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Influence of the nature of the exudates released by different marine algae on the growth, trace metal uptake and exudation of *Emiliania huxleyi* in natural seawater

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Abstract

Marine phytoplankton are known to release metal complexing ligands but little is known about the effect of exudates on the biological behaviour of the different microorganisms, including their toxicity and influence on trace metal availability. In this study, cultures of *Emiliania huxleyi* grown in filtered seawater, enriched with nitrate and phosphate as well as its own exudates and those of *Phaeodactylum tricornutum*, *Porphyra* spp. and *Enteromorpha* spp., were used to investigate the effects of algal exudates on algal growth, uptake (extracellular adsorption plus intracellular uptake) of Cu, Pb, Cd, Zn, Fe, Mn, Ni and Co, and extent of exudation. Cathodic and anodic stripping voltammetry (CSV and ASV) were used to determine metals, both in the medium and taken up by the algae, and total complexing organic ligands in the medium. Among these ligands, thiol compounds (cysteine-like and glutathione-like) were quantified in the exudates of different origins and during the growth of *E. huxleyi* in media enriched with them. An improvement of the final cell yield of *E. huxleyi* was caused by the addition of *Enteromorpha* exudates (the richest in glutathione-like compounds), and growth inhibition (a decrease of final cell yield and growth rate) was caused by the addition of *P. tricornutum* exudates (the richest in cysteine-like compounds). The nature and concentration of the organic compounds present in the culture medium also influenced trace metal uptake and the concentration and composition of the exudates produced by *E. huxleyi*. Therefore, it can be speculated that a bloom of a species of algae that produces large amounts of specific exudates may favour or inhibit the local growth of other algal species and, in an extreme situation, change the biodiversity. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Cathodic stripping voltammetry; Anodic stripping voltammetry; Organic ligands concentration; Thiol compounds concentration; Trace metal speciation

1. Introduction

Phytoplankton affect trace metal chemistry in natural waters by surface reactions, by taking up the metal directly, and by production of extracellular organic matter with metal complexing properties. Complexation of trace metals with organic ligands often ameliorates trace metal toxicity and influence biogeochemistry of these elements in aquatic environments (Moffett et al., 1990). Therefore, the influence of actively-released organic ligands on metal bioavailability and toxicity to algae and metal speciation in natural waters is of great interest.

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While a number of studies have illustrated that many trace metals are strongly complexed by organic ligands, very little is known about the source and chemical characteristics of the organic ligands present at low (nM) concentrations in oceanic water. Most of the chemical studies using in vitro cultures have been carried out in synthetic or natural seawater supplemented with trace metal buffer systems (EDTA and NTA), where the natural organic ligands (including those released during growth) have been ignored (e.g., Lee et al., 1995; Sunda and Huntsman, 1996, 1998). However, organic ligands naturally present in oceanic water can also control the speciation of some trace metals, particularly Cu (Buckley and van den Berg, 1986; van den Berg, 1985; Capodaglio et al., 1990; Bruland, 1992).

For example, anionic polysaccharides that can bind metal ions are released by *Chlorella* species (Kaplan et al., 1987). Carbohydrates represent a significant fraction (15–30%) of marine DOC and are released into seawater in large amounts by phytoplankton, including *Emiliania huxleyi* (Biersmith and Benner, 1998). The charged structure and relative lipophilicity of organically complexed metals and organic contaminants may alter rates of transport across the lipid membranes of cells (Simkiss and Taylor, 1989). Short-term uptake experiments using the coastal diatom *Thalassiosira weissflogii* demonstrated that low molecular weight, lipophilic, organic Cu, Cd and Pb complexes enter the cell by diffusion across the plasma membrane (Phinney and Bruland, 1994). Biogenically produced organic compounds contain both hydrophilic and hydrophobic groups and trace metals may be bound to functional groups associated with the hydrophobic "backbone" of the organic macromolecules. This makes it difficult to predict the bioavailability of organically complexed metals to marine organisms and their subsequent behaviour once they have been taken up (Carvalho et al., 1999). In the last few years, increasing evidence has been found that, besides free metal ions and lipid soluble metal complexes (Croot et al., 1999), the labile fraction of metal-complexes in the diffusion layer of the organisms is also bioavailable (e.g., Sunda, 1989; Hering and Morel, 1990; Tubbing et al., 1994; Azenha et al., 1995). Literature about toxic or nutritional properties of exudates of a specific algal species to other species is practically absent.

