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Sources and sinks of dissolved phytochelatin in natural seawater

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Abstract

We modeled dissolved phytochelatin concentrations by studying its exudation from phytoplankton and its removal from seawater. Exudation rates were obtained from experiments in which a marine diatom, *Thalassiosira pseudonana*, was grown in Cd-containing medium. Dissolved phytochelatin in the culture medium was measured as a function of time, and a first-order model was fit to the data to derive exudation rate constants. A synthetic phytochelatin standard was used to study phytochelatin removal from natural seawater. The addition of Cd or Cu, as well as decreasing temperature, significantly decreased phytochelatin removal rates. An acclimation period followed by rapid removal was observed; subsequent incubations showed no acclimation. Using rate constants derived from these experiments and some assumptions regarding the particulate phytochelatin pool, we used a model to calculate the steady state concentration of dissolved phytochelatin in the field and obtained values that are close to field measurements of dissolved phytochelatin in the Elizabeth River estuary, Norfolk, Virginia, where metal concentrations are high.

Phytochelatins are small sulfhydryl-containing peptides synthesized from glutathione by eukaryotic marine phytoplankton in response to some metals (*see* review by Ahner and Morel 1999). In algae, phytochelatins have the structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ where $n = 2$–4, whereas in higher plants, longer chain lengths have been observed (Grill et al. 1985). Phytochelatins play an important role in metal detoxification as intracellular chelators (Rauser 1995). When released from algal cells to the surrounding seawater by exudation (Lee et al. 1996) or by cell breakage, they can influence the biogeochemical cycling of metals in natural seawater. However, the concentrations of dissolved phytochelatins and their sources and sinks in natural seawater have yet to be quantified.

Intracellular phytochelatins have been measured in marine phytoplankton cultures that were grown under conditions ranging from low to inhibiting free-ion concentrations of many metals (Ahner and Morel 1995; Ahner et al. 1995, 2002). Measurable increases are observed in response to even very low levels of metals such as Cd, Cu, Pb, and Zn in many species of algae. In general, phytochelatin production increases with increasing metal concentrations. However, there are antagonistic effects among some metals (Ahner et al. 1997; Wei et al. 2003), and phytochelatin production can also depend on nutrient supply and light intensity (Rijstenbil et al. 1998; Ahner et al. 2002). Field studies of particulate phytochelatin in coastal waters (Ahner et al. 1997; Tang et al. 2000; Wei et al. 2003) and open ocean waters (Ahner et al. 1998) have shown that particulate phytochelatin concentration ranges from 2 to 50 μmol Σ γ-Glu-Cys g^{-1} chlorophyll *a* (Chl *a*)—where Σ γ-Glu-Cys = 2 (n = 2) + 3 (n = 3) + 4 (n = 4)—and have confirmed that phytochelatins in phytoplankton are ubiquitous in surface seawater.

In the dissolved phase, the speciation of many biologically active trace metals is controlled by organic complexation with strong ligands of recent biological origin (Bruland et al. 1991). The specific structures of these ligands have yet to be identified for any metal. Dissolved phytochelatin (i.e., extracellular) is a potential component of metal-binding ligands in natural seawater (particularly for metals such as Cd, Cu, and Zn), but because it is likely present in only very low concentrations, it has yet to be definitively quantified in natural seawater. Nevertheless, there are reasons to suspect that phytochelatin plays an important role in metal speciation. For example, phytochelatin export was reported in Cd-stressed laboratory cultures of the marine diatom *Thalassiosira weissflogii* (Lee et al. 1996) and electrochemical analyses showed that concentrations of unidentified dissolved thiols covaried with Chl *a* concentrations in coastal surface seawater (Al-Farawati and van den Berg 2001).

In this paper, we report the kinetics of phytochelatin exudation of *Thalassiosira pseudonana,* a marine diatom, that was growing exponentially in a medium containing an intermediate level of Cd, and we derive a simple model of exudation that assumes a fixed exudation rate constant at a given Cd concentration. Also, with a synthetic phytochelatin standard, phytochelatin removal rates were measured in natural seawater under various conditions. With derived exudation and removal rate constants, and some assumptions regarding the particulate phytochelatin pool, a model of dissolved phytochelatin concentration was developed. The model results are compared with measurements made in the Elizabeth River estuary, Norfolk, Virginia, in which dissolved phytochelatin and trace metals were measured in filtered seawater.

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Materials and methods

Phytochelatin exudation—T. pseudonana (CCMP 1335; Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine) was cultured axenically in continuous light (120 μmol photons m^{-2} s^{-1}) at 19 ± 1°C in Aquil, a defined minimal medium developed specially for studies of trace metal nutrition and toxicity in marine algae (Price et al. 1988/1989). Equimolar Cd–ethylenediaminetetraacetic acid (EDTA) was added at a concentration of 3 μmol L^{-1} to achieve the desired free Cd^{2+} ion concentration of pCd 10 (pCd = $-\log[Cd^{2+}]$) on the basis of equilibrium calculations by MINEQL (Westall et al. 1976). Near the end of exponential growth, the algal cells were collected on Nuclepore Track-Etch Membrane filters (Whatman, pore size 1 μm) that had been soaked in 1 mol L^{-1} HCl and rinsed with Milli-Q water (Millipore), and they were resuspended in 2 liters of fresh Aquil medium containing the same level of Cd.

While the culture continued growing exponentially, intracellular phytochelatin, dissolved phytochelatin, and total Chl *a* concentrations were measured every 3 or 4 h over 2–3 d. To do this, cells were filtered onto GF/A filters at low vacuum pressure (<34 kPa). The filters were homogenized in 10 mmol L^{-1} methanesulfonic acid (MSA) and centrifuged at 4°C, and the supernatant was retained (Ahner et al. 1995). Both the culture extract and the culture filtrate were prepared for phytochelatin analysis by high-performance liquid chromatography (HPLC) as described below. Chl *a* was measured by dimethyl sulfoxide (DMSO)–acetone–water extraction followed by fluorescent measurement (Shoaf and Lium 1976).

