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Cu and Pb accumulation by the marine diatom *Thalassiosira weissflogii* in the presence of humic acids

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**Environmental context.** Dissolved organic matter protects aquatic microorganisms from toxic metals by complexing and decreasing the concentration of the biologically reactive species such as free metal ions. However, there are some cases of enhancement of toxic effects when humic acids are present, which is thought to be due to effects of adsorbed humic acids on cell membranes. For a marine diatom, humic acids adsorbed to cell surfaces enhanced metal adsorption, whereas intracellular metal contents decreased as a result of metal binding by humic acids. These findings suggest that the diatom wall, the frustule, presents a barrier against direct effects of adsorbed humic acids on the plasma membrane.

**Abstract.** Metal complexation by dissolved organic matter, as humic acids, is considered to decrease metal bioavailability by lowering the free metal ion concentration. However, dissolved organic matter adsorption on cell surfaces can modify cell membrane properties, which can also influence metal uptake. Copper and lead accumulation and internalisation by the marine diatom *Thalassiosira weissflogii* were studied in the absence and presence of humic acids, and adsorption of humic acids to cell surfaces was evaluated. Both Pb and Cu intracellular concentrations decreased in the presence of humic acids according to labile metal concentrations measured by anodic stripping voltammetry, whereas total (intracellular plus adsorbed) metal content was enhanced in the presence of humic acids, probably owing to enhanced metal plus humics adsorption to cell surfaces. The results of the present work stress the importance of differentiating between intracellular and total cellular metal in bioavailability studies, and suggest that the silica frustule of diatoms represents a barrier against humic acids effects on cell membranes.

**Additional keywords:** anodic stripping voltammetry, DOM, metal bioavailability.

**Introduction**

Humic substances are complex mixtures of refractory heterogeneous compounds naturally existing as a result of organic matter degradation. They account for 40–60% of the dissolved organic matter (DOM) in natural waters and for 5–25% of dissolved organic carbon (DOC) in surface oceans and are very commonly used as model DOM compounds in environmental studies.

The most considered property of DOM with regard to metal bioavailability has been its metal-binding capacity, which affects the free-ion concentrations of many metals in aquatic environments. Based on the chemistry of the medium and if several assumptions are fulfilled, it is generally accepted that metal bioavailability depends on the free metal ion activity (‘free ion activity model’, FIAM). The presence of both inorganic and organic ligands determines the proportion of total dissolved metal occurring as free ion. In seawater, though, only DOM and organic colloids control variations in metal speciation, given that the major inorganic composition is practically constant.

It has been shown that DOM can also affect metal bioavailability by direct interaction with cell surfaces. The amphiphilic character of the humic acid molecules confers surfactant properties on them and favours their aggregation in solution and their accumulation on abiotic and biotic surfaces. DOM adsorbed to cell surfaces can modify cell membrane characteristics, as surface charge and membrane permeability. DOM has been shown to enhance sodium uptake by *Daphnia magna* and to enhance Pb bioavailability, and these effects have been attributed to the adsorption of DOM to cell surfaces. An enhanced Pb uptake by freshwater microalgae has been shown to occur in the presence of several DOM types, when compared with the same free Pb concentrations in the absence of DOM. To explain these observations, the possibility of formation of a ternary complex between the Pb, humic substance and algal surface, as well as changes in speciation of metals adsorbed to the cell wall were proposed. An increase in Pb uptake and toxicity to marine invertebrates was observed in the presence of humic acids (HA), when
comparing with the same total dissolved Pb concentrations in the absence of HA. This increased Pb uptake was thought to be due to DOM adsorption to biological surfaces. However, such an increase was not observed for Cu either in freshwater or in seawater.

The humic and fulvic acids used in the cited studies include soil-derived commercially available humic substances and those extracted from freshwater sources. Soil-derived HA present higher hydrophobicity and other distinct properties compared with waterborne HA and their effects on biological membranes have been shown to be higher than that of aquatic DOM.