The goal of this work was to assess whether exudates produced by different eukaryotic algae influence the biological behaviour of a particular algal species. *E. huxleyi* was selected for this purpose because it is by far the most abundant of the coccolithophores on a global basis and is extremely widespread, occurring in all except the polar oceans. The study was carried out in natural coastal seawater (in which the background levels of organic ligands and different trace metals had been previously determined). This seawater was enriched with nitrate and phosphate, without added chelators, metals or vitamins to minimise any changes in the natural speciation (Leal et al., 1999). As sources of exudates, we choose four algal species: the microalgae *E. huxleyi* (a coccolithophore) and *Phaeodactylum tricornutum* (a diatom) and the macroalgae *Porphyra* spp. (red algae) and *Enteromorpha* spp. (green algae). All are abundant in the marine environment. Diatoms occupy a variety of habitats and are often the most abundant photosynthetic organisms in marine waters. *Porphyra* and *Enteromorpha* macroalgae were chosen because they are very abundant on the Portuguese Coast. They also have a worldwide distribution, being found in the Antarctic, temperate and tropical waters. We quantified the organic ligands as a whole and speciated different thiols (by CSV) in the natural seawater and that enriched with exudates released by the different algae. Based on the composition of the media, speciation calculations of some metals were carried out as the chemical forms of the elements affects the response of the algae (Karman and Jak, 1997). *E. huxleyi* growth, trace metal uptake (extracellular adsorption plus intracellular uptake) and production of exudates were monitored in the seawater cultures enriched with exudates from the different sources. As far as we know, this is the first paper on the influence of exudates produced by different eukaryotic algae on the growth of a particular algal species. This subject is important because the algal growth response in the presence of exudates may be directly related to the exudates and/or to the chemical conditions resulting in the presence of those exudates. Recent studies have demonstrated that the compounds released by phytoplankton have strong capability to bind trace metals, thus changing their speciation, bioavailability and toxicity. However, information about the direct effects (e.g., growth stimulation or inhibition) of organic

compounds liberated by an algal species is very scarce.

2. Materials and methods

The experimental design is summarised in Fig. 1 and more details are given in the following sections.

2.1. Reagents

The water used for reagent preparation and rinsing was deionised with a conductivity of < 0.1 μS cm^{-1}. Reagents were AnalaR-grade unless indicated otherwise. The metal standard solutions used in the voltammetric determinations were prepared by dilution of the atomic absorption spectrometry standard solutions (BDH, Spectrosol grade) with 0.01 M HCl. Salicylaldoxime (SA, 0.01 M; Aldrich) was prepared monthly in 0.1 M HCl. 8-Hydroxyquinoline (oxine, 0.1 M; Merck) was prepared in 0.15 M HCl; a solution of 0.01 M oxine was prepared monthly by dilution. Dimethylglyoxime (DMG, 0.1 M; Sigma) was prepared in NaOH. Catechol (0.1 M; Sigma) was prepared monthly in water. Ammonium pyrrolidinedithiocarbamate (APDC, 0.025 M; Sigma) was prepared in water. Working standards of cysteine, thioacetamide (both from Sigma) and glutathione (from Aldrich) were prepared daily from 0.1-M stock solutions that were prepared weekly. One molar boric acid (Aldrich) and 0.35 M ammonia (borate buffer) were used in the voltammetric determinations; 100 μl of this buffer in 10 ml seawater (0.01 M) gave a pH of 8.3 (NBS pH scale). One molar piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES, Sigma) was also used in the voltammetric determinations. One-hundred microliters of this buffer in 10 ml seawater (0.01 M) gave a pH of 6.9 (NBS pH scale). All the metal, ligands and buffer solutions were stored at 4 °C.