To examine the effects of Cd^{2+} and Zn^{2+} levels on phytochelatin exudation, additional *T. pseudonana* cultures were grown in standard Aquil medium and in Aquil with elevated Cd^{2+} and Zn^{2+} ion concentrations. Additions of equimolar Cd-EDTA and Zn-EDTA were used to increase trace metal concentrations above the standard Aquil levels on the basis of equilibrium calculations made by MINEQL. Free metal ion concentrations were set as follows (expressed as pMe = $-\log[Me^{2+}]$): pCd = 12, 11, 10, and 9 and pZn = 8 and 7 (for total metal additions, *see* details in Wei et al. 2003). The cultures were acclimated to metal conditions for ~20 generations. Near the end of exponential growth in the final batch culture, filtered medium was collected for analysis of dissolved phytochelatin concentration (about 4 d after inoculation).

Phytochelatin removal—Experiments to study phytochelatin removal from seawater were performed by adding synthetic phytochelatin (n = 2, 99% purity, Cell Essentials) to synthetic ocean water (SOW; Price et al. 1988/1989) and aliquots of unfiltered natural seawater collected from Eel Pond, Woods Hole, Massachusetts, Dec 2000 (refrigerated for >1 week). Preliminary experiments showed that exposure to fluorescent light of 20 μmol photons m^{-2} s^{-1} (usual laboratory light) did not affect phytochelatin removal. Therefore, light exposure was not controlled during the experiments. Experiments were performed at room temperature (22 ± 1°C) unless otherwise stated. Assuming first-order kinetics, the removal rate constants were calculated from the decrease of phytochelatin concentration over time.

Microbial acclimation with respect to phytochelatin removal was examined by performing sequential incubations: seawater (Eel Pond water as described above) was incubated with 100 nmol L^{-1} phytochelatin for 2–3 d until phytochelatin was below detection, then an aliquot of the incubated seawater was transferred to a subsequent incubation, to which an additional 100 nmol L^{-1} phytochelatin was added (a dilution factor of ~10 was used for the inoculation). In a control experiment, no phytochelatin was added during the first incubation (but samples were prepared in the same tubes and maintained for the same time period at 22°C), and an aliquot was transferred to a second incubation, to which 100 nmol L^{-1} phytochelatin was added.

The effect of trace metals on phytochelatin removal was examined by adding $CdCl_2$ or $CuSO_4$ or equimolar Cd-EDTA to the incubations, along with the phytochelatin standard and an aliquot of seawater preincubated with phytochelatin. Concentrations of Cd and Cu were 20 times that of phytochelatin (i.e., 2 μmol L^{-1} of $CdCl_2$ or $CuSO_4$) to increase the fraction of phytochelatin bound to a metal ion. In a control incubation, 2 μmol L^{-1} of Cd-EDTA was added.

The effect of temperature on the degradation rate was also examined by performing some experiments at 8 ± 0.5°C compared with those performed at room temperature (22 ± 1°C).

To determine the concentration dependency of removal rates, initial phytochelatin concentrations were varied from 10 to 100 nmol L^{-1}. Additions were made to fresh unfiltered natural seawater that was collected from San Diego Bay, California (March 2002), and sent by overnight mail to Ithaca, New York, and used immediately.

The pH of SOW or natural seawater was not altered by the different treatments described above. Small subsamples (<1 ml) were taken at time intervals that were dependent on the experiment (every 2–4 h for most natural seawater incubations or every 12 h for SOW experiments). Samples were centrifuged at 13,000 × g and 1°C for 10 min in a microcentrifuge (Biofuge Fresco, Heraeus Instruments), and the supernatant was analyzed for phytochelatin concentration.

At the end of some experiments, microbial cell densities in incubation solutions were estimated with viable plate counts. Plates were made from autoclaved seawater containing 1.5% (w/w) agar. Bacterial colonies were examined and counted after 3 to 5 d of incubation in the dark at room temperature. The resulting cell numbers (10^5–10^6 cells ml^{-1}) are approximate because of insufficient replication of plates.

Dissolved phytochelatin in field samples—Samples were collected from the Elizabeth River estuary during May 2000. Small volumes of seawater were filtered through GF/F filters, and the filtrate was frozen immediately and stored in a −20°C freezer. In the laboratory, duplicates of thawed seawater were directly derivatized and analyzed as described in the next section. GF/F filtered seawater samples were also collected from Eel Pond during June 2001 and from the contaminated upstream region of the Elizabeth River estuary

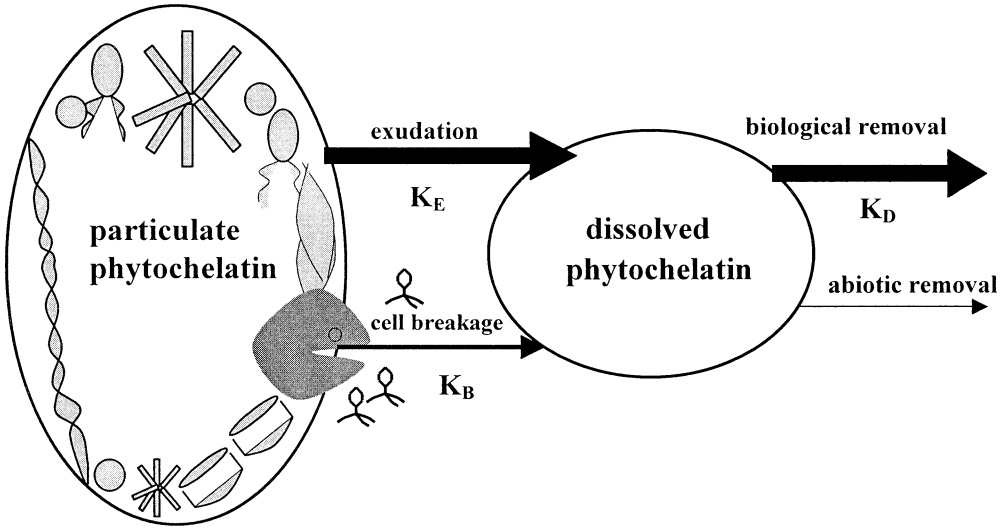

Fig. 1. A model of dissolved phytochelatin in natural seawater. Source terms include active exudation and passive release because of cell lysis or breakage. Dissolved phytochelatin is subject to abiotic and biological removal in natural seawater. Values of rate constants associated with each source and sink are summarized in Table 2. Weight of arrows reflects approximate magnitude of each pathway under moderate metal stress.

during April 2002. Water samples from these two sites were derivatized immediately following GF/F filtration and stored in the freezer before HPLC analysis. Sampling site locations in the Elizabeth River estuary are given in an earlier paper (Wei et al. 2003).

