Si was not used for the dinoflagellate *P. minimum*. The temperature, light intensity, and LD cycle were the same as those used for the algal stock cultures. During the acclimation period, the cell density was enumerated by a Coulter Counter every 24 h. After the cells arrived at the mid-exponential phase, they were collected by centrifugation (4000 g, 15 min) and then resuspended into the toxicity test media. For the toxicity tests of nutrient starved cells, the algae were subcultured into the -N or -P medium for 1 or 2 days before the resuspension into the toxicity test media. After the acclimation, the cells had been slightly N or P-starved based on their cell growth, intracellular nutrient and chlorophyll a contents, which would get even more inhibited throughout the following toxicity tests. The toxicity tests lasted 48 or 96 h with two time points (i.e., 24 and 48 h or 48 and 96 h for *T. weissflogii* and *P. minimum*, respectively, as the former grew much faster than the latter). At each time point, the PAM fluorescence induction curve was obtained with a PAM 101/103 fluorometer (Heinz Walz GmbH, Effeltrich, Germany), using methods described by Miao et al. [158]. $\Phi_M$, which is indicative of the maximum energy conversion efficiency in PS II, was calculated from the fluorescence induction curve [56]. At the same time, the cell number was counted and $\mu$ was calculated with Eq. 4-1.

5.3.4 Nutrient concentrations in the medium and phytoplankton

Dissolved nutrient concentrations were quantified colorimetrically at the beginning and end of the experiments. Nitrate plus nitrite ($\text{NO}_3^- + \text{NO}_2^-$) was
determined using the methods described by Wood et al. [160]. Dissolved inorganic phosphorus and silicon concentrations were measured following the methods of Hager et al. [161] and Armstrong et al. [162]. N (or P) concentration was not determined in the -N (or -P) toxicity tests. Similarly, the Si concentration was not measured in the *P. minimum* tests.

At the end of each toxicity test, a 100 ml sample from each bottle was filtered through a pre-combusted 25 mm Whatman GF/F membrane and stored frozen (-20°C) for the measurement of C and N contents in the phytoplankton. The filters were then dried at 50 °C for 24 h before the elemental analyses. The C and N contents were quantified with a CHNS/O elemental analyzer (Perkin Elmer PE 2400 Series II) using acetanilide (C₈H₈NO) as the standard. The cellular phosphorus content was also measured for *P. minimum*. For this purpose, another 100 ml sample was passed through a pre-combusted GF/F filter, rinsed twice with 2 ml of 0.17 mol l⁻¹ Na₂SO₄, soaked in 2 ml of 0.017 mol l⁻¹ MgSO₄, and dried at 60 °C. The organic matter was mineralized by combustion in a muffle furnace at 460 °C for two hours and then the orthophosphate was extracted with 5 ml of 0.2 mol l⁻¹ HCl in an 80 °C water bath for 30 min. The orthophosphate concentration was determined with a spectrophotometer after neutralization [163, 164]. The efficacy of our digestion procedure was high, as the yields of phosphate from 15 organic phosphorus compounds were 95-103% with this method [163].

5.3.5 **Cellular Cd accumulation and subcellular fractionation**

For each Cd concentration treatment, two of the four or five replicate bottles were spiked with 2-8 nmol l⁻¹ radioactive ¹⁰⁹Cd (in 0.1 mol l⁻¹ HCl) additionally, to trace the cellular Cd accumulation and subcellular fractionation. The HCl added with ¹⁰⁹Cd was neutralized with 1 mol l⁻¹ Suprapure NaOH. At each time point
during the toxicity test, a 10 ml aliquot was filtered through a 1 μm polycarbonate membrane for the measurement of radioactivity in the cells. The metal weakly adsorbed on the cell walls was removed by Ti-technique wash [74, 75]. A 1 ml aliquot was also taken out for the measurement of total radioactivity in the uptake medium. Intra-Cd was calculated as follows:

\[
\text{Intra-Cd (μmol mol C}^{-1}) = \frac{\text{(radioactivity mol C}^{-1} \times \text{total metal concentration μmol l}^{-1})}{\text{(the radioactivity of medium l}^{-1})}
\]

(5-1)

At the end of the toxicity tests, a 100 ml aliquot was removed from each bottle, filtered through a 3 μm polycarbonate membrane, and rinsed with 0.22 μm filtered artificial seawater three times. Then, the algal cells were resuspended into 10 ml 8-hydroxyquinoline-5-sulphonate (HQS, 1 mmol l\(^{-1}\) with sea water as the solvent) for 10 min to remove the weakly cell-surface-adsorbed Cd [15, 40]. Although different reagents have been used to remove the ads-Cds in the measurement of Cd accumulation and the subcellular fractionation, their removal efficiency is similar [88]. After that, the cells were centrifuged at 4000 g for 15 min. The supernatant was then decanted and 1 ml was put into a vial for radioactivity count. The pellet was resuspended into 3 ml of 0.02 mol l\(^{-1}\) Tris-buffer with 0.15 mol l\(^{-1}\) NaCl (pH = 8) and homogenized by sonication four times for 20 s with 15 s intervals. This sonication technique was efficient in breaking the cells of T. weissflogii (checked under the microscope) and has been widely used by other researchers for the same diatom species [165]. The homogenate was then ultra-centrifuged at 235,000 g for 30 min. The supernatant and pellet were isolated and differentiated as soluble and insoluble fractions, respectively. The subcellular fractionation was performed at 4 °C throughout. The radioactivity of these three fractions was then counted and the Cd content in each fraction was calculated as ads-, sol-, and insol-Cd. The
radioactivity of $^{109}$Cd was determined by a Wallac gamma detector at 88 keV. The counting time was adjusted to result in propagated counting errors of < 5%.

5.3.6 Calculation of IC50 and statistical analysis

The IC50 of Cd$^{2+}$, intra-, ads- and sol-Cd were estimated for the two toxicity endpoints $\mu$ and $\Phi_M$. A linear interpolation method was used for the estimation with the help of ICPIN software (Version 2.0, USEPA, Duluth, MN, USA). Any 'significant' difference was based on the results of one-way ANOVA with post hoc multiple comparisons (software used: SPSS 11.0 by SPSS, Chicago, USA). Significant difference was accepted at $p < 0.05$.

5.4 Results

5.4.1 Particulate nutrient concentration and nutrient uptake

Cellular C, N, and P contents were measured at the end of the toxicity test (Table 5-1). The cellular P content was not measured in experiments with the diatoms. They were generally higher at higher [Cd$^{2+}$] for both algal species. For example, the cellular C content of the +NP T. weissflogii in treatment E was about 2× higher than in treatment A, but it then decreased in treatment F as a result of membrane permeability change or cell lysis by Cd toxicity. At the same time, the cellular N and P contents were significantly lower under nutrient-starved conditions no matter which nutrient was limited ($p < 0.05$). However, the -N cells were found to have a higher cellular C content than those under +NP condition at the same Cd level. The cellular C contents of the -P cells were similar to the +NP cells. Significant difference was also observed for the cellular nutrient contents between the two algal species ($p < 0.05$). The dinoflagellate P. minimum had a much higher cellular nutrient content than the diatom T. weissflogii under the same conditions.
The nutrient ratios of N:C and P:C in the algae are also shown in Table 5-1. Both were close to the Redfield ratio (0.151 mol mol\(^{-1}\) for N:C and 9.4 mmol mol\(^{-1}\) for P:C) under the +NP condition. When the cells were under the -N (or -P) condition, the N:C (or P:C) then decreased. At the same time, the N:C of the -P cells was lower than that of the +NP cells but higher than the -N cells. A similar trend was also observed for the P:C of the -N cells. No consistent trend was observed for N:C and P:C at different [Cd\(^{2+}\)], except that their ratios were generally the highest at the highest [Cd\(^{2+}\)].

The initial and final dissolved inorganic N, P, and Si concentrations in treatments A-F were also measured for both algal species under different nutrient conditions (data not shown). A notable decrease in N (4-35%), P (3-31%), and Si (16-68%) concentrations was observed after 48 or 96 hours of incubation. At the same time, the final nutrient concentrations were higher at higher [Cd\(^{2+}\)], although the initial cell density in the high-Cd treatments was similar or even higher than those low-Cd treatments. The algal N, P, and Si uptake rates were then calculated based on the decrease in the dissolved nutrient concentration during the toxicity test, which was normalized to the average particulate carbon concentration (Fig. 5-1). Although not statistically significant (p > 0.05), there was a slight decrease in the N and P uptake rates with an increase of [Cd\(^{2+}\)], except at the highest [Cd\(^{2+}\)]. The N uptake rate in treatment A was 0.58 ± 0.10 and 0.23 ± 0.13 mol mol C\(^{-1}\) d\(^{-1}\) for +NP T. weissflogii and P. minimum, respectively, and it then decreased to 0.29 ± 0.06 and 0.12 ± 0.04 mol mol C\(^{-1}\) d\(^{-1}\) in treatment E. Similarly, there was about 1.8× and 2.8× decrease of P uptake rate by the two +NP algae as [Cd\(^{2+}\)] increased from 3.4×10\(^{-11}\) to 1.95×10\(^{-6}\) mol l\(^{-1}\), respectively. A more remarkable and consistent decrease was observed for Si (p < 0.05). For the +NP T. weissflogii, the Si uptake
rate was 0.11 and 0.04 mol mol C⁻¹ d⁻¹ in treatments A and E, respectively. The nutrient uptake rates of treatment F increased abruptly, indicating cell lysis and loss of biomass (i.e., decrease of cellular carbon content). Among different nutrient conditions (i.e., +NP, -N, and -P conditions), the nutrient uptake by algae was higher for the +NP cells. In treatment A of T. weissflogii, for example, the N uptake rate in the +NP cells was about 3.6× higher than in the -P cells and the P uptake rate in the +NP cells was about 2.4× higher than in the -N cells. Similarly, the Si uptake rate was 0.11 mol mol C⁻¹ d⁻¹ under the +NP condition, and decreased to 0.04 and 0.05 mol mol C⁻¹ d⁻¹ under the -N and -P conditions, respectively. Furthermore, both N and P uptake rates of P. minimum were lower than those of T. weissflogii under similar conditions, e.g., the P uptake rate was 0.019 and 0.005 mol mol C⁻¹ d⁻¹ in treatment A for +NP T. weissflogii and P. minimum, respectively.

5.4.2 Cd accumulation and subcellular fractionation

When normalized on the basis of the particulate carbon concentration, intra-Cd increased with an increase of [Cd²⁺] in the media (Fig. 5-2). A linear log-log relationship between the intra-Cd and [Cd²⁺] was observed for T. weissflogii, which followed the Freundlich isotherm (Eq. 4-3). In contrast, a biphasic Cd accumulation (i.e., a high 1/e at low Cd level and a low 1/e at high Cd level) was observed in P. minimum. Therefore, the intra-Cd of P. minimum was lower than that of T. weissflogii at high [Cd²⁺] while it was higher at low [Cd²⁺] (p < 0.05). For example, when [Cd²⁺] was 1.70×10⁻⁹ mol l⁻¹, the intra-Cd was 4.02 and 10.5 μmol mol C⁻¹ for +NP T. weissflogii and P. minimum, respectively, after 48 h exposure. When the [Cd²⁺] was increased to 1.95×10⁻⁶ mol l⁻¹, a reverse trend was observed and the intra-Cd was 162 and 113 μmol mol C⁻¹ for the two species. Furthermore, the intracellular Cd accumulation by the -N cells was lower than that by the +NP
cells ($p < 0.05$). The intra-Cd was 0.435 \( \mu \text{mol mol C}^{-1} \) in treatment A of the +NP \( T. \) weissflogii, while it was 0.132 \( \mu \text{mol mol C}^{-1} \) for -N cells at the same Cd level after 24 h exposure. A similar trend was also observed for \( P. \) minimum. The intra-Cd of treatment A was 0.369 and 0.219 \( \mu \text{mol mol C}^{-1} \) for the +NP and -N cells, respectively, after 48 hours. However, this accumulation difference for the different nutrient-conditioned cells became smaller with the increase of \([\text{Cd}^{2+}]\). The intracellular Cd accumulation in the +NP \( T. \) weissflogii was about 4.0x higher than that of the -N cells for treatment A, which then decreased to 2.3x for treatment C and slightly lower than the -N cells for treatment E after 48 hours. In contrast, the Cd accumulation in the -P cells was similar to that of the +NP cells irrespective of the ambient Cd levels.