The natural seawater used in this study originated from the sea surface, 150 m from the coast at Póvoa do Varzim (Northwestern Portugal). The seawater was pumped continuously from the ocean through a PVC pipe at a rate of 100 l s^{-1} and collected at the end of the discharge pipe directly to cleaned 25-l HDPE containers. In the laboratory, the seawater (35 ‰ salinity, pH 8.0) was immediately filtered (0.1 μm polycarbonate membrane, Millipore) and stored in 50-l HDPE containers. Seawater was subsampled in 500-ml HDPE bottles, stored in the dark at 4 °C and used for experiments within 1 week. The seawater used in the two sets of experiments (I and II) was collected at different times from the same site.

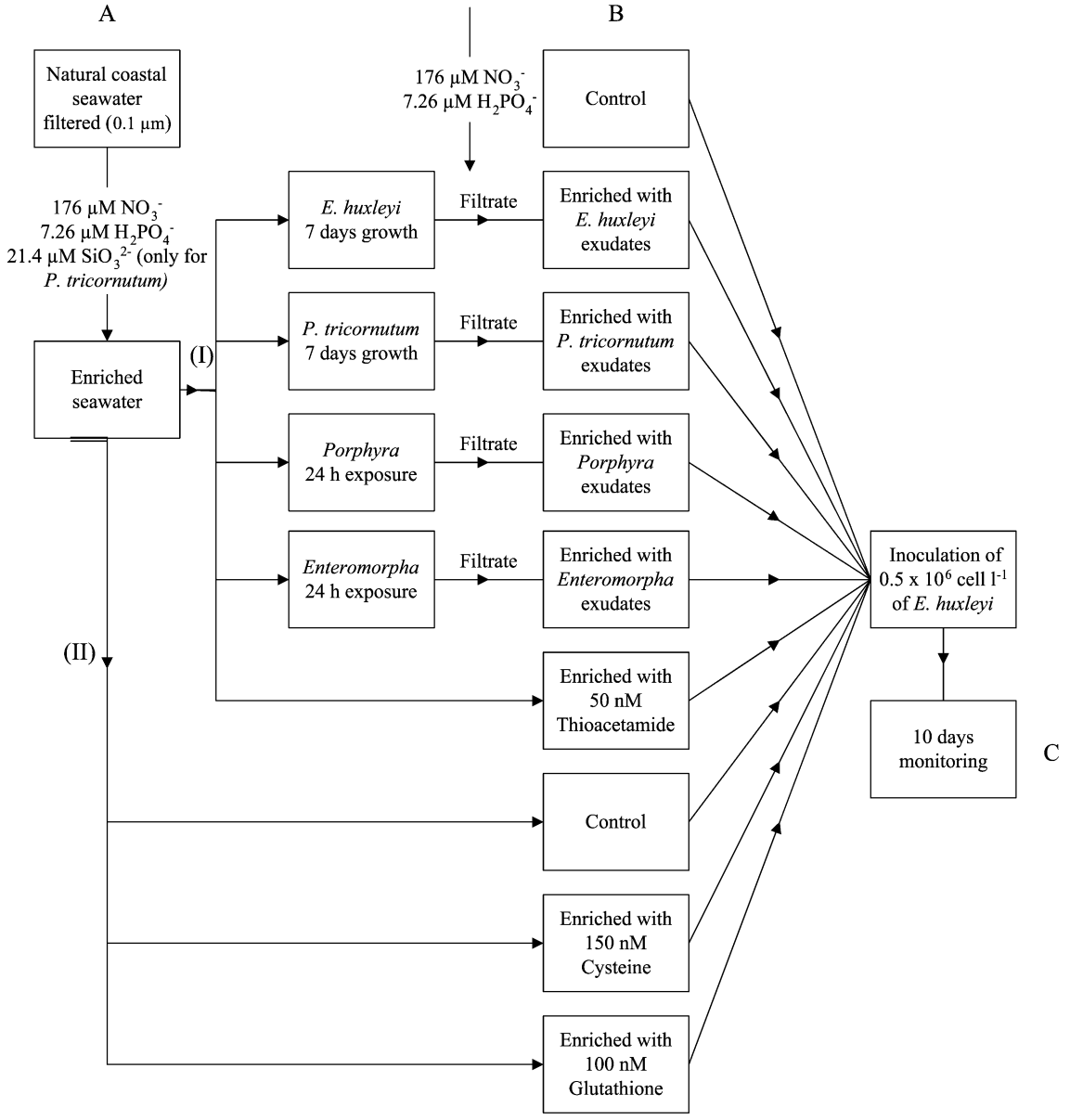
2.2. Cleaning of materials and culture media