To help elucidate the potential mechanism by which the commercially available, soil-derived HA enhanced Pb bioavailability to marine organisms, the effect of humic acids on Cu and Pb bioaccumulation is studied in the present work using the marine diatom Thalassiosira weissflogii. Diatoms present a silica wall, the frustule, which is a rigid lidded box composed of amorphous opaline silica with organic coatings, penetrated by pores of various sizes and types, which are thought to function as passageways for entry into or exit from cells of gases, nutrients, or other materials. The frustule could potentially prevent HA from reaching the cell membrane, and therefore the chosen organism, T. weissflogii, may be a suitable biological model to investigate the effects of HA on medium chemistry without the interference of their effects on cell membrane properties caused by potential adsorption on the plasma membrane.

Experimental methods

Cell culture, reagents and exposure

Stock solutions of 1000 mg L\(^{-1}\) of Pb(NO\(_3\))\(_2\) and Cu(NO\(_3\))\(_2\) (Panreac Quimica SA, Barcelona, Spain) were used. Humic acids were obtained from Aldrich (Fluka, Aldrich; Steinheim, Germany). A concentrated stock of 1 g L\(^{-1}\) was prepared by dissolving the acid form of HA in 7 \times 10\(^{-3}\) M NaOH. This stock was sonicated for 30 min and filtered through 0.45-μm pore-size filters. Experimental solutions were prepared in artificial seawater (ASW) with pH 8.0 ± 0.1 and salinity 34.3 ± 0.3 ppt. It was verified that neither HA nor metal additions altered the pH of the solutions.

The diatom Thalassiosira weissflogii (clone ACTIN, strain CCMP 1336) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). T. weissflogii was cultured in Erlenmeyer flasks with modified f/2 medium prepared in 0.22-nm-filtered seawater (FSW). The modification consisted in the non-addition of EDTA. FSW and the stocks for f/2 medium (nutrients, vitamins, trace metals and silica) were sterilised by autoclaving. The culture was sonicated for 30 min and filtered through 0.45-μm filters. Experimental solutions were prepared in artificial seawater (ASW) with pH 8.0 ± 0.1 and salinity 34.3 ± 0.3 ppt.

Anodic stripping voltammetry measurement of Pb complexation by HA

A series of increasing quantities of HA (13 additions ranging from 0.25 to 1 and 2 μM) were added to different sets of total Pb concentrations in ASW. Solutions were prepared in 20-mL polystyrene vials and allowed to equilibrate overnight. Electrochemically labile lead (Pb\(_{\text{L}}\)) was measured by square wave anodic stripping voltammetry (SWASV) with a hanging mercury drop electrode (HMDE), an Ag/AgCl reference electrode and a Pt-rod auxiliary electrode held in a Metrohm 663 VA polarographic stand coupled to an Eco-Chemie AutoLab PGSTAT10 potentiostat (Metrohm, Herisau, Switzerland). Measurements were performed in a Teflon cell thermostatted at 20°C.

The cell was preconditioned with 10 mL of each sample for 5 min, then the solution was discarded and the remaining 10 mL of the sample was used for measurements. After 250 s of purge with N\(_2\), a deposition potential of −0.65 V was applied and Pb was accumulated on a mercury drop of 0.52 mm\(^2\). Deposition time varied depending on Pb concentration: 30 s for solutions containing less than 1 μM of Pb and 10 s for higher Pb concentrations. Solutions were stirred at 3000 rpm during Pb accumulation and an equilibration time of 5 s was used before the voltage scan. For the voltage scanning (from −0.65 to −0.25 V), a square wave with an amplitude of 25 mV, a frequency of 25 Hz and a scan increment of 2 mV were applied. Three voltammograms were recorded for each solution and labile Pb concentrations ([Pb\(_{\text{L}}\)]) were calculated as [Pb\(_{\text{L}}\)] = \(I_\beta/S\), where \(I_\beta\) is the arithmetic mean of the peak current of the three voltammograms and \(S\) is the slope of the calibration curve obtained from the measurement of Pb solutions in ASW in the absence of HA.

Complexation curves were adjusted to a simple complexation model previously described. Assuming one type of ligand and a stoichiometry of 1:1, a simple complexation model is obtained from the equation that describes the equilibrium and the mass balance equations:

\[
[Pb'] = \frac{-a + \sqrt{a^2 + 4} [Pb_{T}] / K'}{2}
\]

where \(a = (-[Pb_T] + L + 1/K')\), Pb\(_{T}\) is the total dissolved Pb concentration, and \(L = [HA] \times N\), K\(_2\) is the conditional stability constant, and \(N\) (μmol Pb g\(^{-1}\) HA) expresses the number of Pb binding sites present per gram of HA.