Cd subcellular fractionation in the two algal species at the end of the toxicity test is shown in Fig. 5-3. For the diatom \( T. \) weissflogii, most of the accumulated Cd was distributed in the insoluble fraction at low \([\text{Cd}^{2+}]\). In treatment A under the +NP conditions, 70\% of the accumulated Cd was in the insoluble fraction, 20\% in the soluble fraction, and 10\% adsorbed on the cell surface. The subcellular distribution changed with an increase of \([\text{Cd}^{2+}]\). When the \([\text{Cd}^{2+}]\) reached 1.95x10^{-6} \text{ mol L}^{-1} (Treatment E), 42\% of the cellular accumulated Cd was in the cell-surface-adsorbed fraction, 47\% in the insoluble fraction, and 11\% in the soluble fraction, suggesting that more Cd was distributed in the cell-surface-adsorbed fraction while less Cd was in the insoluble fraction with the increase of \([\text{Cd}^{2+}]\). At the same time, the Cd proportion in the soluble fraction increased slightly first and then decreased. For \( P. \) minimum, similar fractions of Cd were distributed in the soluble and insoluble pools while much less was in the cell-surface-adsorbed pool at low \([\text{Cd}^{2+}]\). For example, 45\%, 45\%, and 9.8\% were distributed in the soluble, insoluble, and
cell-surface-adsorbed fractions at the lowest [Cd$_{\text{2+}}$] under the +NP condition. The Cd proportion in both soluble and insoluble fractions was slightly suppressed whereas its distribution in the cell-surface-adsorbed fraction was significantly stimulated at high [Cd$_{\text{2+}}$] ($p < 0.05$). In treatment E, 18% of the Cd accumulated was in the cell-surface-adsorbed fraction, 39% in the soluble fraction, and 43% in the insoluble fraction. However, there was no consistent trend for Cd subcellular distribution under the different nutrient conditions in both species. The Cd distribution in the soluble fraction may have been overestimated in treatment F since cells might lyse and the radioactivity count in each fraction was low.

5.4.3 Changes of $\mu$ and $\Phi_M$ at different Cd concentrations

The relative changes of $\mu$ and $\Phi_M$ are shown in Figs. 5-4 and 5-5 for T. weissflogii and P. minimum, respectively. They are plotted against [Cd$_{\text{2+}}$] in the medium, intra-, ads-, and sol-Cd. Both $\mu$ and $\Phi_M$ decreased at high Cd levels. The IC50 for $\mu$ and $\Phi_M$ were calculated from the four different types of Cd concentration and are listed in Table 5-2. For T. weissflogii, the [Cd$_{\text{2+}}$]-IC50 (i.e., IC50 was calculated from [Cd$_{\text{2+}}$]) of the +NP cells was much higher than that for the nutrient starved cells (e.g., 11.6× and 5.3× higher than the -N cells for $\mu$ and $\Phi_M$, respectively). A similar trend was observed when the intra- and ads-Cd were used for the estimation of IC50, but the difference was smaller than with [Cd$_{\text{2+}}$]. The difference was even smaller when the sol-Cd was used (< 2.5× difference for the different nutrient-conditioned cells). For example, the sol-IC50 (IC50 was calculated based on sol-Cd) of $\Phi_M$ ranged from 36 to 42 mol mol C$^{-1}$ for the diatoms under +NP, -N, and -P conditions. For P. minimum, the IC50s under different nutrient conditions were similar to each other no matter which Cd concentration was used in their estimation. At the highest [Cd$_{\text{2+}}$] the cells may lyse, which can lead to
the underestimation of Cd accumulation. Therefore, the accumulated Cd concentration measured may be significantly lower than at the lower Cd levels \( (p < 0.05) \) and some of the data points at this Cd level were thus neglected for the calculation of IC50. At the same time, \( P. \) minimum grew slowly and \( \mu \) showed a large variation among different \([\text{Cd}^{2+}]\). The growth of -N cells was even slower and sometimes negative. Their relative change is therefore not shown. The insol-Cd was not used in the toxicity prediction since it was not a better predictor than the other two fractions.

The relative changes of \( \mu \) and \( \Phi_M \) with intra-Cd at different time points are compared in Figs. 5-6 and 5-7 for \( T. \) weissflogii (24 and 48 h) and for \( P. \) minimum (48 and 96 h), respectively. For \( T. \) weissflogii, \( \mu \) was calculated during the first and second 24 h of the whole toxicity test period. \( P. \) minimum grew slowly and the cell density was rather constant during the first 48 h of testing, thus only the relative change of \( \Phi_M \) is shown. Both \( \mu \) and \( \Phi_M \) were inhibited to a greater extent with an increase in exposure time than what was expected from the intra-Cd increase. In the toxicity test of +NP \( T. \) weissflogii, \( \Phi_M \) of treatment F was suppressed by 27% after 24 h exposure while it was suppressed by 92% after 48 hours, although the intra-Cd per unit C was comparable at both times. Similarly, there was a 75% decrease in \( \mu \) after 24 hours while there was a 100% decrease after 48 hours.

5.5 Discussion

5.5.1 Interactions between macronutrients and Cd

Our study found that exposure to high \([\text{Cd}^{2+}]\) suppressed macronutrient uptake (i.e., N, P, and Si) by phytoplankton. Inhibition of N and P uptake by trace metals has been reported for plants and phytoplankton [31, 153, 154, 166]. Singh and
Yadava [153] speculated that the inhibition on N uptake was due to metal-induced depletion of ATP and reductant pools required for the NO₃⁻ reducing system in photoautotrophic organisms. Alternatively, exposure to high metal concentrations may alter the plasma membrane permeability as a consequence of membrane functionality loss [167, 168]. Similar mechanisms may also contribute to the P and Si uptake inhibition at high [Cd²⁺].

Although N and P uptake was inhibited at high [Cd²⁺], i.e., less N and P were removed from the solution, the cellular contents of these nutrients (pg cell⁻¹) increased. As the cellular nutrient concentration was positively related to the nutrient uptake rate but negatively related to μ, it was possible that the decrease of μ with increasing [Cd²⁺] was quicker than that of the nutrient uptake rate, resulting in a higher cellular nutrient content at higher [Cd²⁺]. Although not examined in this study, the potential increase in cell size with the increase of [Cd²⁺] may also contribute to the higher nutrient content per cell at high Cd level [169]. The carbon fixation rate was not measured in this study, but a similar trend is likely to occur for C, since the N:C and P:C remained relatively constant at different [Cd²⁺]. Furthermore, as μ of *P. minimum* was much lower and its cell size was larger than those of *T. weissflogii*, its cellular nutrient content per cell was higher, although its nutrient uptake rate was lower than that for *T. weissflogii*. Payne and Price [135] observed a higher cellular N content at high Cd levels as a result of Cd detoxification. However, this was likely caused by the decrease in μ and the increase in the cell size at a high Cd level.

Similar to the influence of Cd exposure, N and P starvation also suppressed the assimilation of each other, as the uptake rate of N (or P) under P- (or N-) starvation was much lower than under the nutrient-enriched condition. At the same time, the
Si uptake rate was also inhibited by N and P starvation. This inhibition effect was further evidenced by the lower cellular N (or P) content under P (or N) starvation as compared with the +NO$_3^-$ condition at the same [Cd$^{2+}$], even though $\mu$ decreased under the nutrient-starved condition. Similar results were also observed by Riegman et al. [170]. In their study, *Emiliania huxleyi* was limited by P to different extents while the cellular N concentration was kept relatively constant. As $\mu$ of *Emiliania huxleyi* was lower when the cells were more limited by P, the N uptake rate decreased with increasing P starvation. In contrast, C fixation was less suppressed by N and P starvation in our study. There was a similar or slightly higher cellular C content under -P and -N conditions as compared with the +NP condition at the same Cd level. Our results demonstrate the need to examine the nutrient stoichiometry in phytoplankton under metal stress or nutrient-limited conditions.

On the other hand, cellular nutrient contents can influence Cd accumulation in phytoplankton. Wang and Dei [32, 33] found that N addition strongly increased Cd uptake by different algal species while P addition had no influence. Similar results were also observed in this study for the diatom *T. weissflogii* and the dinoflagellate *P. minimum*. It was hypothesized that the inhibition of Cd accumulation under the -N condition was due to the low concentrations of protein ligands for metal binding and internalization, as N is involved in protein synthesis. In our study, however, the suppression of Cd accumulation under the -N condition became smaller with an increase of [Cd$^{2+}$], implying that intracellular Cd accumulation was less influenced by the N-containing ligands at high Cd levels.

### 5.5.2 Cellular Cd accumulation and subcellular fractionation
Intra-Cd increased with an increase of [Cd\(^{2+}\)] in *T. weissflogii* and conformed with the Freundlich isotherm, similar to our previous study [158]. In contrast, a biphasic Cd accumulation was observed for *P. minimum*. Therefore, the intra-Cd of *P. minimum* was higher than that of *T. weissflogii* in treatments A-C but was lower in treatments D-F. It is possible that *P. minimum* has two Cd transport pathways with different affinities (a high 1/e for the first three metal concentrations and a low 1/e for the residual in Eq. 4-3). Cd may be internalized mainly through the high affinity system when its ambient concentration is low, while the low affinity system is used under a high concentration of Cd [171].

Relatively few subcellular fractionation studies have been conducted in phytoplankton [15, 172, 173]. No study has been performed to predict metal toxicity through subcellular distribution. Our subcellular fractionation results are consistent with Ng et al. [173] for the same metal in the same algal species. Moreover, Chang and Reinfelder [172] also noted that the proportion of ads-Cu increased with an increase of ambient Cu concentration. Nevertheless, the subcellular metal distribution may be highly metal- and algal-species specific, e.g., >95% of cellular Cd was in the soluble fraction of *Skeletonema costatum* and *Tetraselmis suecica* [15] and Ag was evenly distributed in the three fractions of *T. weissflogii* [173].

### 5.5.3 Toxicity endpoints (μ and Φ\(_M\)) and Cd toxicity prediction

Cell-specific growth rate, μ, is the most prevalent endpoint used in toxicity tests, since any toxic effect may finally be reflected in cell growth changes. PAM fluorescence measurement is a relatively new technique and has now been applied in the toxicity studies of plants, freshwater algae, and marine phytoplankton [56, 119, 158]. This technique was shown to be more efficient and sensitive than the
typically used endpoints (e.g., $\mu$, C fixation, ATP synthesis, respiration) in ecotoxicity tests [56, 120], but our previous study showed that $\mu$ and PAM parameters have a similar sensitivity to trace metal toxic effects in marine phytoplankton [158]. Similar results were also found in this study. Whenever inhibition was observed for $\Phi_M$, there was also a decrease of $\mu$. However, PAM parameters may offer more advantages than $\mu$ as marine phytoplankton endpoints besides the examination of toxicity mechanisms, especially when cell growth is slow (e.g., cell growth under nutrient-starved conditions). When $\mu$ is low, a small variation due to some unknown reasons will result in a large fluctuation in its relative value. Therefore, the toxicant effects may be under- (or over-) estimated if only $\mu$ was used as the endpoint. However, under this condition $\Phi_M$ is less affected (e.g., $\Phi_M$ was 0.76 and 0.61 under $+NO_3^-$ and $-N$ conditions, respectively, while $\mu$ was 0.97 and 0.14 d$^{-1}$ at the lowest [Cd$^{2+}$] in this study) and thus may serve as a better endpoint.

Sunda and Guillard [7] found that Cu toxicity to the two algal species *Thalassiosira pseudonana* and *Nannochloris atomus* was similar at the same [Cu$^{2+}$] with different total Cu concentrations. They suggested that the free metal ion is the only metal species directly taken up by the organisms and that metal toxicity is dependent on the free metal ion concentration. These and subsequent observations led to the formulation of the well-known Free-Ion Activity Model or FIAM. One of the key assumptions of the model is that the algal surface is in equilibrium with the exposure solution and that metal internalization is the slow step in metal uptake. A number of exceptions to the model have however been identified [1] and it is clear that the free metal ion is not always a good predictor of metal toxicity. As intracellular metal accumulation and the subcellular metal distribution in the cells are
more directly related to the metal toxicity in phytoplankton, it was thus hypothesized that metal concentration at cellular or subcellular level may serve as a better toxicity predictor than the free metal ion in the bulk solution. Recently, De Schamphelaere et al. [14] did find that Cu toxicity in algae can be better predicted by the cell-surface-adsorbed or the internal metal concentrations as compared with the free metal ion concentration.