Measurement of phytochelatin with HPLC—Extracted or dissolved thiols were acidified with MSA, adjusted to pH 9.0 with a borate buffer amended with diethylenetriaminepentaacetic acid (DTPA), reduced with dithiothreitol (DTT), and, following a 10-min incubation, derivatized with the fluorescent label monobromobimane (mBBr; Ahner et al. 2002). Derivatized samples, stable for up to 2 weeks, were stored in a refrigerator or freezer until HPLC analyses were performed. Using a Beckman chromatograph equipped with a Supelco Discovery® RP-Amide C16 column (4.6 × 250 mm) and a Gilson 121 filter fluorometer (310–410 nm excitation, 475–650 nm emission), we were able to measure as little as 0.17 ± 0.02 pmol of the phytochelatin–mBBr derivative with a 100-μl injection. The HPLC buffers and elution gradient are described in detail elsewhere (method C, Wei et al. 2003). Use of a small-bore column (2.1 × 250 mm, same material) with a flow rate of 0.4 ml min^{-1} and the same buffers and injection volume permitted the analysis of lower concentrations of dissolved phytochelatin with a detection limit approaching 0.01 pmol (or 0.1 nmol L^{-1}). The elution gradient used with this column was as follows: 2% B (pure acetonitrile) for 8 min, a linear increase to 10% B over 30 min, constant at 10% B for 10 min, a linear increase to 15.5% B over 15 min, a linear increase to 18% B over 5 min, a linear increase to 60% B over 1 min, held at 60% B for 8 min to flush column, a linear decrease to 2% B over 1 min, and a final 7 min at 2% B to re-equilibrate the column (buffer A is 25 mmol L^{-1} KH_2PO_4/H_3PO_4, pH 3.2).

Modeling dissolved phytochelatin in natural seawater—A model incorporating the sources and sinks of dissolved phytochelatin in natural seawater was developed. Possible sources of dissolved phytochelatin include the active exudation from algae at a rate constant dependent on the degree of metal stress and the passive release during cell breakage because of viral lysis or grazing. We assume the dominant sink of dissolved phytochelatin is microbial uptake, degradation, or both in natural seawater. The proposed model of phytochelatin turnover, including fluxes, sources, and sinks, is depicted in Fig. 1. Equation 1 can be used to describe the concentration of dissolved phytochelatin.

$$\frac{dC_d}{dt} = 0.001 \times K_E X + 0.001 \times K_B C_p X - K_D C_d \quad (1)$$

C_d is the dissolved phytochelatin concentration (nmol L^{-1}), t is time (h), X is algal biomass (μg Chl *a* L^{-1}), K_E is the phytochelatin exudation rate constant (μmol h^{-1} g^{-1} Chl *a*), K_B is the rate constant that defines phytochelatin release into seawater because of cell breakage (h^{-1}), and C_p is the particulate phytochelatin concentration (μmol g^{-1} Chl *a*). K_D is the phytochelatin removal rate constant (h^{-1}), which we assume is dominated by biological processes. A unit conversion factor of 0.001 is included where necessary.

If we assume steady state with respect to dissolved phytochelatin (i.e., $dC_d/dt = 0$), then the model can be used to estimate dissolved phytochelatin concentrations under various conditions in the field.

Results

Phytochelatin exudation—Following filtration and resuspension into identical aliquots of fresh sterile Cd-containing growth medium, cultures of *T. pseudonana* continued to

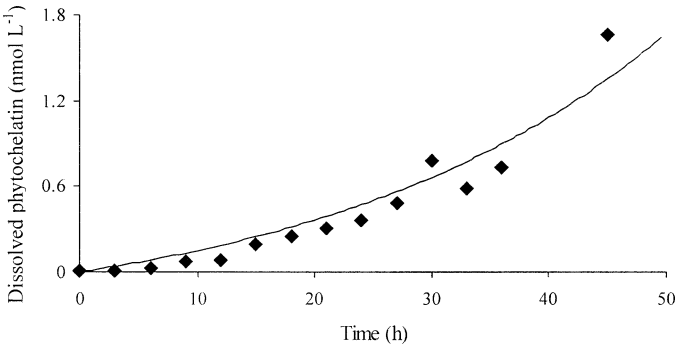

Fig. 2. Phytochelatin concentration (nmol L^{-1}) as a function of time in the growth medium of a *T. pseudonana* culture that has been resuspended in fresh growth medium containing pCd 10. Measured model parameters used to derive the model fit are the specific growth rate, $\mu = 0.034\ h^{-1}$, and the initial biomass, $X_0 = 0.020\ \mu g$ Chl *a* ml^{-1}.

grow exponentially at the same growth rate (data not shown). The intracellular phytochelatin and other major low–molecular mass thiols remained constant during the experiment (data not shown). Dissolved phytochelatin in the culture medium increased from below detection to 1.6 nmol L^{-1} over 45 h (Fig. 2). Glutathione, which is an abundant intracellular low–molecular mass thiol in algae, was also detected in the culture medium. The molar ratio between dissolved glutathione and phytochelatin was significantly different from the intracellular ratio and decreased during the experiment (from ~400 to ~4), supporting our conclusion that, in culture experiments, dissolved phytochelatin is a result primarily of export rather than cell leakage, which would presumably lead to more constant ratios.

Because the cells had been exposed to a constant concentration of Cd for 20–30 generations, we assumed that the exponentially growing cells were exuding phytochelatin at a fixed rate constant, K_E (μmol h^{-1} g^{-1} Chl *a*). Thus, the phytochelatin concentration in the culture medium can be modeled with Eq. 2.

$$\frac{dC_d}{dt} = K_E X \tag{2}$$

Because $X = X_0 e^{\mu t}$, Eq. 3 can be derived with the assumption that at $t = 0$, $C_d = 0$.

$$C_d = \frac{K_E X_0}{\mu}(e^{\mu t} - 1) \tag{3}$$

The measured data fits well with the proposed model ($r = 0.96$, $n = 14$; Fig. 2). The exudation rate constant for *T. pseudonana* growing in medium containing pCd = 10 was determined to be 0.64 μmol h^{-1} g^{-1} Chl *a*. The rate constant derived from a replicate experimental culture was 0.62 μmol h^{-1} g^{-1} Chl *a* (data not shown).