Eqn 1 was adjusted to the complexation data by least-squares non-linear regression using Sigma-Plot 2002 for Windows (SPSS Inc., Chicago, IL, USA).

In order to study the lability and electroactivity of the Pb–HA complexes, pseudopolarograms were constructed by measuring the Pb accumulated on the mercury drop at different deposition potentials. Four solutions with 1 μM Pb and different HA additions were chosen for the pseudopolarograms. For each of them, 30 voltammograms were recorded at 30 different deposition potentials (varied in small steps from −0.25 to −1 V).
Metal uptake experiments

ASW solutions enriched with Pb and Cu in the absence or presence of increasing additions of HA were prepared in 50-mL polypropylene vials and allowed to equilibrate overnight. Each experimental solution was divided into five 10-mL polypropylene vials. Two of them were used for determination of initial metal concentrations and the rest were used to expose the algae (three replicates per treatment). After 1 h exposure, cell suspensions were isolated by 1-min centrifugation at 3500 g at 25°C (Hettich Rotanta 460R) and washed with ASW. The obtained pellet was used to determine total cellular metal (\(\{\text{M}_{\text{tot}}\}\)). To determine intracellular metal content (\(\{\text{M}_{\text{int}}\}\), cells were additionally washed during 10 min with ‘isotonic washing agent’ (IWA), composed of 23.5 g L\(^{-1}\) NaCl, 0.2 g L\(^{-1}\) KCl, 0.01 M EDTA and NaOH to pH 8. Control vials with experimental solutions in the absence of cells were used to obtain metal background concentrations that were subtracted from the metal content of the pellets.

Adsorbed metal (\(\{\text{M}}_{\text{ads}}\)) was calculated by difference between \(\{\text{M}}_{\text{cell}}\) and \(\{\text{M}}_{\text{int}}\).

Blank vials and reference material (CRM414 – marine plankton) were included within the samples and dried at 60°C during 24 h. Samples were digested with 250 μL of ultrapure HNO\(_3\) and heated at 90°C on a hotplate during 1 h. After digestion, samples were diluted with ultrapure water and analysed by inductively coupled plasma mass spectrometry (ICP-MS; Thermo Elemental, Cheshire, UK). Metal concentrations in the blanks in the different analysis batches ranged between 0.22 and 0.85 nM for Pb and between 2.81 and 4.24 nM for Cu. These values were in all cases less than 10% of metal present in the samples. The mean percentage recovery of the reference material CRM414 (n = 5) was between 95 and 105% in all batches.

Electrochemically labile [Cu\(^{+}\)] and [Pb\(^{+}\)] in the experimental solutions were measured by SWASV. The precise conditions for measurement of [Cu\(^{+}\)] are described elsewhere.\(^{[21]}\) Measured metal concentrations were used for all calculations instead of nominal values, given that there were metal losses due to adsorption to the polypropylene vial walls (Cu and Pb concentrations decreased due to metal adsorption to the vials).

Evaluation of humic acids adsorption to Thalassiosira weissflogii

Samples of ASW with different concentrations of HA (from 2.5 to 30 mg L\(^{-1}\)) were prepared and allowed to equilibrate for 24 h in the dark. Algal suspensions containing 1.5 × 10\(^6\) cells mL\(^{-1}\) were exposed during 1 h to these solutions.

Quantification of humic acids adsorption to Thalassiosira weissflogii was made by determining the difference between the absorbance at 340 nm (\(\text{abs}_{340}\)) of the supernatant after centrifugation for 5 min (3500 g; 25°C) of the samples in the absence and presence of algae. Absorbance was measured using a Beckman DU 640 (Fullerton, CA, USA) spectrophotometer.