In our study, the -N and -P T. weissflogii were more sensitive to Cd exposure, as their [Cd$^{2+}$], intra- (IC50 was calculated based on intra-Cd), and ads-IC50 (IC50 was calculated based on ads-Cd) were significantly lower than for the +NP cells ($p < 0.05$). This trend was further evidenced by the different inhibitions of $\mu$ and $\Phi_M$ for the different nutrient-conditioned T. weissflogii at the same or similar [Cd$^{2+}$], intra-, and ads-Cd. $\mu$ was inhibited by 64%, 89%, and 84% for the +NO$_3$,-N, and -P cells, respectively, at [Cd$^{2+}$] 1.95×10$^{-6}$ mol l$^{-1}$. Cultures of Thalassiosira pseudonana grown under -N conditions had lower levels of glutathione (GSH) and PCs [155], and nutrient limitation constrained PC synthesis in some marine algae under metal stress [174]. Therefore, when the cells were under N- or P-starvation, the cellular N and P contents would be lower and there would be less N and P for the metal detoxification and the cells would be more sensitive to metal exposure [135, 175]. However, when the Cd concentration in the soluble fraction was used to predict its toxicity, much less difference was observed between the different nutrient-conditioned T. weissflogii. And the difference between the maximum and minimum IC50 for the different nutrient-conditioned cells was the smallest, when its estimation was based on sol-Cd (2.4× for sol-IC50 vs. 5.0 – 11.7× for [Cd$^{2+}$], intra-, and ads-IC50). Therefore, Cd toxicity to T. weissflogii under different nutrient conditions can be better predicted with sol-Cd, as compared with [Cd$^{2+}$], intra- and
ads-Cd. The significantly lower \( p < 0.05 \) relative changes of \( \mu \) in treatments B and C of -N \textit{T. weissflogii}, as compared with +NP cells, may not be due to Cd toxicity but to some other unknown factors, as the cell growth under the -N condition was very slow (i.e., < 0.25 d\(^{-1}\) in all treatments). In contrast, no inhibition effects were observed in treatments A-E of \textit{P. minimum}, while the cells died in treatment F. Potential toxicity differences between different nutrient treatments were thus not observed. As \textit{P. minimum} was highly lysed in treatment F and the radioactivity count was low, its sol-Cd was probably underestimated and the toxicity prediction with sol-Cd was compromised.

It may be argued that metal toxicity prediction at subcellular level is less practical and convenient than the FIAM in the water criteria control. However, this will not be the case if the relationship between the ambient physicochemical characteristics, physiology of the organisms and metal distribution at the sensitive binding sites is well-established in the future. Indeed, given the very different metal handling strategies displayed by different aquatic organisms, it may be more advantageous to use the distribution in the sensitive subcellular pools to predict the metal toxicity to a specific organism under specific conditions. Subcellular fractionation research is still in its infancy and certainly more work needs to be performed before a relatively accurate subcellular distribution approach can be developed. In this study, the different intracellular pools were only operationally (i.e., insoluble fraction is the mixture of different organelles and membranes while the soluble fraction is mostly the cytosol) but not functionally defined. Further study needs to be performed for more mechanistic explanations.

The observation that even intra-Cd may not be a good predictor of metal toxicity can be further evidenced in the time lag effects of Cd toxicity to the
phytoplankton. The relative changes of $\mu$ and $\Phi_M$ with intra-Cd are significantly different ($p < 0.05$) at the two different time points, even at comparable intra-Cd levels. It appears that the internalized metals need some time to reach the toxic binding sites (e.g., subcellular redistribution) and trigger the detrimental effects on PS as well as on cell growth. Thus, caution is required when comparing toxicity data with different exposure times even if intracellular concentration is used as the predictor. In our study, subcellular distribution was not measured at the first time point and it may be different from that in the end. Therefore, the relative changes in $\mu$ and $\Phi_M$ with Cd concentrations in different subcellular pools at different time points were not compared.

In conclusion, Cd accumulation inhibited the uptake of macronutrients by the diatom *T. weissflogii* and the dinoflagellate *P. minimum*. The cellular nutrient contents per cell increased with [Cd$^{2+}$], likely due to a more abrupt decrease in $\mu$ than in the nutrient uptake rate. In addition, the possible contribution of any change in cell size can not be discounted. Similarly, macronutrient assimilation was also suppressed by N and P starvation. In contrast, Cd accumulation could be strongly decreased under -N condition, but this effect was smaller with an increase of ambient Cd concentration, suggesting that Cd internalization at high metal levels is less influenced by N-containing ligands. However, Cd accumulation in -P cells was similar to that in +NO$_3^-$ cells. The relationship between intra-Cd and [Cd$^{2+}$] follows the Freundlich isotherm for *T. weissflogii*, while there is a biphasic accumulation for *P. minimum*. Thus the intra-Cd of *P. minimum* is higher than that of *T. weissflogii* at low Cd levels while lower at high Cd levels, which may explain why *P. minimum* is more tolerant of Cd stress. Furthermore, most of the accumulated Cd is distributed in the insoluble fraction in *T. weissflogii* while equal amounts are distributed in the
soluble and insoluble fractions of *P. minimum* at low Cd levels. Relatively small percentages of Cd are in the cell-surface adsorbed fraction for both species, but more Cd is distributed into this fraction with an increase of [Cd$^{2+}$]. For Cd toxicity prediction with different types of Cd concentrations, sol-Cd is the best predictor of Cd inhibition of $\mu$ and $\Phi_M$ under different nutrient conditions.
Table 5-1. The cellular C, N, and P contents (pg cell\(^{-1}\)) as well as N:C (mol mol\(^{-1}\)) and P:C (mmol mol\(^{-1}\)) in *T. weissflogii* and *P. minimum* under nutrient-enriched (+NO\(_3\)^-) and starved (-N) conditions for treatments A-F. Values are means ± SD (n = 2)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>[Cd(^{2+})] (mol l(^{-1}))</th>
<th><em>T. weissflogii</em></th>
<th><em>P. minimum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T. weissflogii</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>N</td>
</tr>
<tr>
<td>+NO(_3)^-</td>
<td>A 3.40×10(^{-11})</td>
<td>112±4.14</td>
<td>23.6±0.57</td>
</tr>
<tr>
<td></td>
<td>B 1.70×10(^{-9})</td>
<td>119±2.60</td>
<td>25.0±0.83</td>
</tr>
<tr>
<td></td>
<td>C 1.70×10(^{-8})</td>
<td>115±1.42</td>
<td>24.0±0.26</td>
</tr>
<tr>
<td></td>
<td>D 1.74×10(^{-7})</td>
<td>131±2.03</td>
<td>24.2±1.10</td>
</tr>
<tr>
<td></td>
<td>E 1.95×10(^{-6})</td>
<td>220±9.49</td>
<td>30.2±0.75</td>
</tr>
<tr>
<td></td>
<td>F 1.20×10(^{-5})</td>
<td>110±0.37</td>
<td>21.7±0.60</td>
</tr>
<tr>
<td>-N</td>
<td>A 3.40×10(^{-11})</td>
<td>153±4.05</td>
<td>5.84±0.05</td>
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<tr>
<td></td>
<td>B 1.70×10(^{-9})</td>
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</tr>
<tr>
<td></td>
<td>C 1.70×10(^{-8})</td>
<td>174±6.14</td>
<td>6.86±0.02</td>
</tr>
<tr>
<td></td>
<td>D 1.74×10(^{-7})</td>
<td>190±1.65</td>
<td>8.44±0.04</td>
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<tr>
<td></td>
<td>E 1.95×10(^{-6})</td>
<td>178±12.0</td>
<td>9.62±0.03</td>
</tr>
<tr>
<td></td>
<td>F 1.20×10(^{-5})</td>
<td>152±3.29</td>
<td>9.37±0.01</td>
</tr>
<tr>
<td>-P</td>
<td>A 3.40×10(^{-11})</td>
<td>109±2.79</td>
<td>13.4±0.03</td>
</tr>
<tr>
<td></td>
<td>B 1.70×10(^{-9})</td>
<td>105±2.57</td>
<td>14.7±0.01</td>
</tr>
<tr>
<td></td>
<td>C 1.70×10(^{-8})</td>
<td>109±2.26</td>
<td>15.5±0.02</td>
</tr>
<tr>
<td></td>
<td>D 1.74×10(^{-7})</td>
<td>121±7.32</td>
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<td>E 1.95×10(^{-6})</td>
<td>195±14.6</td>
<td>21.5±0.02</td>
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<tr>
<td></td>
<td>F 1.20×10(^{-5})</td>
<td>79.1±1.17</td>
<td>12.4±0.03</td>
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Table 5-2. Estimation of the IC50 of the cell-specific growth rate ($\mu$) and the maximum PS II quantum yield ($\Phi_M$) for *T. weissflogii* (TW) and *P. minimum* (PM) under nutrient-enriched (+NO$_3^-$), N- (-N), and P- (-P) starved conditions based on [Cd$^{2+}$] (mol l$^{-1}$), intra-, ads-, and sol-Cd (µmol mol C$^{-1}$). Values are means ± SD (n = 2).

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatments</th>
<th>IC50</th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[Cd$^{2+}$] ($\times 10^6$)</td>
<td>intra-Cd</td>
<td>ads-Cd</td>
<td>sol-Cd</td>
</tr>
<tr>
<td>TW</td>
<td>+NO$_3^-$</td>
<td>1.40 ± 0.06</td>
<td>141 ± 2.3</td>
<td>52.0 ± 1.7</td>
<td>28.4 ± 1.3</td>
</tr>
<tr>
<td>$\mu$</td>
<td>-N</td>
<td>0.12 ± 0.01</td>
<td>28.5 ± 3.1</td>
<td>6.52 ± 0.55</td>
<td>12.0 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>-P</td>
<td>0.65 ± 0.07</td>
<td>65.4 ± 1.6</td>
<td>15.9 ± 0.89</td>
<td>19.5 ± 0.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.81 ± 0.06</td>
<td>381 ± 3.04</td>
<td>202 ± 1.81</td>
<td>41.7 ± 0.31</td>
</tr>
<tr>
<td>$\Phi_M$</td>
<td>-N</td>
<td>1.28 ± 0.1</td>
<td>132 ± 8.0</td>
<td>30.8 ± 1.71</td>
<td>35.8 ± 1.34</td>
</tr>
<tr>
<td></td>
<td>-P</td>
<td>3.17 ± 1.1</td>
<td>170 ± 7.25</td>
<td>72.7 ± 36.2</td>
<td>40.3 ± 14.3</td>
</tr>
<tr>
<td></td>
<td>+NO$_3^-$</td>
<td>6.00 ± 0.59</td>
<td>191 ± 3.4</td>
<td>63.4 ± 4.1</td>
<td>69.2 ± 0.5</td>
</tr>
<tr>
<td>$\mu$</td>
<td>-P</td>
<td>1.01 ± 0.20</td>
<td>147 ± 11.3</td>
<td>43.0 ± 5.9</td>
<td>71.1 ± 5.9</td>
</tr>
<tr>
<td>PM</td>
<td>+NO$_3^-$</td>
<td>6.71 ± 0.11</td>
<td>195 ± 0.66</td>
<td>69.2 ± 0.99</td>
<td>69.9 ± 0.10</td>
</tr>
<tr>
<td>$\Phi_M$</td>
<td>-N</td>
<td>6.66 ± 0.11</td>
<td>243 ± 0.45</td>
<td>118 ± 1.97</td>
<td>N.D.</td>
</tr>
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<td></td>
<td>-P</td>
<td>6.76 ± 0.03</td>
<td>124 ± 0.15</td>
<td>89.6 ± 0.10</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Note: N.D. means not determined.
Fig. 5-1. The uptake rates of N, P, and Si for *T. weissflogii* and *P. minimum* in treatments A-F under different nutrient conditions. TW+NO$_3^-$, TW-N, and TW-P signify nutrient-enriched, N- and P-starved *T. weissflogii*. PM+NO$_3^-$, PM-N, and PM-P signify nutrient-enriched, N- and P-starved *P. minimum*. A-F refers to [Cd$^{2+}$] shown in Table 5-1. Values are means ± SD (n = 2 or 3).
Fig. 5-2. Increase of intracellular accumulated Cd concentration (intra-Cd) with an increase of [Cd$^{2+}$] for the two algal species *T. weissflogii* and *P. minimum* under nutrient-enriched (+NO$_3$) and N- (-N) and P- (-P) starved conditions at the two time points. Values are means ± SD (n = 2).
Fig. 5-3. The relative distribution of Cd in soluble (sol), insoluble (insol), and cell-surface-adsorbed (ads) fractions of *T. weissflogii* and *P. minimum* under nutrient enriched (+NO$_3^-$), N- (-N) and P- (-P) starved conditions at the end of the toxicity tests. A-F refers to [Cd$^{2+}$] shown in Table 5-1. Values are means ± SD (n = 2).
Fig. 5-4. The relative change in the cell-specific growth rate ($\mu$) and the maximum PS II quantum yield ($\Phi_M$) with an increase in $[\text{Cd}^{2+}]$ (mol l$^{-1}$), intracellular Cd concentration (intra-Cd, $\mu$mol mol C$^{-1}$), and Cd concentrations in the cell-surface-adsorbed (ads-Cd, $\mu$mol mol C$^{-1}$) and soluble (sol-Cd, $\mu$mol mol C$^{-1}$) fractions for *T. weissflogii* at the end of the toxicity tests. Values are means ± SD (n = 2 or 3).
Fig. 5-5. The relative change in the cell-specific growth rate ($\mu$) and the maximum PS II quantum yield ($\Phi_M$) with an increase in $[\text{Cd}^{2+}]$ (mol l$^{-1}$), intracellular Cd concentration (intra-Cd, $\mu$mol mol C$^{-1}$), and Cd concentrations in the cell-surface-adsorbed (ads-Cd, $\mu$mol mol C$^{-1}$) and soluble (sol-Cd, $\mu$mol mol C$^{-1}$) fractions for *P. minimum* at the end of the toxicity tests. Values are means $\pm$ SD ($n$ = 2 or 3).
Fig. 5-6. The relative change in the cell-specific growth rate ($\mu$) and the maximum PS II quantum yield ($\Phi_M$) with an increase in intracellular Cd concentration (intra-Cd) at the two different time points for *T. weissflogii*. Values are means ± SD (n = 2 or 3).
Fig. 5-7. The relative change in the maximum PS II quantum yield ($\Phi_M$) with an increase in intracellular Cd concentration (intra-Cd) at the two different time points for $P.\ minimum$. Values are means ± SD ($n = 2$ or $3$).
Chapter 6

