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Phytochelatin production by marine phytoplankton at low free metal ion concentrations: Laboratory studies and field data from Massachusetts Bay

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ABSTRACT **Phytochelatins are small metal-binding polypeptides synthesized by algae in response to high metal concentrations. Using a very sensitive HPLC method, we have quantified phytochelatins from phytoplankton in laboratory cultures at environmentally relevant metal concentrations and in marine field samples. Intracellular concentrations of phytochelatin, in the diatom *Thalassiosira weissflogii*, exhibit a distinct dose–response relation with free Cd^{2+} concentration in the medium—not with total Cd^{2+}—and are detectable even when the free Cd^{2+} concentration is less than 1 pM. In Massachusetts Bay, phytochelatin levels (normalized to chlorophyll a) in the particulate fraction are similar to those measured in laboratory cultures exposed to picomolar free Cd^{2+} concentrations and exhibit a decreasing seaward trend. Incubations of natural samples with added Cd^{2+} confirmed the induction of the peptides by this metal. Ambient phytochelatin concentrations thus appear to provide a measure of the metal stress resulting from the complex mixture of trace metals and chelators in natural waters.**

First identified in fission yeast (1), phytochelatins have now been found to be ubiquitous in plants, algae, and many fungi (2–5). Like metallothioneins—the primary metal-binding peptides in animals and prokaryotic organisms—they detoxify intracellular metals by binding them through a thiolate coordination. Phytochelatins are enzymatic products (6) with the amino acid composition $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where n = 2–11 (2). The enzyme, phytochelatin synthase, is activated *in vitro* in the presence of metals and deactivated when the metals have been bound by the newly synthesized phytochelatins (6, 7). Phytochelatin synthase adds the γ-Glu-Cys from glutathione, (γ-Glu-Cys)-Gly, to another glutathione (to make n = 2) or to another phytochelatin chain to increase the repeating unit from n to $n+1$. Longer chain lengths bind metals more tightly *in vitro* (7), but their cellular roles are otherwise unknown.

On the basis of the laboratory data, plants growing in areas contaminated by metals are expected to contain phytochelatins. The presence of these peptides may serve as a bioindicator of metal exposure (8). Indeed, two field measurements have documented elevated phytochelatin concentrations in plants growing near mine tailings, areas of extreme metal pollution (9, 10).

Extrapolating laboratory data on phytochelatin production by phytoplankton in culture to natural waters is not straightforward. All laboratory experiments to date have been performed at very high metal concentrations (20–1000 μM) (11, 12). Such concentrations are 10,000 to 100,000 times greater than the total dissolved concentrations of metals in surface seawater [e.g., Cd^{2+} = 2–4 pM and Cu^{2+} = 0.5 nM (13, 14)], including those near sewage outfalls (15). Even control cultures, to which metals are not deliberately added, may contain higher metal concentrations than seawater because of metal impurities in the chemical reagents used to prepare media and in the culture flasks. The problem is further complicated by the presence of unidentified natural chelating agents in coastal (16) and oceanic waters (13, 14, 17, 18), which may dramatically alter the chemical speciation of the metal and hence its biological availability. The biological effects of metals on phytoplankton are known to depend usually on the free rather than on the total metal concentrations (19, 20), but this has not been studied with regard to phytochelatin induction. Thus, the complexity of the metal chemistry in natural waters precludes a simple extrapolation of laboratory data, and direct field measurements are needed to demonstrate the existence of phytochelatin in natural phytoplankton populations.

For this study we designed an analytical measurement technique for phytochelatins to establish the phytochelatin response of phytoplankton in culture at environmentally relevant Cd^{2+} concentrations. We also demonstrate that the response is indeed controlled by the free rather than the total Cd^{2+} concentration. Finally, we present data on phytochelatin concentrations in natural samples from Boston Harbor and Massachusetts Bay that exhibit a systematic, decreasing seaward trend away from metal sources in the harbor.