With the above model and single measurements of dissolved phytochelatin near the end of exponential growth, exudation rate constants were estimated for *T. pseudonana* cultures that were growing exponentially at different Cd and Zn concentrations (Table 1). The rate constant estimated from the endpoint measurement of the culture grown at pCd 10 and pZn 10.9 agrees remarkably well with that obtained from the previously described experiment (0.72 μmol h^{-1} g^{-1} Chl *a* vs. an average of 0.63 μmol h^{-1} g^{-1} Chl *a*). From these estimates, it is possible to conclude that exudation rate constants increase with increasing Cd concentrations, and it also appears that increasing Zn increases rate constants at low Cd concentrations. It is notable that although Zn ameliorates intracellular phytochelatin concentrations at pCd 10 by 50% because of an antagonistic effect between Zn and Cd on phytochelatin production (Wei et al. 2003), it does not appear to dampen exudation rate constants at pCd 10. Further experimentation will be necessary to obtain rate constants with enough precision to make more definitive comparisons.

Table 1. Estimated phytochelatin exudation rate constants for *T. pseudonana* grown in medium with a range of Cd and Zn exposure (pMe = $-\log[Me^{2+}]$). For conversions (to μmol h^{-1} $cell^{-1}$), an average cellular content of 0.26 pg Chl *a* $cell^{-1}$ can be used (Ahner et al. 2002). Rate constants are estimated by the model described in the text and a single measurement of dissolved phytochelatin made in a batch culture close to the end of exponential growth.

	Exudation rate constant (μmol h^{-1} g^{-1} Chl *a*)				
	no added Cd	pCd 12	pCd 11	pCd 10	pCd 9
pZn 10.9	0.05	0.04	0.08	0.72	2.7
pZn 8	0.08	0.13	0.14	0.99	3.2
pZn 7	0.13	0.22	0.27	0.75	1.9

Phytochelatin loss from SOW and unfiltered natural seawater—Experiments in which 100 nmol L^{-1} phytochelatin was added to SOW with and without the addition of NaN_3 (1 mmol L^{-1}) or metals were performed. Derived first-order removal rate constants from these experiments were statistically indistinguishable; therefore, the results were averaged to yield a rate constant of $0.0026 \pm 0.0005\ h^{-1}$. This rate constant corresponds to a half-life of 280 ± 45 h ($n = 6$), which represents a quasi-abiotic rate constant because aseptic techniques were not strictly followed during the experiment. When phytochelatin was added to unfiltered natural seawater, removal rate constants increased by nearly one order of magnitude (0.027 h^{-1}, half-lives of 25 h) compared with those measured in SOW. This is likely because of greater microbial activity in natural seawater than in SOW.

Microbial acclimation in phytochelatin removal experiments—The removal of 100 nmol L^{-1} synthetic phytochelatin from unfiltered seawater repeatedly displayed two distinct phases. An initial phase of slow removal was observed from 0 to 40 h, followed by rapid removal from 40 to 65 h (Fig. 3A). During subsequent incubations, into which an aliquot of seawater from the previous removal experiment was transferred, rapid losses were observed immediately (Fig. 3B), and rates were comparable to those measured during the rapid phase of the first incubation. In a control experiment, in which no phytochelatin was added during the first incubation, a low removal rate comparable to that of the slow phase in the initial incubation was observed during the first 40 h (Fig. 3B). This result suggests that the rapid re-

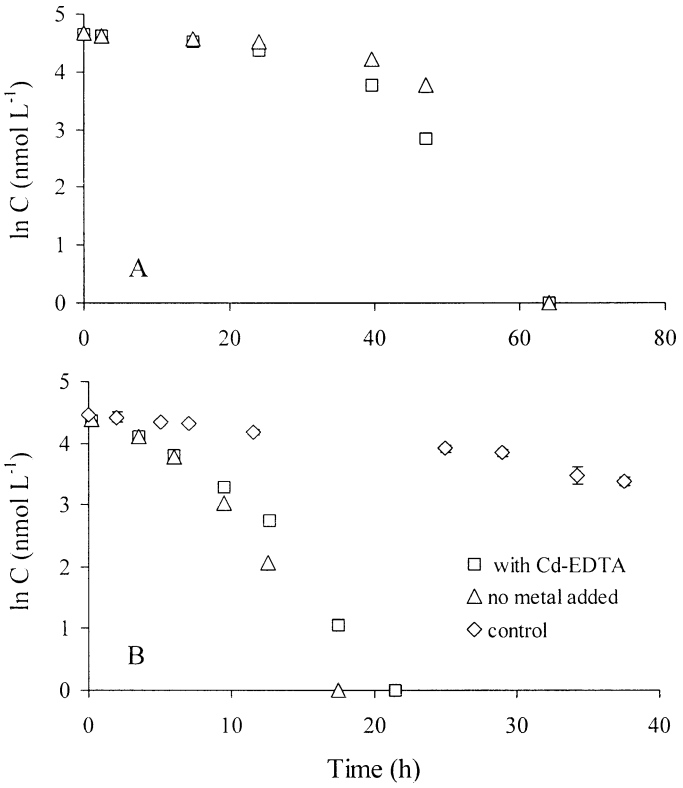

Fig. 3. The natural log of phytochelatin concentration, *C* (nmol L^{-1}), as a function of time following its addition to unfiltered seawater from Eel Pond, Woods Hole, Massachusetts, December 2000, and incubation at 22°C. The first incubation was done with and without Cd-EDTA (A; note that the data points at ~60 h overlap). Subsequent incubations to which an aliquot of the first incubation was added along with phytochelatin, again with and without Cd-EDTA (B). Also shown are control experiments receiving phytochelatin and an aliquot of an incubation of unfiltered seawater at 22°C containing no phytochelatin addition. Error bars shown for the control experiment indicate the range of duplicate incubations.

moval observed in subsequent transfers was the result of microbial acclimation, as opposed to nonspecific enhancement of microbial activity resulting from the 3-d room temperature incubation. Calculated initial removal rate constants during the lag phase were five to six times lower than those in subsequent incubations (I1 and I2, respectively; Fig. 4); calculated half-lives were 26 ± 3 h and 4.6 ± 0.9 h, respectively (assuming first-order kinetics).