Cu accumulation, toxicity, and the synthesis of LMW thiols in different nutrient-conditioned diatoms

6.1 Abstract

In this study, the toxicity of Cu to a marine diatom Thalassiosira weissflogii was examined under different nutrient conditions (+NO$_3^-$, -N, -P, +NH$_4^+$ conditions). The macronutrients themselves showed a remarkable interaction for their assimilation by phytoplankton. Accordingly, the cellular N (P) contents under the -P (-N) condition were lower than those under the nutrient-enriched conditions. Meanwhile, the +NH$_4^+$ cells had higher cellular N and P contents than did the +NO$_3^-$ cells. Strong metal-nutrient interactions were also observed. High Cu exposure inhibited the nutrient assimilation. P uptake by +NO$_3^-$ cells was suppressed by 86.7% at the highest Cu level as compared with the control treatment. On the other hand, Cu accumulation was induced in the NO$_3^-$-exposed (+NO$_3^-$ and -P) cells suggesting that Cu may be directly or indirectly involved in the intracellular NO$_3^-$ reduction. Subcellular Cu distribution was also examined and Cu toxicity to different nutrient-conditioned cells was predicted with [Cu$^{2+}$] and intra-Cu as well as its distribution in different subcellular compartments. Intra-Cu was found to be the best predictor as it accounted for most of the difference for the relative changes of $\Phi_m$ with [Cu$^{2+}$] between different nutrient-conditioned cells. The synthesis of intracellular cysteine and five LMW thiols was not obviously induced at high Cu levels possibly due to the existence of some other Cu detoxification mechanisms. This was further evidenced by the much lower cysteine and LMW thiol contents in -N cells with a similar Cu tolerance to the nutrient-enriched cells. Kinetic studies of thiols and cysteine should be performed for a fuller understanding.
6.2 Introduction

Cu toxicity to marine phytoplankton has received extensive attention in the past few decades. As an essential metal, Cu is an important cofactor in plastocyanin, cytochrome c and ascorbate oxidase [52]. However, it would be toxic when highly accumulated in algae due to non-specific competition for important coordination sites with other essential metals. Several models have been proposed to predict metal toxicity with the free metal ion concentration (FIAM) [7, 8] or with metals adsorbed on the surface of organisms (BLM) [9, 10]. However, these models are based on several assumptions and a number of exceptions have been documented [1, 13]. Exploration of a more accurate model is thus warranted. De Schamphelaere et al. [14] attempted to relate intracellular or cell-surface-adsorbed metal concentration to the metal’s toxicity. Recently, we demonstrated that sol-Cd can better predict its toxicity in phytoplankton under different nutrient conditions as compared with the Cd distribution in other subcellular fractions as well as intra-Cd [176]. However, whether this conclusion can be extended to other trace metals remains unknown.

Nitrogen limitation and phosphorus limitation are both frequently observed in marine waters [177], which may influence metal assimilation by phytoplankton. N limitation can strongly suppress metal accumulation in both laboratory cultures and natural phytoplankton communities while P has little effect [32, 34, 176]. The algal biochemical composition is also different under different nutrient conditions [22]. Therefore, different nutrient-conditioned phytoplankton may have different metal sensitivity. Metal toxicity may be affected not only by the ambient nutrient concentration but also by different N sources (NO₃⁻ vs. NH₄⁺). As NH₄⁺ is preferred over NO₃⁻ by phytoplankton and its assimilation could save energy used for
NO$_3^-$ reduction [178], more energy may be available for metal detoxification, which leads to the hypothesis that phytoplankton exposed to NH$_4^+$ are more metal-tolerant. On the other hand, nutrient uptake could also be affected by the metal concentration in the environment. The inhibition of nutrient uptake by Cd has already been observed in a previous study [176]. We are interested in determining if Cu has the same inhibition effect.

PCs are LMW thiols with a general formula $\gamma$-glu-cys$_n$-gly, where n is the number of $\gamma$-glu-cys groups and the carboxyl in the functional chain of glutamate is bound with the amino group of the cysteine ($\gamma$ linkage) [179]. PC$_{2-4}$ are the predominant PCs in phytoplankton. PC synthesis can be strongly induced by several metals and Cd is the most effective activator [38], suggesting that PC may play an important role in metal detoxification. Trace metals are bound to PC through coordination with the sulphydryl group in cysteine with a sulfur to metal ratio ranging from 2 to 4. However, both the induction of PCs and their metal binding stoichiometry are specific to the metal and phytoplankton species. Accordingly, their effect on metal detoxification may be different for different metal and algal species [38, 155, 180, 181]. GSH is another important LMW thiol involved in the alleviation of intracellular oxidative stress and is the substrate for PC synthesis [155, 182]. Examination of intracellular PC and GSH contents as well as their precursors (i.e., cysteine and $\gamma$-glu-cys [$\gamma$GC]) would be helpful in determining the potential Cu tolerances of different nutrient-conditioned algae.

In this study, we examined Cu toxicity to a marine diatom Thalassiosira weissflogii under different macronutrient conditions. Intracellular Cu accumulation and its subcellular distributions were examined. The main objectives of our study were to examine: 1) interactions between the uptake of macronutrients and Cu in
phytoplankton, 2) the effects of macronutrients on Cu toxicity to the cells, 3) whether the intracellular or subcellular Cu concentration can better predict its toxicity to phytoplankton, and 4) whether PCs and GSH play an important role in Cu detoxification.

6.3 Materials and methods

6.3.1 Phytoplankton and culture conditions

An axenic culture of the diatom *Thalassiosira weissflogii* (CCMP 1048) was obtained from the Provasoli-Guillard Center for the Culture of Marine Phytoplankton, Bigelow Laboratory, West Boothbay Harbor, ME, USA. The cells were maintained under sterile conditions in f/2 medium [73] at 19 °C with a light illumination of 170 μmol photons m⁻² s⁻¹ in a 14:10 LD cycle. The pH value of the medium was 8.2 ± 0.1. Seawater for the culture was collected 10 km off East Hong Kong to minimize any anthropogenic influences and was filtered through a 0.22 μm Poretic membrane before use.

6.3.2 Media for Cu toxicity tests

Artificial sea water was used for the toxicity test media. It was similar to the Aquil medium [159] as described by Miao and Wang [176]. After the artificial sea water was prepared, it was passed through a chelex-100 ion exchange resin to remove the background trace metals. N, P, Si and vitamins were spiked at the f/2 levels, while the trace metals were at the f/10 levels except for Cu. N and P were not added in the -N and -P experiments, respectively. NO₃⁻ was used as the N source in the +NO₃⁻ and -P experiments while it was replaced by the same amount of NH₄⁺ in the +NH₄⁺ experiments. All the nutrient stocks except for the trace metals were also passed through the chelex resin. μ of *T. weissflogii* grown in natural and
artificial sea water were compared. No significant difference was observed ($p > 0.05$).

There were six treatments (i.e., treatments A-F) in each toxicity test with two replicates each and the dissolved Cu concentration (nominal) was 0.008, 5, 10, 50, 100, and 110 µmol l\(^{-1}\), respectively. One hundred µmol l\(^{-1}\) NTA was added to keep the [Cu\(^{2+}\)] constant. The pH value of the media was adjusted to 8.2 ± 0.1 with 1 mol l\(^{-1}\) Suprapure NaOH (Merck, Darmstadt, Germany). [Cu\(^{2+}\)] was calculated by the MINEQL+ software package (Version 4.5 from Environmental Research Software, Hallowell, ME, USA) with the updated thermodynamic constants and the calibration set on ionic strength. It was \(1.00 \times 10^{-13}\), \(7.94 \times 10^{-11}\), \(2.00 \times 10^{-10}\), \(2.00 \times 10^{-9}\), \(7.94 \times 10^{-8}\), \(2.51 \times 10^{-6}\) mol l\(^{-1}\) for treatments A to F, respectively. All the media were equilibrated for about 24 hours before use. Trace metal clean technique was used throughout the experiment. All the bottles (polycarbonate) and beakers (polypropylene) were soaked in 1 mol l\(^{-1}\) HCl until one day before the toxicity test and rinsed with Milli-Q (18.0 MΩ) water at least five times.

### 6.3.3 Toxicity tests by PAM-fluorometry

Toxicity tests were performed on the four different nutrient-conditioned (i.e., +NO\(_3\)\(^{-}\), -N, -P, and +NH\(_4\)\(^{+}\)) cells. After reaching the mid-exponential phase in their development, the cells were collected and resuspended into the toxic media. In the -N and -P experiments, the +NO\(_3\)\(^{-}\) cells were grown in the -N and -P media for one and two days, respectively, before their resuspension in the toxic media. The test lasted 48 hours with two time points (24 and 48 h). At each time point, PAM fluorescence induction curves were obtained with a PAM 101/103 fluorometer (Heinz Walz GmbH, Effeltrich, Germany) as described by Miao et al. [158]. $\Phi_M$ was revealed by the induction curves [56]. The cell number at the beginning and
each time point of the toxicity test was also counted. \( \mu \) was then calculated with the Eq. 4-1.

6.3.4 Nutrient measurements and Cu quotas at cellular and subcellular levels

At the beginning and end of each experiment, nitrate plus nitrite (\( \text{NO}_3^- + \text{NO}_2^- \)) in the +\( \text{NO}_3^- \) and -\( \text{P} \) media was determined using the methods described by Wood et al. [160]. Similarly, the concentrations of ammonium (\( \text{NH}_4^+ \)) in the +\( \text{NH}_4^+ \) medium and dissolved inorganic phosphorus in all except the -\( \text{P} \) medium were also measured [161, 183]. Meanwhile, the cellular organic C, N, and P contents at different Cu levels were quantified at the end of each experiment following the method in our previous study [176].

At the end of each experiment, the intra-Cu was measured in treatments A-F. For this measurement, a 100 ml sample from each bottle was filtered through a 3\( \mu \text{m} \) polycarbonate membrane, rinsed with artificial sea water three times and then resuspended into 10 ml HQS (1 mmol l\(^{-1}\) with sea water as the solvent) for 10 min to remove the weakly cell-surface-adsorbed Cu [15, 40]. The cells were then collected by centrifugation, digested in 1 ml suprapure \( \text{HNO}_3 \) (70\%, v/v), and diluted with Milli-Q water to 7\% (v/v) after the digestion was complete. Another 100 ml sample from each bottle was also harvested for Cu subcellular fractionation. The cell-surface-adsorbed Cu (cell-surface-adsorbed fraction) was firstly removed as in the intra-Cu measurements. However, the cells were subsequently resuspended into 3 ml of 0.02 mol l\(^{-1}\) Tris-buffer with 0.15 mol l\(^{-1}\) NaCl (pH = 8) and homogenized by sonication four times for 20 s with 15 s intervals. The homogenate was further ultra-centrifuged at 235,000 g for 30 min. The supernatant and pellet were isolated and differentiated as the soluble and insoluble fractions, respectively. The subcellular fractionation was performed at 4 \(^{\circ}\)C. The cell-surface-adsorbed fraction
in the HQS was acidified with a final 7% HNO₃ concentration. At the same time, the soluble and insoluble fractions were dried first and then digested with suprapure HNO₃ before final dilution to 7%. The Cu concentrations were quantified with inductively coupled plasma atomic emission spectrometry (ICP-AES) for the high metal concentration treatment or ICP-MS for the low metal concentration treatment. Blanks were used throughout the experiments and they fell below the detection limit.