MATERIALS AND METHODS

For laboratory experiments, the marine diatom *Thalassiosira weissflogii*, clone Actin, was cultured in the chemically defined artificial seawater medium Aquil (21) at several Cd^{2+} concentrations. Medium is treated with Chelex-100 to remove trace metal impurities, and the background total Cd^{2+} concentration is estimated to be <1 nM, equivalent to a free Cd^{2+} concentration of <0.1 pM. Free Cd^{2+} concentrations in the medium were adjusted by equimolar Cd^{2+} EDTA additions as discussed by Price *et al.* (21). The resulting free Cd^{2+} concentrations were calculated with the thermodynamic equilibrium model MINEQL (22). Cells were grown at a constant 20°C with continuous light at 120 $\mu E \cdot m^{-2} \cdot s^{-1}$ in acid-cleaned polycarbonate bottles. Growth was monitored daily with a Coulter Counter, and rates were calculated from the regression lines of logarithmic cell concentration vs. time. Late exponentially growing cells ($\approx 4.0 \times 10^4$ cells per ml; 500 ml) were harvested by gentle filtration (<5 psi; 1 psi = 6.89 kPa) onto Whatman GF/F filters and stored in liquid nitrogen.

Harvested cells were placed directly from storage into 10 mM methanesulfonic acid at 70°C for 2 min to denature proteases. This mixture was then homogenized with a Wheaton Scientific Teflon pestle and glass grinding tube on ice. The homogenate was then centrifuged in a Beckman Mi-

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crofuge E for 10 min. Supernatant was retained for derivatization with the fluorescent tag monobromobimane (23, 24) and separation with ion-pair reverse-phase chromatography. Analyses were performed on a Beckman HPLC equipped with a Gilson 121 fluorometer and an Alltech Associates (2.1 × 250 mm) C_{18} 5-μm reverse-phase column using an acetonitrile gradient (from 18% to 90%) buffered at pH 4.2 by 42 mM acetic acid with 0.64 mM of the ion-pairing reagent tetraoctyl ammonium bromide. The system was optimized for maximum sensitivity by adjusting the particular ion pair employed, the column diameter, and the sample injection loop size (200 μl). The detection limit is ≈0.3 pmol per injection.

For field experiments, samples of surface seawater (see map of Fig. 1 for location) were dispensed into acid-cleaned polycarbonate bottles. The particulate fraction was collected by gentle filtration (<5 psi) onto 25-mm Whatman GF/F filters. Chlorophyll samples were processed as described by Parsons *et al.* (25) and were measured on a Turner Designs (Sunnyvale, CA) fluorometer. Samples for phytochelatin analysis were treated like the laboratory phytoplankton samples, the only difference being the use of an Alltech Associates Adsorbosphere HSC_{18} 3-μm (2.1 × 250 mm) reverse-phase column.

Incubation experiments were performed on water collected from the University of Massachusetts Boston pier (arrow on map of Fig. 1). Water was collected into 10 2-liter acid-cleaned polycarbonate bottles by using an acid-cleaned Teflon line and a battery-operated Masterflex peristaltic pump. Incubations were done in a 10°C refrigerator with attenuated light. Aliquots were sampled from the duplicate bottles at 6, 18, and 42 hr.

RESULTS AND DISCUSSION

Over a range of free Cd^{2+} concentrations, from no added Cd^{2+} to 1 nM, phytochelatins exhibited a distinct dose–response (Fig. 2). Each of the oligomers (n = 2, 3, and 4) increased systematically with increasing metal concentrations, the dimer being dominant in all culture extracts. Internal phytochelatin levels varied by 2 orders of magnitude over the range employed, while the growth rate of the culture remained practically unchanged. It is notable that even with no added Cd^{2+} and at natural (nonpolluted) Cd^{2+} concentrations, $[Cd^{2+}] < 1$ pM (13), phytochelatin was present in significant amounts in the cells, as noted previously by Price and Morel (26). This suggests the possibility that, besides detoxification, phytochelatins may serve in intracellular