Humic acid adsorption to the algae was described by a Henry adsorption isotherm:

\[
\{\text{HA}}_{\text{ads}} = K_{\text{HA}}^{\text{ads}} \times [\text{HA}]_{\text{diss}} 
\]

\(\{\text{HA}}_{\text{ads}}\) being the quantity of HA adsorbed to cells (expressed in mg of HA cell\(^{-1}\)) and \([\text{HA}]_{\text{diss}}\) the concentration of HA remaining in solution after algae exposure, i.e. in equilibrium, expressed in mg L\(^{-1}\).

\(K_{\text{HA}}^{\text{ads}}\) was calculated according to the equation:

\[
K_{\text{HA}}^{\text{ads}} = (m - b)/(b \cdot D) 
\] (3)

where \(m\) is the slope of the linear regression of \(\text{abs}_{340}\) measured in the supernatant of centrifuged control samples (without algae) vs. initial HA concentration (in mg L\(^{-1}\)); \(b\) is the slope of the linear regression of \(\text{abs}_{340}\) of the supernatant of centrifuged samples with algae vs. initial HA concentration; and \(D\) is the algal density in cells L\(^{-1}\).

The error of \(K_{\text{HA}}^{\text{ads}}\) was obtained from propagation of the standard error of the slopes \(m\) and \(b\).

Description of metal accumulation, internalisation and adsorption

Cellular, intracellular and adsorbed Pb were modelled using a hyperbolic equation:

\[
\{\text{Pb}}_{\text{algae}} = \frac{[\text{Pb}]_{\text{max}} 	imes [\text{Pb}]}{K_{\text{M}} + [\text{Pb}]} 
\]

where \([\text{Pb}]_{\text{algae}}\) represents cellular Pb (\([\text{Pb}}_{\text{cell}}\)), intracellular Pb (\([\text{Pb}}_{\text{int}}\)) or adsorbed Pb (\([\text{Pb}}_{\text{ads}}\)) expressed in nmol cell\(^{-1}\); \([\text{Pb}]_{\text{max}}\) is the asymptotic maximum for metal accumulation in the pool considered; \(K_{\text{M}}\) is the apparent half-saturation constant; and [Pb] is the dissolved metal concentration.

In the case of Cu, a trend towards a plateau was not observed in the range of concentrations used, and therefore a linear equation was used for description of \([\text{Cu}}_{\text{cell}}\). \([\text{Cu}}_{\text{int}}\) and \([\text{Cu}}_{\text{ads}}\).

Linear and non-linear fittings were done by least-squares regression analysis using Sigma-Plot 2002 for Windows (SPSS Inc.).

Results and discussion

Pb complexation by HA in ASW

Fig. 1 shows the ASV-[Pb\(^{+}\)] measured at different total Pb concentrations in the presence of increasing HA concentrations. There is a clear decrease in [Pb\(^{+}\)] with HA additions. Data were
fitted to Eqn 1, and complexation parameters (log $K'$ and $N$) were obtained. The parameters $N$ and log $K'$ present a high correlation coefficient, meaning that their values are dependent, and similar fittings could be obtained with higher log $K'$ values together with lower $N$ values. Based on the complexation of Cu and HA,$^{[21]} N$ was constrained to 230 for comparison purposes. The parameters thus estimated with the standard errors from the fittings were: log $K'_{\text{HA}} = 5.65 \pm 0.11$ and $N = 230 \pm 43$. The model explains 99.2% of data variability.

Pb complexation by HA was weaker than that of Cu, given that log $K'_{\text{PbHA}}$ was 5.65 compared with 6.53 obtained for Cu$^{[21]}$ under similar experimental conditions. Cu is in general more strongly complexed by humic substances than Pb, as shown in other studies.$^{[27–29]}$