6.3.5 Cysteine and LMW thiols in the cells

At the end of each experiment, the concentrations of cysteine and LMW thiols were determined in treatments A-F following the method described by Wei et al. [184]. For this purpose, another 100 ml sample was taken from each bottle, filtered through a 25 mm combusted GF/F membrane and immediately stored at -140°C. Before the analysis, the samples were placed directly into 2 ml 70°C methanesulfonic acid (MSA, 10 mmol l⁻¹) to denature the proteins that may break the LMW thiols. The mixture was then homogenized with a tissue grinder (pyrex) and the homogenate was centrifuged at 13,800g for 10 minutes. An aliquot of 0.8 ml supernatant was collected and its pH value was adjusted to 9.0 with the addition of 100 mmol l⁻¹ borate buffer (final concentration-9.32 mmol l⁻¹) that also contained 10 mmol l⁻¹ diethylenetriaminepentaacetic acid (DTPA, final concentration-932 μmol l⁻¹). The supernatant was then reacted with excess 15 mmol l⁻¹ dithiothreitol (DTT, final concentration-50.0 μmol l⁻¹) for 10 min to reduce disulfides formed when preparing the cell homogenate. The mixture was then labeled with 50 mmol l⁻¹ fluorescence tag monobromobimane (mBrB, final concentration-537.8 μmol l⁻¹). After 10 min, excess15 mmol l⁻¹ DTT (final concentration-161 μmol l⁻¹) was added to react with the remaining mBrB. The sample was finally acidified with 1 mol l⁻¹ MSA (final
concentration-21.5 mmol l\(^{-1}\)) to stabilize the reaction mixture and it was transferred to an autosampler vial for HPLC analysis.

The different thiols and cysteines labeled with mBrB in the samples were separated in a Supelco Discovery RP Amide-C16 HPLC column (4.6×250 mm) and detected with a Waters 2475 multi-\(\lambda\) fluorescence detector (excitation wavelength-380 nm, emission wavelength-470 nm). The elution time was 40 minutes with two solvents (25 mmol l\(^{-1}\) KH\(_2\)PO\(_4\) with pH 3.2 and pure acetonitrile). The percentage gradient of acetonitrile was as follows: 8% in 0-2 min, 8-12.5% in 2-12 min, 12.5-16.4% in 12-14.7 min, 16.4-18.4% in 14.7-25 min, 18.4-40% in 25-31 min, 40-80% in 31-34 min, 80% in 34-36 min, 80-8% in 36-37 min, and 8% in 37-40 min. The fraction of KH\(_2\)PO\(_4\) solution was adjusted with acetonitrile. All the percentage gradients during this period were changed linearly. Six standards (i.e., cysteine [Sigma-Aldrich], \(\gamma\)GC [Sigma-Aldrich], GSH [Sigma-Aldrich], PC\(_{2,4}\) [AnaSpec, San Jose, CA, USA]) dissolved in 10 mmol l\(^{-1}\) MSA were used in the sample quantification.

6.3.6 Calculation of IC\(_{50}\) and statistical analysis

The median inhibition concentrations IC\(_{50}\) of Cu\(^{2+}\), intra-, ads- and sol-Cu (Cu concentration in the soluble fraction) were estimated for the two toxicity endpoints, \(\mu\) and \(\Phi\_M\). A linear interpolation method was used in the estimation with the help of ICPIN software (Version 2.0, USEPA, Duluth, MN, USA). Any 'significant' difference was based on the results of one-way or two-way ANOVA with post-hoc multiple comparisons (software used: SPSS 11.0 by SPSS, Chicago, USA). Significant differences were accepted at \(p < 0.05\).

6.4 Results
6.4.1 Cellular organic nutrient contents and their uptake rates

The cellular organic C, N, and P contents were measured at the end of the exposures and the results are listed in Table 6-1. When $[\text{Cu}^{2+}]$ increased, both C and N contents increased at first and leveled off or even decreased at higher Cu levels. There was about $1.35 \times$ increase for the organic C content of the +NO$_3^-$ cells as the $[\text{Cu}^{2+}]$ increased from $1.0 \times 10^{-15}$ to $2.0 \times 10^{-9}$ mol l$^{-1}$ followed by an abrupt decrease. Similarly, the N content in the +NH$_4^+$ cells was increased by 75.5% from treatments A to D and then decreased by 30.3% in treatment F. In contrast, the P content remained relatively constant at different Cu levels except at the highest $[\text{Cu}^{2+}]$ in the –N and –P cells.

Under the different nutrient conditions, the C contents of +NH$_4^+$ and +NO$_3^-$ cells were comparable to each other while they were significantly higher under -P and -N conditions ($p < 0.05$) in treatments A-C. When $[\text{Cu}^{2+}]$ was further increased (treatments D-F), no consistent trend was observed for the cellular C contents of different nutrient-conditioned cells except that they were higher in the +NH$_4^+$ cells. On the other hand, the cellular N and P contents of the nutrient-starved cells were much lower than those of the nutrient-enriched cells (+NO$_3^-$ and +NH$_4^+$) regardless of which nutrient was limited. Meanwhile, the N content in the -P cells was significantly higher than that in the –N cells ($p < 0.05$). Similar trend was also observed for P content in the –N cells as compared with the –P cells. Furthermore, the +NH$_4^+$ cells had higher N and P contents than the +NO$_3^-$ cells at the same Cu level.

The C:N and C:P ratios in different nutrient-conditioned cells at each Cu level were also calculated (Table 6-1). As expected, they were close to the Redfield ratios (6.6 for C:N and 106 for C:P) under the +NO$_3^-$ condition. Although cultured
in the nutrient-enriched media, the $+\text{NH}_4^+$ cells had a significantly lower C:N and C:P than the $+\text{NO}_3^-$ cells had ($p < 0.05$). For the $-\text{N}$ and $-\text{P}$ cells, both ratios were much higher than the Redfield ratio with C:N (C:P) of the $-\text{N}$ ($-\text{P}$) cells higher than the $-\text{P}$ ($-\text{N}$) cells. No consistent trend was observed for C:N and C:P at different $[\text{Cu}^{2+}]$ except that they were sometimes decreased in treatments E and F.

The initial and final dissolved inorganic N and P concentrations in the toxicity media were also measured (data not shown). The dissolved nutrient concentrations at the end of the tests increased with increasing $[\text{Cu}^{2+}]$, even though the initial cell density was similar or even higher in the high $[\text{Cu}^{2+}]$ treatments. The N and P uptake rates were then calculated based on their decreases during the 48h period (Fig. 6-1). Both uptake rates were significantly inhibited at high Cu levels ($p < 0.05$). The N uptake rate was 0.26 ± 0.11 mol mol C$^{-1}$ d$^{-1}$ in treatment A under the $+\text{NO}_3^-$ condition, then decreased to 0.11 ± 0.09 mol mol C$^{-1}$ d$^{-1}$ in treatment C and even became negative in treatment F. Similarly, there was about 15× decrease in the P uptake rate as $[\text{Cu}^{2+}]$ increased from $1.00\times10^{-13}$ to $2.51\times10^{-6}$ mol l$^{-1}$ under the $+\text{NH}_4^+$ condition. In the different nutrient-conditioned cells, the $-\text{P}$ ($-\text{N}$) cells had a lower N (P) uptake than the $+\text{NO}_3^-$ and $+\text{NH}_4^+$ cells had. The P uptake rate for the $-\text{N}$ cells was 0.005 ± 0.002 mol mol C$^{-1}$ d$^{-1}$ as compared to 0.021 ± 0.004 and 0.022 ± 0.006 mol mol C$^{-1}$ d$^{-1}$ for the $+\text{NO}_3^-$ and $+\text{NH}_4^+$ cells, respectively, at the lowest Cu level. Although no consistent difference was observed in the nutrient uptake rates of the nutrient-enriched cells with different N sources, $+\text{NH}_4^+$ cells have a slightly higher uptake rate than the $+\text{NO}_3^-$ cells had in most treatments, especially in the N uptake. Its uptake rate for the $+\text{NH}_4^+$ cells was about 1.5× higher than the $+\text{NO}_3^-$ cells in treatment A.

6.4.2 Cu accumulation and subcellular distribution
The intra-Cu under different nutrient conditions was measured at the end of the tests as shown in Fig. 6-2. The concentrations had already been calibrated to the cellular organic C content. As expected, intra-Cu increased linearly with an increase in [Cu^{2+}] in the log-scale and it was getting a little bit leveled off at the highest [Cu^{2+}]. A significant difference was observed in the intra-Cu of different nutrient-conditioned cells at the same Cu level (p < 0.05). The intra-Cu in the +NO_3^- and −P cells were higher than those in the −N and +NH_4^+ cells. It was 36.5 ± 0.913 and 42.9 ± 0.236 μmol mol C^{-1} for the +NO_3^- and −P cells while 12.0 ± 0.201 and 17.3 ± 3.71 μmol mol C^{-1} for the −N and +NH_4^+ cells, respectively, when [Cu^{2+}] was 7.9×10^{-11} mol l^{-1}.

The Cu distribution in the three subcellular compartments was shown in Fig. 6-3. The results of treatment A are not shown due to the low Cu concentrations and the interference of reagents used in the subcellular fractionation when measured by ICP-MS. More Cu was distributed in the cell-surface-adsorbed fraction with increasing [Cu^{2+}] and then leveled off at high Cu levels. In treatments B and F of −N experiment, 35.6% and 60.1% of the accumulated Cu were adsorbed on the cell surface, respectively. On the other hand, a reversed trend was observed in the Cu distribution in the soluble fraction while no consistent trend was observed in its distribution in the insoluble fraction. As [Cu^{2+}] increased from 7.94×10^{-11} to 2.51×10^{-6} mol l^{-1}, its distribution in the soluble fraction decreased from 34.2% to 19.0% in the −N cells. And its distribution in the insoluble fraction ranged from 30.2 to 20.9% for treatments B-F in the same experiment.

6.4.3 \textbf{Φ}_M and \textbf{μ} at different Cu levels

The relative changes of the two endpoints, \textbf{Φ}_M and \textbf{μ}, with [Cu^{2+}] in the media are shown in Figs. 6-4 and 6-5. Both were strongly inhibited at high Cu levels.
\( \Phi_M \) at the highest \([\text{Cu}^{2+}]\) was 11.2-37.0\% of that at the lowest \([\text{Cu}^{2+}]\) for the different nutrient-conditioned cells after 48h. \( \mu \) reached zero in treatments E and F. The cell density was unchanged or even decreased during the experimental period in these two treatments. On the other hand, the relative changes of \( \Phi_M \) with \([\text{Cu}^{2+}]\) in the \(+\text{NO}_3^-\) and \(-\text{P}\) cells were similar to each other. However, \( \Phi_M \) was less (more) inhibited in the \(-\text{N} (+\text{NH}_4^+)\) cells. It was suppressed by 57.7\% and 59.2\% in the \(+\text{NO}_3^-\) and \(-\text{P}\) cells while it was suppressed by 20.8\% and 83.8\% in the \(-\text{N}\) and \(+\text{NH}_4^+\) cells when \([\text{Cu}^{2+}]\) was \(7.94 \times 10^{-8}\) mol l\(^{-1}\). The PAM fluorescence measurement was repeated in separate experiments with similar results obtained (data not shown). The \( \Phi_M \) response difference between the different nutrient-conditioned cells was not observed for the cell growth. \( \mu \) in treatment D were 13.2\%, 6.02\%, 8.52\%, and 0.00\% of those in treatment A for the \(+\text{NO}_3^-, -\text{N}, -\text{P},\) and \(+\text{NH}_4^+\) cells, respectively. The relative changes in the two endpoints (\( \Phi_M \) and \( \mu \)) were also plotted against the different types of Cu concentration: intra-Cu, ads-Cu, and sol-Cu. The \( \Phi_M \) response difference under different nutrient conditions was much less obvious when these three types of Cu concentrations were used, especially with intra-Cu. However, they had little effect on \( \mu \).