Phytochelatin removal in the presence of metals—The addition of $CdCl_2$ or $CuSO_4$ resulted in removal rate constants that were on average about five times lower (Fig. 4). The addition of Cd-EDTA (total 2 μmol L^{-1}, which corresponds to roughly 80 nmol L^{-1} [Cd^{2+}]) did not significantly affect phytochelatin removal (two-sample *t*-test, $p > 0.5$, comparing removal rate constants without metals and those with Cd-EDTA). Thus, we incorporated the rate constant from the Cd-EDTA experiment into our average microbial value to obtain a rate constant of 0.18 ± 0.03 h^{-1} (I2, $n = 4$; Fig. 4) and a corresponding half-life of 4.1 ± 0.9 h. We do not know whether the rate decreases found in the presence of metals

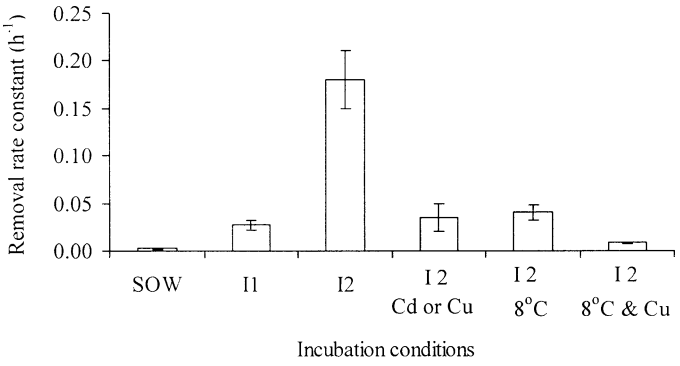

Fig. 4. Removal rate constants of phytochelatin under different experimental conditions with water collected from Eel Pond, Woods Hole, Massachusetts, December 2000. Error bars indicate the standard deviations for independent measurements. I1 and I2 indicate first and subsequent incubations, respectively. Experiments have been grouped as described in the text. The average phytochelatin removal rate constant in SOW is 0.0026 h^{-1} (quasi-abiotic), and those in Eel Pond water are 0.03 and 0.18 h^{-1} for the first and second incubations, respectively. Removal rate constants for second incubations of phytochelatin in Eel Pond water with Cd or Cu addition, or at 8°C are 0.04 h^{-1}, and that at 8°C with Cu addition is 0.008 h^{-1}.

were a result of toxicity of the uncomplexed metals to the microbial community (because we added metals in excess of phytochelatin), decreased degradability of phytochelatin–metal complexes, or a combination of both.

Effect of temperature on phytochelatin removal—Phytochelatin removal rate constants at 8°C were about four times lower than those at 22°C (Fig. 4). At low temperature, the presence of $CuSO_4$ decreased the removal rate constant by about the same factor as it did at room temperature (Fig. 4).

Effect of initial phytochelatin concentration on removal rate—To examine the effect of initial concentration on phytochelatin removal rates, different amounts of phytochelatin were added to unfiltered natural seawater from San Diego Bay. Again, phytochelatin was not degraded rapidly until after a period of microbial acclimation (Fig. 5A). Longer acclimation periods were observed with higher initial phytochelatin concentrations. First-order removal rate constants were estimated from linear regressions through data points following acclimation; they increase with increasing initial concentration of phytochelatin (Fig. 5B). The rate constant calculated for the addition of 100 nmol L^{-1} is fairly similar to that measured in incubations of Eel Pond water (I2; Fig. 4) with the same initial concentration (0.12 h^{-1} and 0.18 h^{-1}, respectively).

An alternative approach to modeling this system is to assume that a small subpopulation of bacteria, able to efficiently degrade phytochelatin, grows to a sufficient density (proportional to the initial phytochelatin concentration) and subsequently consumes the phytochelatin. The growth of the subpopulation can be modeled with Eq. 4.

$$\frac{dX_b}{dt} = \alpha\left(-\frac{dC_d}{dt}\right) \tag{4}$$

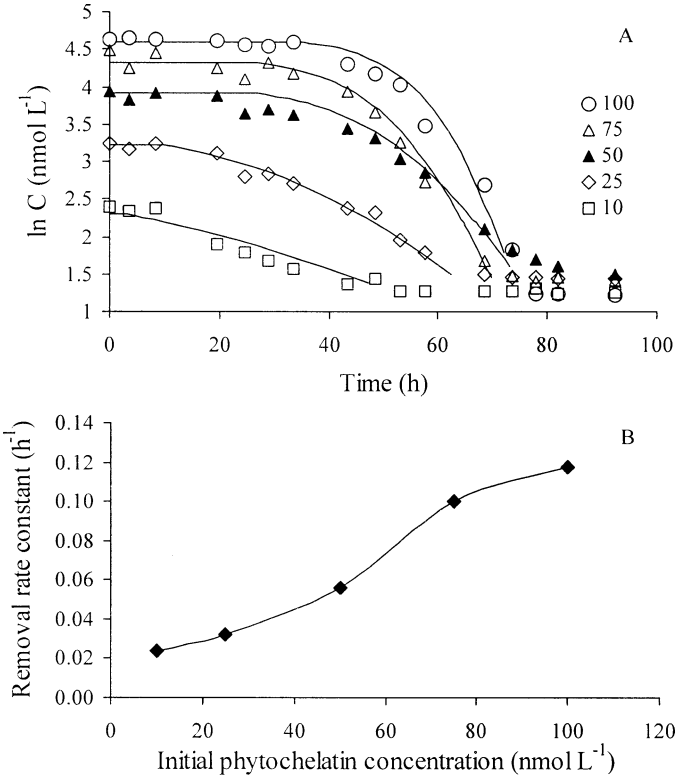

Fig. 5. (A) The natural log of phytochelatin concentration (nmol L^{-1}) over time during a removal experiment in which initial phytochelatin concentrations were varied. The curves show the proposed model (in which a small subpopulation of bacteria, able to efficiently remove phytochelatin, grows to a sufficient density and subsequently consumes the phytochelatin; *see text*) fit to the experimental data. Values of constants used to generate model lines were: $\alpha = 0.6$ cell $pmol^{-1}$, X_b at $t_0 = 5$ cells L^{-1}, $k_s = 110$ nmol L^{-1}, $K = 0.35$ nmol $cell^{-1}$ h^{-1}. Fixed lag times of 34.5, 27, 27, 10, and 3 h were used for modeling loss of phytochelatin for initial concentrations of 100, 75, 50, 25, and 10 nmol L^{-1}, respectively. (B) First-order removal rate constants were also determined for each initial phytochelatin concentration from data points following the initial lag phase, not including data points toward the end of the experiment when rates tapered off. Correlation coefficients (R^2) for the linear regressions through the data sets ranged from 0.97 to 0.999.