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The addition of HA caused changes in the voltammetric response of Pb: a decrease in peak height ($I_p$), an increase in peak width, a shift in peak potential ($E_p$) towards more negative values and an increase in the background current (Fig. 2a). The last three processes are normally related to the adsorption of HA on the electrode surface, which can affect the potential and shape of the signal.$^{[30]}$ However, deviations in $E_p$ towards more negative values with increasing concentrations of HA have also been related to the lability of the complexes. Labile complexes, i.e. complexes that dissociate in the diffusion layer during the deposition step, can contribute to the measured signal. In those cases, the decrease in $I_p$ can be attributed to the slower diffusion coefficient of Pb–HA compared with Pb$^2+$.$^{[31,32]}$ The contribution from dissociation of the complexes was kept to a minimum by using the maximum stirring rate (3000 rpm), which results in a very thin diffusion layer. The use of pseudopolarography can differentiate between labile and inert complexes.$^{[32]}$ For labile complexes, the half-wave potential ($E_{1/2}$) should shift towards more negative values with increasing ligand additions.$^{[32,33]}$ The pseudopolarograms showed that the half-wave potential of the curves of solutions with HA did not shift towards more negative values compared with the pseudopolarograms of solutions with only Pb (Fig. 2b), and therefore the possibility of labile Pb–HA complexes contributing to the measured signal seems unlikely under the applied measurement conditions.

**Humic acid adsorption to Thalassiosira weissflogii**

Humic acid adsorption to *Thalassiosira weissflogii* was first evaluated in untreated (not precentrifuged) solutions of HA in ASW. Direct loss of HA during the centrifugation procedure was observed in control samples (Fig. 3), and therefore, HA adsorbed to cells was obtained by the difference between HA in the supernatant of control samples without algae and samples with algae. The $K_{\text{HA}}^{\text{ads}}$ determined according to Eqn 3 was $1.7 \pm 0.3 \times 10^{-17}$ L cm$^{-1}$.

To elucidate if HA adsorbed to algae surfaces was in particulate form, a second experiment was performed, in which solutions of ASW with HA were precentrifuged 5 min at 3500 g at 25°C to remove large HA aggregates. Algae were exposed to the supernatants, and the measured $K_{\text{HA}}^{\text{ads}}$ was equal to that measured in the untreated HA solutions, $1.7 \pm 0.1 \times 10^{-17}$ L cm$^{-1}$. Therefore, it was concluded that HA adsorption to *Thalassiosira weissflogii* is comparable in the presence or absence of large HA aggregates in solution.

In order to compare them with values reported by other authors with other microorganisms, determined $K_{\text{HA}}^{\text{ads}}$ were transformed into Henry adsorption constants ($K_{\text{H}}$), expressed as HA adsorbed per algal surface unit. The *Thalassiosira weissflogii* cell was considered as a cylinder of 17-μm height and 11-μm diameter and the calculated $K_{\text{H}}$ was $2.1 \pm 0.3 \times 10^{-2}$ cm. To our knowledge,

![Fig. 2.](image)

**Fig. 2.** (a) Effect of increasing additions of humic acids (HA) on the voltammogram of 1 μM Pb in artificial seawater. (b) Pseudopolarograms (i.e. plots of peak intensity, $I_p$, as a function of deposition potential, $E_{1/2}$) of 1 μM Pb in artificial seawater with increasing additions of HA.

![Fig. 3.](image)

**Fig. 3.** Humic acid (HA) concentration determined by measurement of absorbance at 340 nm in: the supernatant of centrifuged solutions of HA in artificial seawater (ASW) in the absence (○), and presence (▲) of $1.5 \times 10^6$ cells mL$^{-1}$ of *Thalassiosira weissflogii* exposed for 1 h. Centrifugation was applied for 5 min at 3500 g at 25°C. The straight line represents the calibration of non-centrifuged solutions.
this is the first paper evaluating and quantifying DOM adsorption to marine phytoplankton cells; therefore, we can only carry out comparisons with published values for freshwater species. Different $K_H$ values have been reported in the literature for humic and fulvic acids, varying from one species to another, and as a function of pH. Slaveykova and coworkers have reported $K_H$ values of $9 \times 10^{-3}$ cm for humic and fulvic acids adsorption to *Chlorella kessleri* at pH 6,\cite{11,20,34} approximately half of those obtained for *T. weissflogii* in the present study.\cite{10} Knauer and Buffle\cite{10} reported much higher adsorption at low pH (pH 4–5), reaching $K_H$ values as high as 0.3 cm for *Chlamydomonas reinhardtii*.