The IC50 was then estimated based on the different Cu concentration types for \( \Phi_M \) and \( \mu \). Consistent with Figs. 6-4 and 6-5, the \([\text{Cu}^{2+}]-\text{IC50}\) (i.e., the IC50 was calculated based on \([\text{Cu}^{2+}]\)) for \( \Phi_M \) showed remarkable differences between different nutrient-conditioned cells. It was about 142\times higher for the \(-\text{N}\) cells as compared with the \(+\text{NH}_4^+\) cells. However, this difference was much smaller when the other three types of Cu concentration were used for the estimation. And intra-IC50 (i.e., IC50 was calculated based on the intra-Cu) ranged from 279 to 1099 \( \mu \)mol mol C\(^{-1}\) under different nutrient conditions. In contrast, the IC50s for \( \mu \) of different
nutrient-conditioned cells was comparable to each other regardless of the type of Cu
concentration used.

6.4.4 Cysteine and LMW thiols

The cellular LMW thiols and cysteine contents normalized against the organic
C content in the algae are shown in Fig. 6-6. Although PC₄ was also measured in
our study, its concentration was well below the detection limit and is not shown. Of
the four LMW thiols and one amino acid, GSH had the highest cellular content while
the contents of cysteine, γGC, PC₂ and PC₃ had the same order of magnitude under
the same nutrient conditions. At the lowest [Cu²⁺], the GSH content in the +NO₃⁻
cells was 65,142 ± 1674 nmol mol C⁻¹ while it was 1077 ± 103, 3472 ± 17.3, 812 ±
46.4, and 2291 ± 118 nmol mol C⁻¹, for cysteine, γGC, PC₂ and PC₃, respectively.
At the same time, the contents of all the LMW thiols and cysteine in the −N cells
were much lower than those in the other nutrient-conditioned cells. Although less
obvious, a similar trend is also observed for the −P cells as compared with +NO₃⁻
cells. In treatment A the GSH content of +NO₃⁻ cells was about 27.9× and 2.93×
higher than the −N and −P cells, respectively. A notable difference was also
observed in the cells grown with different N sources (i.e., +NO₃⁻ and +NH₄⁺ cells) at
the same Cu level. However, this difference was Cu concentration and thiols
species dependent and no consistent results were obtained.

As the contents of LMW thiols, cysteine and intra-Cu were all available, the
ratios of LMW thiols and cysteine to intra-Cu were then calculated for different
nutrient-conditioned cells (Fig. 6-7). The concentrations of PC₂ and PC₃ were
combined with each other and expressed as the concentration of γ-glu-cys group (Σ
γ-glu-cys = 2×PC₂ content + 3×PC₃ content). All the ratios decreased with the
increasing [Cu²⁺] in the toxicity media. Under the +NO₃⁻ condition, the Σ γ-glu-cys
to intra-Cu ratio was $1.41 \pm 0.629$ in treatment A and it decreased to $2.27 \times 10^{-3} \pm 7.91 \times 10^{-5}$ in treatment F. As GSH had the highest concentrations in the cells, the GSH to intra-Cu ratio was also higher than the others under the same conditions. At the same time, all the ratios in -N cells were significantly lower than those in the other nutrient-conditioned cells at the same Cu level ($p < 0.05$).

6.5 Discussion

6.5.1 Cu-macronutrient interactions

As with Cd [176], nutrient uptake by the algae was strongly inhibited by a high Cu accumulation, which may be due to the metal-induced depletion of ATP and the alteration of plasma membrane permeability [153, 167, 168]. However, this inhibition effect was not observed with cellular N and P contents, which even increased with an increase of $[\text{Cu}^{2+}]$ at first. The cellular nutrient content is known to be positively related to the nutrient uptake rate while negatively to $\mu$. Therefore, it was the cell growth inhibition which contributed to the constant or higher cellular nutrient contents at higher Cu levels. The P uptake rate of the +NO$_3$ cells decreased by 43.3% as $[\text{Cu}^{2+}]$ increased from to $1.0 \times 10^{-13}$ to $2.0 \times 10^{-10}$ mol l$^{-1}$. Concurrently, there was about 42.2% decrease in $\mu$, which could thus compensate for the decrease in the P uptake and the cellular content remained constant in the algae. At the same time, the potentially higher cell size at high $[\text{Cu}^{2+}]$ may also influence the nutrient contents per cell in algae [169]. Although the C fixation rate was not measured, it may also be suppressed by high Cu exposure as its cellular content showed similar trends to N at different $[\text{Cu}^{2+}]$ and the nutrient ratios (C:N and C:P) remained unchanged in most of the treatments. An abrupt decrease in cellular
nutrient contents at the highest Cu levels may be due to the change in cell membrane permeability and cell lysis.

Macronutrients themselves can also interact with each other in their assimilation by phytoplankton. N (P) uptake rates under the -P (-N) conditions were much lower than those under the +NO₃⁻ and +NH₄⁺ conditions. Accordingly, when P (N) was depleted, the cellular N (P) contents were lower than the nutrient-enriched cells at the same Cu level, even though the cell growth was inhibited. The C fixation may also be suppressed by the nutrient starvation in spite of the higher cellular C contents in the –N and –P cells, which was mainly due to a more abrupt decrease of μ as compared with the C fixation rate. ATP and protein production were strongly inhibited under the nutrient-starved conditions [22, 185]. Therefore, nutrient assimilation, as an active/energy-dependent process [186], was suppressed. The nutrient uptake was also different in nutrient-enriched cells with different N sources. The cellular N and P contents in the +NH₄⁺ cells were higher than those in the +NO₃⁻ cells with a similar cell growth at the same Cu level. The phytoplankton preferred the reduced form of N (NH₄⁺) and additional energy was required to reduce NO₃⁻ to NO₂⁻ and further to NH₄⁺ when nitrate was used as the N source [178]. Therefore, less energy was available for nutrient assimilation and lower cellular nutrient contents were observed. However, NH₄⁺ induction of nutrient uptake was not observed with C fixation, which was decoupled from the uptake of N and P [187, 188].

On the other hand, Cu accumulation can also be affected by the cellular nutrient status. The intra-Cu of –N and +NH₄⁺ cells was lower than that of +NO₃⁻ and –P cells, especially when [Cu²⁺] was low. Similar results have also been observed previously with Cd accumulation in both laboratory cultures and natural
phytoplankton communities [32, 34]. In those studies, nitrate enrichment was found to induce Cd uptake while P had little effect. The N inhibition of metal accumulation was thus hypothesized to be due to the smaller Cd-uptake-proteins synthesis in the –N cells. However, this hypothesis cannot explain the lower intra-Cu in the +NH$_4^+$ cells observed in this study as well as the inhibition of the protein and ATP in the –P cells while their metal uptake was not affected. It thus seems that Cu accumulation in phytoplankton is dependent on the assimilated N state. When NO$_3^−$ was used as the N source (e.g., +NO$_3^−$ and –P conditions in this study), more Cu was required, suggesting that Cu may be directly or indirectly involved in the reduction of NO$_3^−$ to NH$_4^+$. Further study was needed on the underlying mechanisms.

As compared with intra-Cu, no consistent trend was observed for subcellular distribution of Cu under different nutrient conditions. Although less obvious than Cd, the increasing Cu distribution in the cell-surface-adsorbed fraction at high Cu levels may alleviate the Cu stress. Furthermore, Cu distribution in different subcellular compartments was also different from Cd in the same algal species. Our previous study found that most accumulated Cd was distributed in the insoluble fraction of +NO$_3^−$ cells (e.g., 70% in the insoluble fraction, 20% in the soluble fraction, and the other in the cell-surface-adsorbed fraction) when the ambient Cd concentration was low. The distribution of Cu in the three compartments was similar to each other or at least not as different as Cd. Cu distributions in the cell-surface-adsorbed, soluble, and insoluble fractions were 35.6%, 34.2%, and 30.2%, respectively, under the +NO$_3^−$ condition. Therefore, subcellular metal distribution is metal species specific.

6.5.2 Cu toxicity under different nutrient conditions and its prediction
Two endpoints ($\mu$ and $\Phi_M$) were used in this study to indicate the response of *T. weissflogii* to Cu exposure under different nutrient conditions. $\Phi_M$ had a similar sensitivity to Cu exposure as $\mu$. When there's an inhibition in the cell growth, the maximum quantum PS II yield also decreased. However, a notable difference was observed for the relative change of $\Phi_M$ under different nutrient conditions at the same Cu level while there was no consistent trend for $\mu$. $\Phi_M$ of the $+\text{NO}_3^-$ and $-\text{P}$ cells had a similar response to Cu exposure while $-\text{N}$ ($+\text{NH}_4^+$) cells were less (more) inhibited. Such a difference was also observed in the IC50 estimates. The different responses of $\mu$ and $\Phi_M$ to Cu exposure suggests that the effect of Cu on the PS of different nutrient-conditioned cells was different while this can not be seen in the cell growth. Dzhibladze et al. [189] found that wheat had different PS I reaction center contents and PS II redox states when cultured under $+\text{NH}_4^+$, $-\text{N}$, and $+\text{NO}_3^-$ conditions, which may explain the different responses of $\Phi_M$ to Cu under different nutrient conditions.

Several exceptions to the FIAM and BLM models have been reported [1, 13], thus there is a significant need to explore other more direct and accurate metal toxicity predictors (e.g., intracellular metal concentration and metal distribution in the subcellular fractions). Although intracellular metal accumulation (metal body burden) and subcellular metal fractionation have been extensively examined in different organisms [15-18], few studies have tried to link them with metal toxicity. It was not until recently that some trials have been made [14, 176].

In this study, we tried to predict Cu toxicity to different nutrient-conditioned *T. weissflogii* with intra-Cu concentration as well as its subcellular distribution. When $\Phi_M$ was plotted against $[\text{Cu}^{2+}]$, a significantly different response was found for different nutrient-conditioned cells ($p < 0.05$). Such nutrient-condition-dependent
differences were smaller when ads- and sol-Cu were used in the prediction. And the smallest difference was observed when intra-Cu was used. There is about 142× difference between the highest and lowest [Cu$^{2+}$]-IC50 while it was 3.9× for intra-IC50. The Cu subcellular distribution was only operationally defined in this study and it is possible that the subcellular fractionation defined here was not appropriate to indicate the Cu toxicity on the PS. Therefore, a more functionally-based subcellular fractionation may have a better predictability of metal toxicity. Some techniques are now available such as energy-dispersive X-ray microanalysis (EDAX) and electron energy loss spectroscopy (EELS) [190-192]. However, both techniques have some disadvantages. Further improvement is required for these techniques to be applied to metal toxicity prediction.

6.5.3 Function of LMW thiols and cysteine in Cu detoxification

Five LMW thiols and cysteine were quantified in this study. The cellular PC$_4$ contents were below the detection limit. Among the other four thiols and cysteine, only PC$_2$ showed an increasing cellular content with an increase of [Cu$^{2+}$] first and then it decreased at higher Cu levels. As more Cu accumulated in the algae at higher Cu levels, the ratios of LMW thiols or cysteine to intra-Cu thus decreased and a constant ratio [179] was not observed in our study. The cellular contents of the LMW thiols and cysteine were similar to the results from Ahner et al. [174] for the same algal species at similar [Cu$^{2+}$]. In the control treatment of the +NO$_3$ experiment, the contents of cysteine, γEC, GSH, and Σ γ-glu-cys were, respectively, 1.90, 6.13, 115.1, and 15.0 μmol g$^{-1}$ chlorophyll a as compared to < 5, ~10, ~500, and ~40 μmol g$^{-1}$ chlorophyll a in their study. Ahner et al. [174] found no obvious relationship between [Cu$^{2+}$] and LMW thiols or cysteine production either.
There are several possible reasons for the weak relationship between Cu exposure and thiol synthesis. Firstly, Cu is not efficient in intracellular thiol induction, while Cd was found to be the best activator of PC synthase [38]. The Cu-exposed cells had much fewer PCs as compared with the Cd-exposed cells for the same algal species [174]. Therefore, high Cu exposure may not effectively increase thiol synthesis. Secondly, measuring cellular LMW thiol contents without examining their kinetics (synthesis and elimination rate) may not indicate their real function in metal detoxification, as thiols have rapid turnover in cells [193]. For instance, over 50% of the cellular GSH was converted into PCs after 2h exposure in *Phaeodactylum tricornutum* and 65-70% of the cellular PC disappeared during a 24h period after transfer into metal-free sea water in the same algal species. A mechanism in which metal-PC complexes are actively transported from cytosol to the vacuole and therein undergo a rapid turnover has been proposed for plant cells [194]. Therefore, simultaneous examination of thiol contents as well as their turnover rate is required for a better assessment of their contribution to metal detoxification. Thirdly, sequestration by the LMW thiols may not be a main pathway to detoxify intracellular accumulated Cu. Besides the five LMW thiols measured in our study, a number of other thiols (e.g., [γ-Glu-Cys]₃-β-Ala, [γ-Glu-Cys]₃-Ser, and [γ-Glu-Cys]₃-Glu) have also been identified in some plant species [38], and these other thiols may have some roles in metal detoxification. At the same time, PC synthesis was not observed in Cu-exposed *Prorocentrum micans* [195]. In that study [195], a 25 kD protein was detected after 3h Cu exposure, which may explain the high Cu tolerance of this species. Furthermore, the existence of LMW thiols did not improve the Cu tolerance of *Thalassiosira pseudonana*, although they may synthesize PCs even at low Cu levels [155]. All
this evidence suggests that there may be other mechanisms for Cu detoxification in phytoplankton.