X_b is bacterial biomass of the subpopulation that is capable of rapid phytochelatin removal, and α is a constant coefficient. Phytochelatin removal can then be modeled as a function of bacterial biomass and phytochelatin concentration by a Michaelis–Menten relationship, as in Eq. 5.

$$-\frac{dC_d}{dt} = \frac{v_{max} C_d}{C_d + k_s} \quad (5)$$

The k_s is the half-saturation constant, and v_{max} is the maximum removal rate constant dependent on the phytochelatin-degrading bacterial biomass; that is,

$$v_{max} = KX_b \quad (6)$$

where K is the rate constant at which this subpopulation degrades phytochelatin. Solving these differential equations, we arrive at an analytical solution that can be used with the experimental data to estimate values for the various constants (Fig. 5 caption). We get a reasonable fit of the data with this model by incorporating a fixed lag time for each initial phytochelatin concentration (solid lines, Fig. 5A). The model is also consistent with the previously described phenomena of rapid removal in incubations that included an aliquot of seawater from the previous removal experiment.

One feature of the experimental data that we do not capture with the model is the rapid decrease in removal rates as the phytochelatin concentration approaches ~3 nmol L^{-1}. This is likely because the equations do not take into account that there can exist threshold concentrations below which microbes cannot effectively utilize substrate. We hypothesize that at very low concentrations, it becomes energetically unfavorable for microbes to target phytochelatin directly as a substrate and that the slow removal therefore could be because of nonspecific extracellular peptidases. An estimate of this rate constant can be obtained by taking the slope of the last eight data points from the 10 nM addition experiment (0.0024 h^{-1}, $r = 0.60$).

The applicability of this more complex model to natural systems is somewhat limited because phytochelatin concentrations are likely to be very low in surface seawater. Because of this, we have used a simple first-order removal term in our field model. A reasonable range of values for this rate constant (K_D) on the basis of our results is 0.02 h^{-1} (the first-order rate constant observed initially in the 10 nmol L^{-1} addition experiment) to a lower value of 0.0024 h^{-1} (described above), a rate constant that is essentially the same as the ''quasi-abiotic'' rate constant measured in SOW (Fig. 4).

Dissolved phytochelatin in coastal seawater—Dissolved phytochelatin in the Elizabeth River estuary during May 2000 ranged from 1.5 to 3.6 nmol L^{-1}, with an average of 2.0 nmol L^{-1} (Fig. 6A). Stations 6, 7, and 8 were more contaminated with metals than Stas. 3, 4, and 5 (pCd ~10.5, pZn 7, and pCu 12 vs. pCd ~11.6, pZn 10.2, and pCu 13.4, respectively; Wei et al. 2003). Despite very different metal levels, dissolved phytochelatin concentrations were similar in the stations with high and low metals. Station 3, outermost in the estuary, was the only station that deviated significantly from the average concentration. In April 2002, dissolved phytochelatin concentrations showed somewhat less variability from station to station and averaged 3.4 nmol L^{-1} (Fig. 6B). The stations sampled at this time were near Stas. 7 and 8 and therefore were likely contaminated with high concentrations of metals (John Donat pers. comm.). In Eel Pond during June 2001, the dissolved phytochelatin concentration was below detection (~1 nmol L^{-1} as the result of an analytical problem during this particular trip).

Model of dissolved phytochelatin—The proposed model of phytochelatin turnover, including fluxes, sources, and sinks, is depicted in Fig. 1 (with the weight of arrows reflecting rates under conditions of moderate metal stress). Equation 7 can be used to describe the concentration of dissolved phytochelatin.

$$\frac{dC_d}{dt} = 0.001 \times K_E X + 0.001 \times K_B C_p X - K_D C_d \quad (7)$$

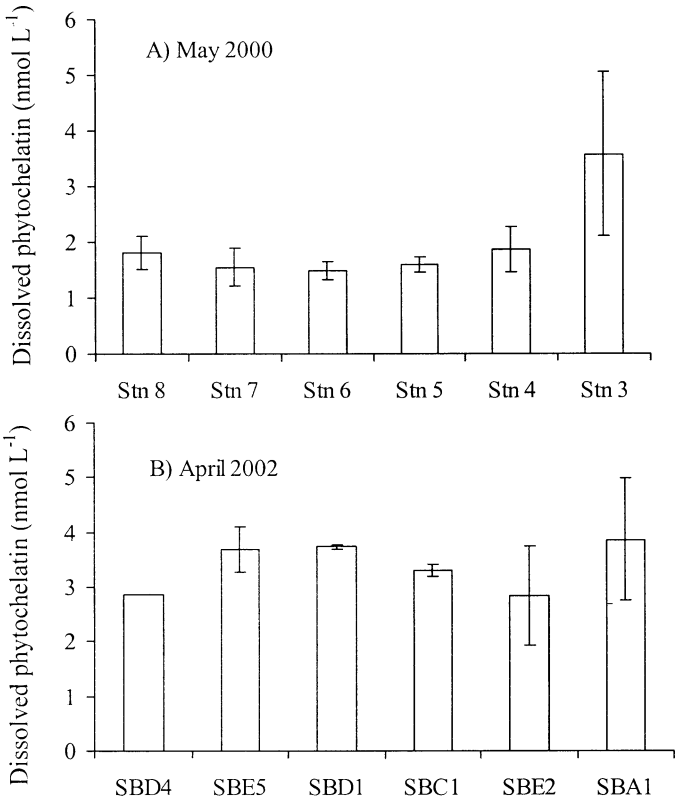

Fig. 6. Dissolved phytochelatin in the Elizabeth River estuary in (A) May 2000 and (B) April 2002. Error bars show the range of duplicate measurements. Sampling site locations are given in an earlier paper (Wei et al. 2003).