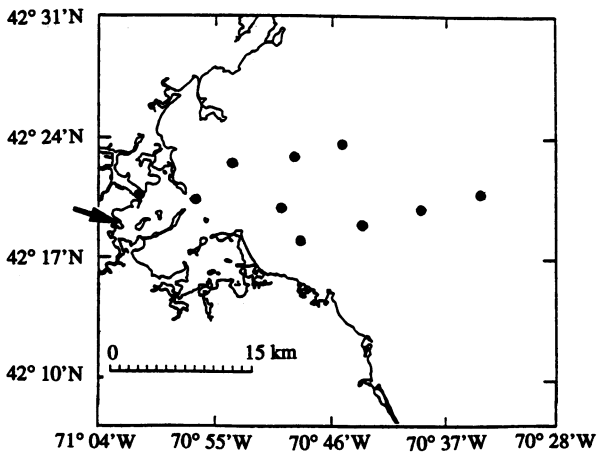

FIG. 1. Map of Boston Harbor and Massachusetts Bay. Sampling stations are indicated by black circles; the arrow shows the sampling location for the incubation experiment.

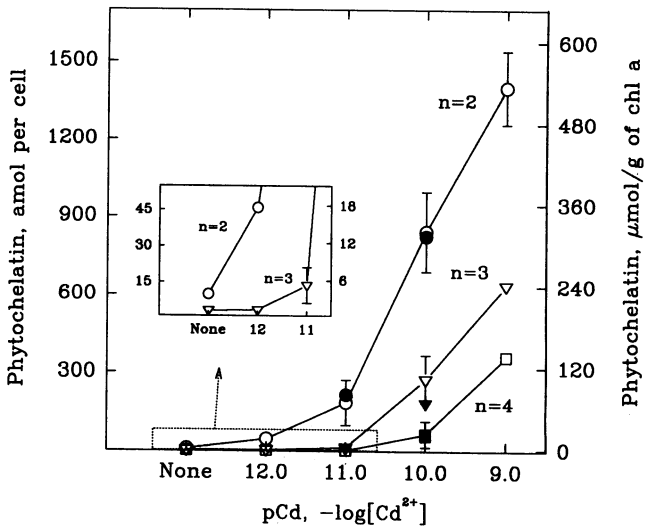

FIG. 2. Phytochelatin concentrations in *T. weissflogii* at various free Cd^{2+} concentrations (pCd = −log $[Cd^{2+}]$); the various chain lengths (n) are labeled: n = 2 (○, ●), n = 3 (▽, ▼), and n = 4 (□, ■). Filled symbols correspond to measurements from cultures in which the total Cd^{2+} concentration was 1 order of magnitude lower and the EDTA concentration was adjusted to obtain the same pCd. Data points representing no added Cd^{2+} and pCd = 12.0 were not duplicated. All other points are the average of duplicates, error bars being within the symbol for several data points. The right vertical axis is calculated by assuming an average of ≈2.6 pg of chlorophyll (chl) a per cell. (*Inset*) Detail of n = 2 and n = 3 values for the three lowest pCd values. Growth rates were 2.3, 2.6, 2.6, 2.6, and 2.3 doublings per day in the order of increasing pCd values.

metal homeostasis as hypothesized by several previous researchers (2, 4, 5). In our experiments so far with other metals, we have found that Cu^{2+}, Pb^{2+}, and Ni^{2+} at free concentrations of 1 nM or less, also induce phytochelatin in *T. weissflogii*, but not to the extent that Cd^{2+} does (unpublished data).

Many studies of the effects of trace metals on phytoplankton have established that the toxicity of a metal is dependent on its free ion concentration rather than its total concentration (19, 20). This is also true of phytochelatin production as demonstrated by the filled data points included in Fig. 2. These data represent cultures that contained 1/10th of the total Cd^{2+} concentration of the others but achieved the same free Cd^{2+} concentration by lowering proportionally the EDTA in the medium. All other total metals were adjusted accordingly to maintain their free ion concentrations constant. The resulting phytochelatin levels are identical to those obtained at higher total Cd^{2+} concentrations but the same free-ion concentrations.