The third possibility is further evidenced by the much lower LMW thiol contents in the -N cells. However, their Cu tolerance was comparable or even higher than the other nutrient-conditioned cells based on the response of the two endpoints. As N is an important component for amino acids, much less thiol and cysteine was produced under the -N condition. Similar results were also obtained by Rijstenbil et al. [155]. In their study, PC was detectable only at the upper Cu level when N:P was low (1.5-7.1) while it was detectable even without Cu addition when N:P was high (12.2-18.4). However, these Cu-binding thiols did not prevent a sharp decrease in cell division rates. It was thus proposed that PC production in the cells may be an indicator of metal stress rather than a contributor to algal species-dependent metal tolerance [182]. In the future, it would be interesting to examine other possible metal detoxification mechanisms in phytoplankton.

In conclusion, strong metal-nutrient and nutrient-nutrient interactions were observed in our study. Nutrient uptake was suppressed at high Cu levels. However, the cellular nutrient contents remained relatively constant or sometimes increased with an increase of [Cu$^{2+}$], as the cell growth inhibition by Cu exposure compensated for the nutrient uptake rate decrease. Macronutrients themselves also affect each other's assimilation by phytoplankton. Accordingly, the cellular N (P) contents under the -P (-N) conditions were lower than those under the nutrient-enriched conditions, even though the cell growth was inhibited under the nutrient-starved conditions. On the other hand, the intracellular Cu accumulation in the NO$_3^-$ exposed (+NO$_3^-$ and -P) cells was higher than that in the -N and +NH$_4^+$ cells, suggesting that Cu may be directly or indirectly involved in NO$_3^-$ reduction.
There is a notable difference in the response of $\Phi_M$ to Cu exposure under different nutrient conditions while the relative changes in $\mu$ were similar to each other. However, the different $\Phi_M$ response nearly disappeared when intra-Cu was used to predict Cu toxicity, suggesting it is a better toxicity predictor for Cu. Although PC$_2$ induction was observed in some treatments, no positive relationship was observed between [Cu$^{2+}$] and the other three thiols as well as with cysteine. It is thus possible that some other Cu detoxification mechanisms exist in phytoplankton.
Table 6-1. The organic C, N, and P contents as well as the ratios of C:N and C:P in different nutrient-conditioned T. weissflogii (+NO₃⁻, -N, -P, and +NH₄⁺) at different Cu levels for treatment A-F, respectively.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>[Cu²⁺] (mol l⁻¹)</th>
<th>C</th>
<th>N</th>
<th>P</th>
<th>C:N</th>
<th>C:P</th>
</tr>
</thead>
<tbody>
<tr>
<td>+NO₃⁻</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.0×10⁻¹³</td>
<td>113±0.111</td>
<td>20.3±0.114</td>
<td>2.70±0.074</td>
<td>6.50±0.021</td>
<td>108±2.04</td>
</tr>
<tr>
<td>B</td>
<td>7.9×10⁻¹¹</td>
<td>126±0.096</td>
<td>20.8±0.061</td>
<td>2.84±0.058</td>
<td>7.05±0.010</td>
<td>114±1.71</td>
</tr>
<tr>
<td>C</td>
<td>2.0×10⁻¹⁰</td>
<td>140±2.67</td>
<td>22.1±0.367</td>
<td>2.83±0.232</td>
<td>7.36±0.013</td>
<td>128±9.17</td>
</tr>
<tr>
<td>D</td>
<td>2.0×10⁻⁰⁹</td>
<td>152±6.14</td>
<td>26.4±1.09</td>
<td>2.97±0.089</td>
<td>6.70±0.003</td>
<td>132±0.975</td>
</tr>
<tr>
<td>E</td>
<td>7.9×10⁻⁰⁸</td>
<td>110±5.03</td>
<td>19.2±1.31</td>
<td>2.54±0.256</td>
<td>6.71±0.107</td>
<td>112±4.35</td>
</tr>
<tr>
<td>F</td>
<td>2.5×10⁻⁰⁶</td>
<td>99.6±0.397</td>
<td>19.4±0.031</td>
<td>2.81±0.088</td>
<td>5.99±0.024</td>
<td>91.7±1.77</td>
</tr>
<tr>
<td>-N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.0×10⁻¹³</td>
<td>168±2.51</td>
<td>5.74±0.312</td>
<td>2.52±0.005</td>
<td>34.2±0.956</td>
<td>172±2.04</td>
</tr>
<tr>
<td>B</td>
<td>7.9×10⁻¹¹</td>
<td>167±1.72</td>
<td>6.26±0.103</td>
<td>2.50±0.218</td>
<td>31.1±0.135</td>
<td>173±12.0</td>
</tr>
<tr>
<td>C</td>
<td>2.0×10⁻¹⁰</td>
<td>165±2.38</td>
<td>6.57±0.074</td>
<td>2.25±0.172</td>
<td>29.3±0.532</td>
<td>190±8.36</td>
</tr>
<tr>
<td>D</td>
<td>2.0×10⁻⁰⁹</td>
<td>168±3.45</td>
<td>7.16±0.353</td>
<td>2.31±0.037</td>
<td>27.4±0.558</td>
<td>188±0.595</td>
</tr>
<tr>
<td>E</td>
<td>7.9×10⁻⁰⁸</td>
<td>114±2.22</td>
<td>7.62±0.152</td>
<td>1.65±0.007</td>
<td>17.5±0.007</td>
<td>178±3.01</td>
</tr>
<tr>
<td>F</td>
<td>2.5×10⁻⁰⁶</td>
<td>87.8±0.921</td>
<td>7.83±0.018</td>
<td>1.82±0.075</td>
<td>13.1±0.118</td>
<td>125±2.71</td>
</tr>
<tr>
<td>-P</td>
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<td></td>
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<tr>
<td>A</td>
<td>1.0×10⁻¹³</td>
<td>131±5.95</td>
<td>12.5±0.136</td>
<td>0.277±0.041</td>
<td>12.2±0.300</td>
<td>1227±89.6</td>
</tr>
<tr>
<td>B</td>
<td>7.9×10⁻¹¹</td>
<td>142±2.44</td>
<td>12.9±0.048</td>
<td>0.307±0.062</td>
<td>12.8±0.122</td>
<td>1223±190</td>
</tr>
<tr>
<td>C</td>
<td>2.0×10⁻¹⁰</td>
<td>156±1.48</td>
<td>14.2±0.313</td>
<td>0.315±0.050</td>
<td>12.8±0.114</td>
<td>1296±138</td>
</tr>
<tr>
<td>D</td>
<td>2.0×10⁻⁰⁹</td>
<td>167±5.63</td>
<td>14.7±0.595</td>
<td>0.326±0.001</td>
<td>13.3±0.065</td>
<td>1327±32.7</td>
</tr>
<tr>
<td>E</td>
<td>7.9×10⁻⁰⁸</td>
<td>107a±3.39</td>
<td>16.7±0.322</td>
<td>0.326±0.002</td>
<td>7.45±0.066</td>
<td>845±23.0</td>
</tr>
<tr>
<td>F</td>
<td>2.5×10⁻⁰⁶</td>
<td>117±3.49</td>
<td>19.2±0.776</td>
<td>0.545±0.019</td>
<td>7.09±0.053</td>
<td>554±25.3</td>
</tr>
<tr>
<td>+NH₄⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.0×10⁻¹³</td>
<td>115±3.80</td>
<td>22.9±0.929</td>
<td>3.71±0.346</td>
<td>5.83±0.030</td>
<td>79.9±3.39</td>
</tr>
<tr>
<td>B</td>
<td>7.9×10⁻¹¹</td>
<td>128±6.16</td>
<td>25.4±0.784</td>
<td>4.13±0.079</td>
<td>5.90±0.071</td>
<td>80.4±3.82</td>
</tr>
<tr>
<td>C</td>
<td>2.0×10⁻¹⁰</td>
<td>147±5.23</td>
<td>27.6±1.34</td>
<td>4.34±0.100</td>
<td>6.21±0.057</td>
<td>87.5±0.78</td>
</tr>
<tr>
<td>D</td>
<td>2.0×10⁻⁰⁹</td>
<td>207±2.69</td>
<td>40.2±0.110</td>
<td>4.54±0.177</td>
<td>6.02±0.044</td>
<td>118±4.33</td>
</tr>
<tr>
<td>E</td>
<td>7.9×10⁻⁰⁸</td>
<td>175±3.00</td>
<td>35.0±1.22</td>
<td>4.03±0.062</td>
<td>5.84±0.073</td>
<td>112±0.138</td>
</tr>
<tr>
<td>F</td>
<td>2.5×10⁻⁰⁶</td>
<td>138±3.18</td>
<td>28.0±0.887</td>
<td>3.87±0.114</td>
<td>5.76±0.035</td>
<td>92.2±3.43</td>
</tr>
</tbody>
</table>
Table 6-2. The estimated IC50 of different nutrient-conditioned phytoplankton (+NO₃⁻, -N, -P, and +NH₄⁺) for different types of Cu concentration ([Cu²⁺], intra-Cu, ads-Cu, and sol-Cu).