K_B is assumed to be equivalent to growth rate (Latasa et al. 1997), and K_D is assumed to be dominated by biological processes. A unit conversion factor of 0.001 is included where necessary. An estimated range of values for each parameter has been derived from published studies or from experiments reported in this paper (Table 2).

Using this model, we calculated steady-state dissolved phytochelatin concentrations in the Elizabeth River estuary and Eel Pond. For the Elizabeth River estuary, K_E values of 0.7 and 0.04 μmol h^{-1} g^{-1} Chl *a* were assigned to stations with high and low metal levels, respectively, with the use of measured free ion concentrations and Table 1. A constant cell breakage rate constant (K_B = 0.025 h^{-1}, an intermediate value from literature data; Table 2) was assumed for all calculations. For the removal rate constant, K_D, we used 0.0024 h^{-1} (the low end of our estimated range). Using the model, we calculate concentrations of 1.6, 2.9, and 2.2 nmol L^{-1} for dissolved phytochelatins at Stas. 8, 7, and 6, respectively, where metal concentrations are high, and 0.2–0.3 nmol L^{-1} at Stas. 5, 4, and 3, where metal concentrations are low. In Eel Pond, the estimated dissolved phytochelatin is ~0.2 nmol L^{-1}.

The calculated values for the high-metal stations are close to field measurements, but those for the low-metal stations in the Elizabeth River estuary are significantly less than measured concentrations, especially Sta. 3, where they differ by a factor of roughly 10. In Eel Pond, with low Cd concentrations, the calculated value is not inconsistent with measurements below detection level.

Discussion

In this study, we measured rates of phytochelatin exudation and removal and formulated a model of dissolved phytochelatin in seawater. The model calculations are consistent with measurements made in a high-metal environment, but not with those made in low-metal environments.

Exudation—We have shown that exudation can be modeled as a first-order process with a Cd-exposed culture of *T. pseudonana.* In addition, we used this model to estimate exudation rate constants for *T. pseudonana* cultures grown at various Cd and Zn concentrations and showed that they are metal dependent. These experiments provide reference values for exudation rate constants, an important parameter in our phytochelatin turnover model, as well as allow an interesting comparison with Cd exudation reported by other research groups.

Phytochelatin is most likely excreted as a phytochelatin–metal complex as part of a metal detoxification mechanism in marine phytoplankton. Lee et al. (1996) first reported phytochelatin and Cd export in Cd-challenged *T. weissflogii,* but insufficient data points were collected to estimate a phytochelatin exudation rate constant. They did report a Cd exudation rate constant of 0.01 h^{-1} or 0.8 μmol h^{-1} g^{-1} Chl *a* (calculated from information provided, and assuming 4 pg Chl *a* cell^{-1} for *T. weissflogii*) for a culture exposed to a free Cd^{2+} ion concentration slightly greater than pCd 10. Thus, the Cd exudation rate constant measured in *T. weissflogii* is very close to the phytochelatin exudation rate constant reported here for the closely related species *T. pseudonana* (0.7 μmol h^{-1} g^{-1} Chl *a* at pCd 10). Sunda and Huntsman (1996) estimated Cd exudation rate constants for *T. pseudonana* exposed to variable concentrations of Cd and Mn. In their study, Cd exudation rate constants were estimated from the difference between net Cd uptake (on the

Table 2. Ranges of the parameters for the model of dissolved phytochelatin in coastal seawater.

Model parameters	Range	References
X (μg Chl *a* L^{-1})	1–20	Ahner et al. 1997; Bruland et al. 2001; Wei et al. 2003
C_p (μmol g^{-1} Chl *a*)	1–25	Ahner et al. 1997; Wei et al. 2003
K_B (h^{-1})	0.0083–0.042	Latasa et al. 1997; Kuipers and Witte 2000; Agusti and Duarte 2002
K_E (μmol h^{-1} g^{-1} Chl *a*)	0.04–3	This study
K_D (h^{-1})	0.0024–0.02	This study

basis of measured cellular Cd:C ratio and specific growth rate) and the predicted Cd uptake (on the basis of a theoretical model of competitive metal uptake kinetics). The estimated Cd exudation rate constants ranged from ~0.1 to ~10 μmol h^{-1} g^{-1} Chl *a* for pCd 10.3 and 8.3, respectively (calculated with data from fig. 14 of Sunda and Huntsman [1996] at pMn 8.3, the Mn^{2+} concentration in Aquil, and the assumption of a C:Chl *a* ratio of 50 for *T. pseudonana*). In our study, the endpoint estimation method resulted in phytochelatin exudation rate constants of 0.08, 0.72, and 2.74 μmol h^{-1} g^{-1} Chl *a* for pCd 11, 10, and 9, respectively (Table 1). Using a very different estimation approach, we have generated phytochelatin exudation rate constants that are entirely consistent with the Cd exudation rate constants estimated by Sunda and Huntsman (1996).

The good agreement between the aforementioned Cd efflux rate constants and phytochelatin efflux, measured and estimated in this paper, further supports a close coupling between phytochelatin and Cd export. However, when cultures are simultaneously exposed to intermediate Cd^{2+} and high Zn^{2+}, an ion that weakly induces phytochelatin synthesis in *T. pseudonana* (Wei et al. 2003) and competes with Cd^{2+} for ion uptake channels (Sunda and Huntsman 1998), the phytochelatin exudation rate constant remains high (Table 1), even though Cd uptake rate, cellular Cd quota and intracellular phytochelatin concentration decrease (Sunda and Huntsman 1998; Wei et al. 2003). It is possible that phytochelatin export is also coupled to Zn export under these conditions. Note that there also appears to be Zn-induced phytochelatin export at low Cd concentration (Table 1), but more work is needed to confirm these results.