It has been shown for freshwater that DOM adsorption to cell surfaces was increased at low pH values.\cite{8,10,14} Not only adsorption, but also aggregation of HA is enhanced at low pH, or in the presence of divalent cations.\cite{35–37} We have previously found that HA aggregation and retention in organic filters is enhanced in seawater in comparison with ultrapure water (P. Sánchez-Marín, V. I. Slaveykova and R. Beiras, unpubl. data). It should be expected also, as occurs at low pH in freshwater, that the quantity of HA adsorbed to phytoplankton could also be higher in seawater than in freshwater at neutral pH. However, DOM adsorption may also be very influenced by cell-wall characteristics and can vary in magnitude from one species to another,\cite{10} and much more data are needed to verify if DOM adsorption to cellular surfaces is different from seawater to freshwater systems. Note too, that the calculated surface area of the frustule may have been underestimated, given its complex morphology, presenting pores and spines.\cite{38}

The fact that HA adsorbs to *Thalassiosira weissflogii* cells suggest that the adsorbed DOM may well affect cell-membrane properties and metal bioavailability, as previously shown for freshwater organisms.\cite{19–19} As well, this observation can have important implications for organic carbon and trace metal cycling in the oceans.

**Pb and Cu accumulation by *Thalassiosira weissflogii* in the absence of HA**

Total, adsorbed and intracellular metal determined in the algae after 1 h exposure to different concentrations of Pb or Cu are presented in Fig. 4 and Fig. 5 (black symbols). For Pb, hyperbolic curves fitted the uptake data better than linear models within the range of concentrations tested.

Metal adsorption to cell walls is expected to be a fast process, occurring in the first minutes of exposure and rapidly attaining equilibrium, whereas internalisation is a slower process that normally follows a linear increase with time until it reaches steady state. Therefore, the ratio $[M]_{\text{ads}}/[M]_{\text{int}}$ will vary as a function of exposure time. For the 1-h exposure, the majority of the metal associated with algae was adsorbed (IWA-extracted), and only...
The intracellular Cu content was 1.6 \times 10^{-7} \text{nmol cell}^{-1} whereas intracellular Pb was 1.5 \times 10^{-7} \text{nmol cell}^{-1}. The quantity of adsorbed metal at the same dissolved metal concentration was 9 \times 10^{-7} \text{nmol cell}^{-1} for Cu and 1.4 \times 10^{-6} \text{nmol cell}^{-1} for Pb. Therefore both metals seem to have very similar internalisation rates for this alga, although Pb adsorption to cell surfaces increases with the amount of metal–HA complexes bound to the cell surface. Thus, it can be expected that the quantity of metal adsorbed to surfaces will increase with the amount of metal–HA complexes bound to cells. Based on the complexation curves obtained by ASV, and on mass balance equations, the quantity of metal that will...
Fig. 6. Adsorbed (cellular minus intracellular) metal on *Thalassiosira weissflogii* cells exposed for 1 h to different concentrations of Pb and Pb + humic acids (HA) (a); or Cu and Cu + HA (b). White circles represent predictions based on labile Pb concentrations according to the model obtained in the absence of HA. Black circles represent predictions according to the hyperbolic equation:

\[
\{M\}_{ads}^{M,HA} = \left\{M\right\}_{ads}^{M} + K_{ads} \cdot [HA]_{diss} \cdot P
\]

where \(\{M\}_{ads}^{M,HA}\) was calculated by substituting measured [Pb'] and [Cu'] in the hyperbolic and linear equations describing adsorption in the absence of HA for Pb and Cu respectively; and \(P\) is the ratio of labile Pb concentrations in the bulk solution and at the cell surface. In that case, the value of \(P\) (estimated from complexation measurements in the bulk solution) would be different at the frustule, and metal adsorption calculated according to Eqn 5 would underestimate metal adsorption.

Conclusions

HA shows the ability to complex dissolved Pb and Cu in seawater, and to adsorb to the surface of microalgae. In a diatom, where a complex cell wall (the frustule) is present outside the plasma membrane, adsorption of HA influences the accumulation of metal outside the cell, adsorbed to its surface, but it does not affect intracellular metal concentrations, which can be successfully predicted by anodic stripping voltammetry measurements of [Cu'] and [Pb'] according to equilibrium-based bioavailability models.
Although previous research results indicated that surface HA adsorption increased Pb internalisation, in diatoms, the frustule might be preventing such an effect.

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