To test the production of phytochelatin in natural phytoplankton populations, we collected samples from Massachusetts Bay in February, April, and June of 1993 (see map Fig. 1). Concentrations of the phytochelatin dimer from ≈2 to 25 μmol/g of chlorophyll a were measured in these samples—values similar to those observed in our laboratory cultures. As might be expected, the highest values were found near the harbor, which receives sewage and riverine inputs, and decreased as we sampled farther from anthropogenic sources of metals (Fig. 3). While they are fairly noisy, the three sets of field data are consistent with each other, and the decreasing seaward trend is unmistakable.

To demonstrate that the elevated phytochelatin concentrations in the natural phytoplankton populations evince exposure to metals, we incubated a water sample from Boston Harbor, presumably rich in uncharacterized metal-binding organic material, with added Cd^{2+}. Addition of 100 nM $CdCl_2$ promoted high, though variable, phytochelatin

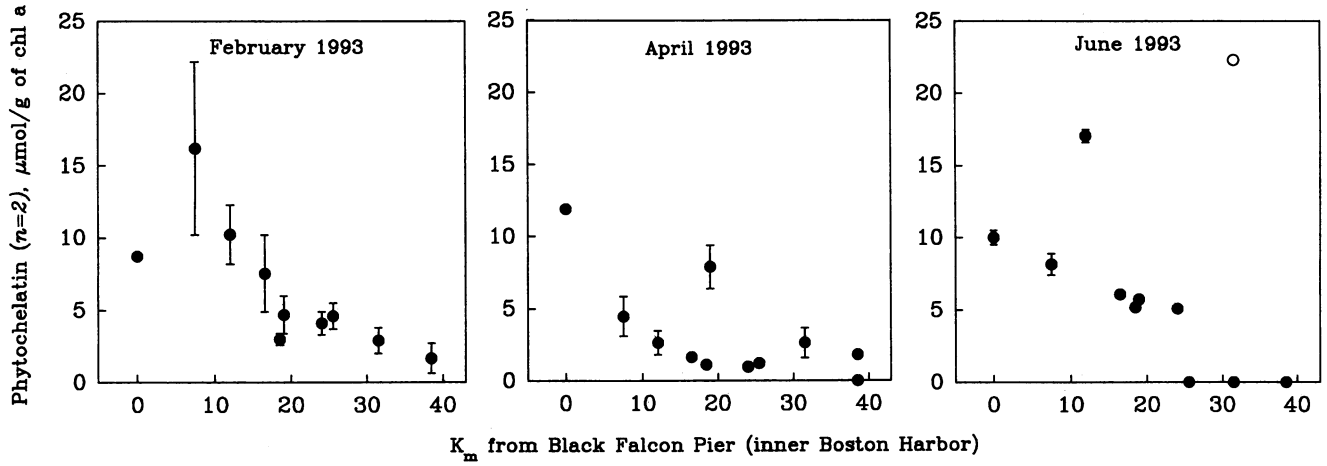

FIG. 3. Phytochelatin (chain length n = 2) concentrations normalized to chlorophyll (chl) a values in particulate samples collected on February 23, April 6, and June 22, 1993, from a seaward transect of Massachusetts Bay plotted as a function of distance from inner Boston Harbor; data points are the average of duplicates and, where not indicated, the error bars are within the symbol. See Fig. 1 for actual location of stations. Points at zero are below the detection limit, and the open circle indicates a sample (the duplicate of which was below detection) in which a low phytochelatin measurement was divided by a very low chlorophyll a value.

concentrations in two separate samples (Fig. 4). A sample amended with 5 nM $CdCl_2$ remained indistinguishable from the control, thus indicating, by comparison to the laboratory data, that <1 pmol of the Cd^{2+} remained uncomplexed. Addition of 100 μM EDTA resulted in a small but perceptible decrease in the algal phytochelatin concentrations (from 1.5 to 0.8 μmol/g of chlorophyll a).