Removal—This is the first report to our knowledge of removal rates for a specific thiol or a specific metal-binding ligand in natural seawater. As a peptide, phytochelatin is subject to very slow chemical hydrolysis. A half-life of 350 yr was reported for the chemical hydrolysis of Gly-Gly at neutral pH, low ionic strength, and room temperature (Radzicka and Wolfenden 1996). The comparatively high rate constants (~0.0026 h^{-1}) of phytochelatin removal measured in SOW are therefore likely due to some microbial or enzymatic activity rather than simple chemical hydrolysis. Not only was the initial NaN_3 concentration (1 mmol L^{-1}) relatively low (perhaps too low to inhibit the growth of some bacteria), but NaN_3 is also subject to slow chemical degradation and thus might lose its inhibitory effect on microbial growth over time. Furthermore, aseptic techniques were not used while removing samples during the 2-week-long phytochelatin removal experiment in SOW.

We can also compare our experiments to those of Pantoja et al. (1997), who studied the biologically mediated hydrolysis of fluorescently tagged di-, tri-, and tetrapeptides in natural seawater. They showed that the hydrolysis rate constant for 50–100 nmol L^{-1} of the tetrapeptide in unfiltered seawater was about 0.3 h^{-1} (on the basis of disappearance; Pantoja et al. 1997), which is fairly close to our phytochelatin removal rate constant after microbial acclimation (0.175 h^{-1}). The estimated half-saturation constant for the tetrapeptide is reported to be ~500 nmol L^{-1} and appears to increase with increasing peptide length (Pantoja et al. 1997). In our model of phytochelatin removal, we found that a half-saturation constant of 110 nmol L^{-1} gave a reasonable fit to the data (Fig. 5A).

Our model provides some evidence that the long acclimation phase during phytochelatin removal experiments is due to the growth of a subpopulation of bacteria capable of degrading phytochelatin. This increase would be small compared with the total bacterial biomass (10^5–10^6 cell ml^{-1}) and thus not directly measurable. Our model predictions of how much X_B increases with time and increasing phytochelatin concentrations are consistent with bioenergetic and stoichiometric calculations (data not shown). An alternative explanation for the long acclimation phase is that extracellular metabolism of phytochelatin results in the release of reactive oxygen species, a phenomenon shown to occur during degradation of glutathione (Accaoui et al. 2000), to which cells have to adapt.

Other removal pathways have not been incorporated into our model. These could include oxidation of the metal-binding sulfhydryl group and photochemical degradation. It remains to be determined whether these other mechanisms might be important under some environmental conditions.

Phytochelatin turnover model—Our model of dissolved phytochelatin in seawater serves two purposes. First, it allows us to evaluate how well we understand the fundamental processes governing the sources and sinks of this specific ligand. Second, it serves as a model for future studies of ligand cycling in natural seawater.

One uncertainty in our model is the rate of release of phytochelatin to the dissolved phase during cell breakage. We assume that total phytoplankton biomass is constant by setting cell breakage from grazing, autolysis, and viral or bacterial lysis equal to the overall cell growth rate, which ranges from 0.2 to 1 d^{-1} (Latasa et al. 1997). This assumption is supported by reports of grazing rates that are very similar to algal growth rates in the field (Latasa et al. 1997; Kuipers and Witte 2000), and cell lysis also has been reported to be of a similar magnitude (Agusti and Duarte 2002). It is likely that the percentage of phytochelatin released will depend on which breakage mechanism predominates in a given system, whereas in our model, we have assumed 100% release of the intracellular phytochelatin to the dissolved phase as a result of this turnover. However, errors in this parameter will only have a significant effect on model results when trace metal concentrations are low because direct export generally dominates as the source of dissolved phytochelatin in high-metal environments (>90% in example calculations for all stations in April 2002).

Exudation rates were obtained through laboratory measurements. We assumed first-order kinetics and that extracellular phytochelatin does not function as a feedback signal with respect to cellular phytochelatin synthesis or exudation, although it is possible that this occurs in the field. In addition, we have only measured exudation rates in one algal species and have focused primarily on Cd because it is generally the most potent inducer of phytochelatin (Ahner and Morel 1995; Rauser 1995). Other metals such as Cu might also stimulate phytochelatin export, but on the basis of some studies with *Emiliania huxleyi*, it seems that other thiols

might replace phytochelatin as the ligand involved in export (Dupont et al. 2004).

We also used laboratory experiments to derive phytochelatin removal rate constants in seawater. Using the low end of our estimated range, we calculated phytochelatin concentrations that are reasonably close to measured values in the high-metal region of the Elizabeth River estuary, but calculations underestimate concentrations in the low-metal stations. Are we possibly justified to use a different degradation rate constant in the less contaminated portion of the estuary? Our laboratory results suggest that removal rates are five times slower in the presence of metals, and it is known that ligand–metal complexes are generally less susceptible to degradation than the free ligand (Satroutdinov et al. 2000; Vandevivere et al. 2001). Removal rate constants in this paper were derived from experiments with natural seawater collected from relatively high metal environments (Eel Pond and San Diego Bay); it is possible that higher removal rates would be observed in more pristine seawater, in which there would be less chelation of the phytochelatin with trace metals. Conversely, rates might be lower in the more pristine environments because there might be fewer microbes adapted to degrade phytochelatin. In addition, temperature variations might affect removal rates.

It is also possible that phenomena not accounted for in our model are responsible for the observed differences between calculated and measured values. For example, the difference between measured and modeled values at Stas. 3, 4, and 5 could be because of the advection of water high in phytochelatin out into the estuary. Although trace metal concentrations decrease rapidly because of scavenging by inorganic and biological particles along the estuary, the slowly degraded phytochelatin might persist in the water column.

Our model serves as a suitable starting point for modeling other metal-binding ligands and is, at least in part, consistent with these first reports of dissolved phytochelatin in the field. In some environments, it is likely that phytochelatin constitutes a significant portion of the dissolved thiol pool. Glutathione and cysteine are also released from algae in response to trace metal additions, but at rates that likely differ from those reported here for phytochelatin. In addition, the removal rates of glutathione and cysteine are also likely to be different from that of phytochelatin. Models for other thiols and other ligands will be necessary to evaluate the relative importance of phytochelatin in controlling metal speciation in natural seawater.

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