<table>
<thead>
<tr>
<th>Nutrient conditions</th>
<th>Endpoints</th>
<th>[Cu²⁺]×10⁸ (mol l⁻¹)</th>
<th>intra-Cu (µmol mol C⁻¹)</th>
<th>ads-Cu (µmol mol C⁻¹)</th>
<th>sol-Cu (µmol mol C⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+NO₃⁻</td>
<td>Φₘ</td>
<td>4.31±0.498</td>
<td>616±41.6</td>
<td>926±58.1</td>
<td>167±5.97</td>
</tr>
<tr>
<td></td>
<td>µ</td>
<td>0.051±0.003</td>
<td>114±3.22</td>
<td>162±6.22</td>
<td>47.4±1.40</td>
</tr>
<tr>
<td>-N</td>
<td>Φₘ</td>
<td>176±8.96</td>
<td>1099±27.1</td>
<td>1982±65.2</td>
<td>623±20.4</td>
</tr>
<tr>
<td></td>
<td>µ</td>
<td>0.02±0.003</td>
<td>34.7±4.19</td>
<td>49.2±4.76</td>
<td>35.6±2.38</td>
</tr>
<tr>
<td>-P</td>
<td>Φₘ</td>
<td>7.51±3.71</td>
<td>827±90.3</td>
<td>616±52.5</td>
<td>201±12.9</td>
</tr>
<tr>
<td></td>
<td>µ</td>
<td>0.015±0.001</td>
<td>66.0±0.432</td>
<td>63.0±0.521</td>
<td>38.6±0.233</td>
</tr>
<tr>
<td>+NH₄⁺</td>
<td>Φₘ</td>
<td>1.24±0.070</td>
<td>279±3.99</td>
<td>306±2.17</td>
<td>55.1±0.647</td>
</tr>
<tr>
<td></td>
<td>µ</td>
<td>0.052±0.003</td>
<td>78.6±3.07</td>
<td>71.7±4.55</td>
<td>39.8±0.115</td>
</tr>
</tbody>
</table>
Fig. 6-1. N and P uptake rates for the +NO$_3^-$, -N, -P, and +NH$_4^+$ cells, respectively. N (P) uptake rate was not calculated for the –N (-P) cells. Data are mean ± SD (n=2).
Fig. 6-2. Increase of intracellular Cu concentration (intra-Cu) with the increase of free copper ion concentration ([Cu$^{2+}$], mol l$^{-1}$) for the $+$NO$_3^-$, -N, -P, and $+$NH$_4^+$ cells, respectively. Data are mean ± SD (n=2).
Fig. 6-3. Cu distribution in the cell-surface adsorbed (ads), soluble (sol), and insoluble (insol) fractions for the +NO$_3^-$, -N, -P, and +NH$_4^+$ cells, respectively. Treatments B-F represent [Cu$^{2+}$] shown in Table 6-1. The data of treatment A was not available as the signal interference with ICP-MS. Data are mean ± SD (n=2).
Fig. 6-4. Relative changes of the photosynthetic system II quantum yield $\Phi_M$ with the free copper ion ([Cu$^{2+}$]) and intracellular Cu concentration (intra-Cu) as well as the Cu concentration in the cell-surface adsorbed (ads-Cu) and soluble fractions (sol-Cu) for the +NO$_3^-$, -N, -P, and +NH$_4^+$ cells, respectively. Data are mean ± SD (n=2).
Fig. 6-5. Relative changes of the cell-specific growth rate $\mu$ with the free copper ion ([Cu$^{2+}$]) and intracellular Cu concentration (intra-Cu) as well as the Cu concentration in the cell-surface adsorbed (ads-Cu) and soluble fractions (sol-Cu) for the $+\text{NO}_3^-$, -N, -P, and $+\text{NH}_4^+$ cells, respectively. Data are mean $\pm$ SD (n=2).
Fig. 6-6. The LMW thiols and cysteine contents for the +NO$_3^-$, -N, -P, and +NH$_4^+$ cells, respectively. Treatments A-F represent [Cu$^{2+}$] shown in Table 6-1. Data are mean ± SD (n=2).
Fig. 6-7. The ratios of cysteine, γGC, GSH, and $\Sigma$ γ-glu-cys to the intracellular Cu concentration (intra-Cu) for the $+\text{NO}_3^-$, -N, -P, and $+\text{NH}_4^+$ cells, respectively. $\Sigma$ γ-glu-cys represents the total γ-glu-cys concentration in PC$_2$ and PC$_3$ (i.e., $\Sigma$ γ-glu-cys = 2×PC$_2$ content + 3×PC$_3$ content). Treatments A-F represent [Cu$^{2+}$] shown in Table 6-1. Data are mean ± SD (n=2).
Chapter 7

General conclusion

7.1 Metal toxicity to marine phytoplankton

7.1.1 Pulse Amplitude Modulated fluorometry

The PAM technique, as a non-intrusive method to study the PS, has been proposed to be a rather rapid and sensitive approach to evaluate pollutant effects on plants and freshwater algae as compared to the frequently used endpoints such as \( \mu \), CO\(_2\) fixation, O\(_2\) evolution, respiration, and ATP levels. It was found in my study (Chapters 4-6) that this technique has a similar sensitivity to the most frequently used endpoint \( \mu \) for the metal toxicity to marine phytoplankton. When there is an inhibition in algal growth, the PAM parameters (\( \Phi_M \) and \( \Phi_M' \)) also decreased.

However, the PAM technique still has several advantages as compared with \( \mu \). Firstly, the metal toxicity mechanisms, especially its influence on the PS, can be investigated with this technique. Secondly, monitoring metal toxicity with this technique is more efficient than the measurement of \( \mu \), for which at least two time points are required. Furthermore, \( \mu \) may be quite low under some conditions (e.g., nutrient-starved condition) or cannot be measured directly (e.g., existent of predators in the objective aquatic system). In this case, metal toxicity can not be properly examined based on the cell growth while there is no problem for PAM technique. Meanwhile, the PAM technique will also be of great benefit in field research work, as several models of portable PAM fluorometer have now been available.

7.1.2 Algal and metal species-specific toxicity difference

Cd, Cu and Zn toxicity to four different algal species, *Dunaliella tertiolecta* (green alga), *Prorocentrum minimum* (dinoflagellate), *Synechococcus sp.* (cyanobacteria), and *Thalassiosira weissflogii* (diatom) was compared in Chapter 4.
Based on the calculated NOECs, *Synechococcus sp.* and *T. weissflogii* were more sensitive than the other two algal species. And *T. weissflogii* was even more sensitive to Cd and Zn than the cyanobacteria *Synechococcus sp.*, which may be due to its different type of toxicity response as compared with the other three algal species. However, *Synechococcus sp.* was still the most sensitive species by taking into account its most abrupt changes in endpoints (μ and PAM parameters). The higher sensitivity of *Synechococcus* to the metals (especially to Cd and Cu) was not due to its lower ability to regulate the metal uptake or eliminate the cellular metal, but to its higher cell surface area to volume ratio and thus higher metal bioaccumulation potentials. Meanwhile, the high tolerance of *D. tertiolecta* to Cu and *P. minimum* to Cd and Zn may result from the regulation of metal uptake or elimination, which leads to a lower cellular metal concentration at the same [M$^{2+}$].

On the other hand, metal toxicity to phytoplankton was also metal species dependent. Among the three metals tested (Cd, Cu, and Zn), Zn was the least toxic, as its NOEC was orders of magnitude higher than those of Cd and Cu for the same algal species. This was further evidenced by the higher cellular Zn accumulation at NOEC as compared with Cd and Cu. Meanwhile, Cd was the most toxic as its cellular concentration at NOEC was lower than those of Cu and Zn.

### 7.1.3 Metal toxicity and its prediction under different nutrient conditions

Cd toxicity under +NO$_3^-$, -N, -P conditions (Chapter 5) and Cu toxicity under +NO$_3^-$, -N, -P, and +NH$_4^+$ conditions (Chapter 6) were also examined. It was found that -N and -P *T. weissflogii* were more sensitive to Cd as their [Cd$^{2+}$]$_{-}$, intra-, and ads-IC50 of the two endpoints μ and Φ$_M$ were lower than those under the +NO$_3^-$ conditions, suggesting that N and P were required in Cd detoxification. However, when sol-Cd was used to predict its toxicity, much less difference was observed.
Therefore, Cd concentration in the soluble fraction may be a better predictor to its toxicity.

As for Cu, the different nutrient-conditioned algae had a similar growth response at the same Cu levels. However, the maximum PS II quantum yield $\Phi_M$ was more inhibited under the $+\text{NH}_4^+$ condition while less inhibited under the $-\text{N}$ condition as compared with the $+\text{NO}_3^-$ and $-\text{P}$ cells. Such a response difference between $\mu$ and $\Phi_M$ to Cu exposure suggested that the effect of Cu on the PS of different nutrient-conditioned cells was different, which can not be indicated from the cell growth. However, the $\Phi_M$ response difference under different nutrient conditions was the smallest when the intra-Cu was used for its toxicity prediction. Failure of Cu subcellular distribution in the prediction of its toxicity under different nutrient conditions may be due to the operationally defined subcellular compartments. A more functionally based subcellular fractionation was needed in the future.

7.1.4 LMW thiols contribution to metal detoxification

LMW thiols, especially PCs, have been proposed to play an important role in Cd detoxification. High metal exposure may induce the PC synthesis and a constant ratio between $\sum \gamma\text{-glu-cys}$ and Cd has been reported. However, it was not the case for Cu. Exposure of $T.\text{weissflogii}$ to high Cu level has little effect on the cellular LMW thiol contents. And the $\sum \gamma\text{-glu-cys}$ to intra-Cu ratio decreased with the increasing [Cu$^{2+}$] in the medium.

The weak relationship between Cu exposure and thiol synthesis may be explained by several possibilities. Firstly, Cu is not efficient for the thiol induction in cells, as Cd is the best PC synthase activator followed by Ag, Bi, Pb, Zn, Cu, Hg and Au. Secondly, measuring the cellular LMW thiol contents without examining their kinetics may not properly illuminate their real function in metal detoxification,
as the thiols turn over rapidly in the cells. Thirdly, intracellular accumulated Cu may not be detoxified mainly through complexation with the LMW thiols examined in this study. Besides the five LMW thiols shown in Chapter 6, a number of other thiols (e.g., \([\gamma\text{-Glu-Cys}]_n\)-\(\beta\)-Ala, \([\gamma\text{-Glu-Cys}]_n\)-Ser, and \([\gamma\text{-Glu-Cys}]_n\)-Glu) have also been identified in some plant species, which may have some roles for metal detoxification. The third possibility may be further evidenced by the much lower LMW thiols contents in the \(-N\) cells, which had a comparable Cu tolerance to the nutrient-enriched cells.

7.2 Metal accumulation in marine phytoplankton

7.2.1 Relationship between \(\mu\) and \(\rho\)

In my study, a strong correlation was observed between \(\mu\) and \(\rho\) of Cd and Zn (Chapter 2). Cd and Zn uptake increased proportionally with the increase of \(\mu\) first and then remained constant. Cell growth was regulated by culturing the diatom \(T. pseudonana\) under different light intensities, LD cycles or temperatures. Exposure to two photosynthetic inhibitors (CCCP and Paraquat) has little effect on the metal uptake suggesting that their uptake was independent of the photosynthetic activity. Meanwhile, no obvious diel trend was observed for the metal uptake and the cell size was kept constant under different light and temperature conditions. Therefore, cell size, photosynthetic activity, and cell cycle were unlikely to account for the variations in metal uptake at different temperatures, irradiances, and LD cycles. And it was through the change of \(\mu\) that \(\rho\) was affected. As both light and temperature could influence the Fe uptake by several other ways (e.g., regulating ambient bioavailable Fe concentration and subcellular Fe distribution etc.) than by changing \(\mu\), the positive relationship observed between \(\mu\) and \(\rho\) of Fe might not be
true. Further studies are needed to examine how metal uptake is affected by cell growth at the cellular and sub-cellular levels.

7.2.2 Fulfilling different Fe requirements under different irradiances and temperatures

Although the Fe requirement was higher at lower light levels, its uptake was decreased and so was $\mu$. The cellular Fe concentration was thus kept constant or slightly increased at low light intensity, which may be explained by several possibilities. Firstly, diatoms may supply enough Fe for extra pigment synthesis under lower irradiance by transporting Fe from other organelles to the PS. Secondly, the coastal diatom *T. pseudonana* may take up luxury Fe. Thus, abundant Fe storage in the cells was sufficient for pigment synthesis even under low light intensity. Thirdly, the irradiance used in our study may be not low enough to result in an increase in intracellular Fe concentration. Contrastively, the higher Fe requirement at higher temperature was fulfilled mainly through the increase of Fe uptake, while $\mu$ remained constant under different temperatures.

A remarkable Fe efflux was for the first time directly measured in my study. Although Fe efflux had little effect on the Fe accumulation trend under different light and temperature conditions, its existence did influence the calculated cellular Fe concentration markedly and cannot be neglected. Although unexpected, two possibilities may explain the notable Fe efflux from the diatoms. On the one hand, the diatom used in our study is a coastal species, which was rarely limited by Fe, and thus highly efficient usage of intracellular Fe was probably not well developed. On the other hand, the high Fe efflux may also have some advantages with respect to other organisms. Since there is a luxury Fe uptake for marine phytoplankton, the cells may first take up much more Fe than their own biological requirements when
the Fe concentration in the environment is high (e.g., episodic aeolian deposition). Subsequently, Fe may be excreted out of the cells in the form of more dissolved and bioavailable species, which can be taken up again by the phytoplankton and other organisms. Thus, the iron luxury uptake in combination with its efflux may serve as a buffer system for Fe and can eliminate the Fe limitation in the ocean.
References


capricornutum, and Chlamydomonas reinhardtii to copper”, Arch. Environ.

[57] O. Bjorkman and B. Demmig-Adams, “Regulation of photosynthetic light
energy capture, conversion, and dissipation in leaves of higher plants”,
Ecophysiology of photosynthesis, Springer-Verlag, Hong Kong, p17-48,
1994.

[58] H. Kautsky and A. Hirsch, “Neue Versuche zur Kohlenstoffassimilation”,
Naturwissenschaften, 19: 964, 1931.

Biochem., 274: 422, 1931.

[60] M. Kitajima and W.L. Butler, “Quenching of chlorophyll fluorescence and
primary photochemistry in chloroplasts by dibromothymoquinone”,

and E.G. Lechner, “Chlorophyll fluorescence as a probe of the
photosynthetic competence of leaves in the field: a review of current

quantum yield of photosynthetic electron transport and quenching of

[63] W.G. Sunda and S.A. Huntsman, “Effect of Zn, Mn, and Fe on Cd
accumulation in phytoplankton: Implications for oceanic Cd cycling”,