Overall, our laboratory and field data exhibit remarkable consistency and support the notion that phytochelatins may be a good quantitative indicator of metal exposure. The similarity between the values obtained in cultures and in natural samples is almost surprising in view of the crudeness of the normalization to chlorophyll a concentrations. The agreement among the three sets of field data shows that the gradient in intracellular phytochelatin concentrations may be a permanent oceanographic feature in Massachusetts Bay,

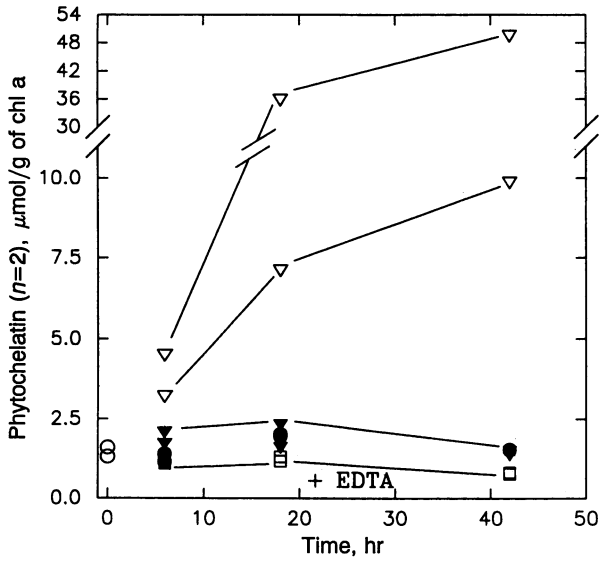

FIG. 4. Phytochelatin (chain length n = 2) concentrations normalized to chlorophyll (chl) a values over time in amended natural seawater samples (duplicate measurements). ○, Initial concentrations; ●, 5 nM $CdCl_2$ addition; ▽, 100 nM $CdCl_2$ addition; ▼, controls; □, 100 μM EDTA addition. No addition was made to the control. Incubation experiments were performed on water collected from the University of Massachusetts Boston pier (arrow on map, Fig. 1) on May 12, 1993.

presumably reflecting a gradient in metal availability. Clearly one expects the position of peak concentrations to be dependent on the tides—which dominate the currents and the mixing regime in the bay and were similar for the three sampling episodes. Because different organisms may respond differently to various metals, it is not possible at this time to say what metals were responsible for the observed phytochelatin gradient. Nonetheless, the similarity in the profiles over the seasons (winter to early summer) when the flora was dominated by different species (27) supports the notion that the phytochelatin response is sufficiently general to provide an overall measure of metal exposure, possibly of exposure to a particular metal such as Cd^{2+}.

Our measurements of phytochelatin concentrations in phytoplankton cultures at background trace-metal concentrations provide a basis to speculate on the extent to which these algal chelators may be a significant source of dissolved complexing agents in seawater. For example, if we consider an open ocean chlorophyll a concentration of 0.1 μg/liter, a phytochelatin concentration of ≈2 μmol/g of chlorophyll a, and a turnover time of the phytoplankton of ≈1 day (assuming all of the phytochelatin to be released to the medium), we can calculate an extracellular turnover time of about 100 days to maintain a concentration of 20 pM in the water column. Phytochelatins, which are subject to oxidation of the thiols and breakdown by proteases, would thus seem unlikely to constitute a major fraction of the mysterious chelators measured by several researchers (13, 14, 17, 18).

While much additional laboratory and field work at relevant metal concentrations remains to be done to elucidate their exact cause, variations in phytochelatin concentration are measurable in field samples and in phytoplankton at environmentally relevant metal concentrations. Thus, phytochelatins appear to provide a measure of the short-term response of the algae to metal stress as imparted by the complex mixture of trace metals and chelating agents in their external milieu. More importantly, perhaps, the study of phytochelatin production *in situ* provides a mechanistic insight into the physiological response of the phytoplankton and may allow us to causally link increases in metal concentrations to their biological and ecological consequences.

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