All materials, including filters, erlenmeyers, polycarbonate bottles and plastics (pipette tips, filtration system, etc.) were acid-cleaned and microwave-sterilised as described before (Leal et al., 1999). The culture media were sterilised by filtration (0.1-μm pore size filter). All sample manipulations were carried out using gloves, in a Class 100 laminar flow hood in a clean room with HEPA filtered air. The absence of bacteria in the cultures was tested before and after experiments I and II using standard bacteriological nonselective growth medium. Bacteria were not observed during the entire growth period. In addition, bacteria were not observed by using a microscope with an oil immersion lens.

2.3. Cultures of algae

Seawater enriched with *E. huxleyi* or *P. tricornutum* exudates was obtained by growing up the respective algae for 7 days. In each case, 1 l of sterilised seawater (filtered through a 0.1-μm pore size filter) was supplemented only with 176 μM nitrate (from sodium nitrate, Merck), 7.26 μM phosphate (from sodium dihydrogenophosphate, Merck) and 21.4 μM silicate (from sodium silicate, Merck) (only in the case of *P. tricornutum*). Then, the seawater was inoculated, in a polycarbonate bottle, with 1 ml of an f/2 (Stein, 1973) stock unialgal, axenic, *E. huxleyi* (Lohmann PCC Nos. 92 and 92d, isolated by Hay and Muller, Plymouth Marine Laboratory) or *P. tricornutum* (from the same origin) culture on day 7 of growth (exponential phase). The initial concentration of algae was approximately 0.5×10^6 cells l^{-1} (*E. huxleyi*) or 1.0×10^6 cells l^{-1} (*P. tricornutum*). The cultures were incubated under continuous (24 h) illumination at 18 °C for 7 days.

Seawater enriched with *Porphyra* spp. or *Enteromorpha* spp. exudates was obtained by submerging a fresh sample of each macroalgae (20 g wet weight of algae per litre of sterilised seawater (filtered through a 0.1-μm pore size filter)) in a polycarbonate bottle for 24 h (enough time for the liberation of a significant



Determined parameters:

A - pH, salinity, total dissolved concentration of Cu, Pb, Cd, Zn, Fe, Mn, Ni, Co, Hg and organic ligands.
B - Total dissolved concentration of Cu, Pb, Cd, Zn, Fe, Mn, Ni, Co, organic ligands as a whole and only those that behaved like cysteine and glutathione in cathodic stripping voltammetry (CSV).
C - After 2, 4, 7 and 10 days of growth: cells number, total dissolved and total cellular concentration of Cu, Pb, Cd, Zn, Fe, Mn, Ni, Co, organic ligands as a whole and those that behaved like cysteine and glutathione in CSV.

Fig. 1. Experimental design of the present study. Two sets of experiments, (I) and (II), were carried out using natural seawater with different concentrations of trace species.

amount of exudate but insufficient for the degradation of the macroalgae) (Vasconcelos and Leal, 2001a) under continuous (24 h) illumination at 18 °C. The intertidal benthic macroalgae used in this study were collected at low tide at Matosinhos beach (northwestern Portugal). The macroalgae were on the surface of the beach rocks and were collected by hand, randomly placed in acid-cleaned plastic bags and transported to the laboratory in seawater collected from the beach. Upon arrival at the laboratory, the algae were washed twice with 3.5% (w/v) sterilised NaCl (filtered through a 0.1-μm pore size filter and of concentration identical to that of the natural environment to avoid osmotic crash).

In all cases, the algae (micro- or macroalgae) were removed by filtration through 0.45 μm cellulose nitrate Millipore filters. The medium was characterised in terms of the concentration of total dissolved metals, total organic ligands and specific thiols (cysteine-like and glutathione-like compounds, which were the only identified). After addition of an extra 176 μM nitrate and 7.26 μM phosphate and sterilisation by filtration, 0.1-μm pore size filter, each of the mentioned media were used to grow up *E. huxleyi* for 10 days at 18 °C under continuous (24 h) illumination. A continuous light source does not mimic the natural conditions where these algae would grow in normally. Nevertheless, it has been observed that the cultures are able to display exponential growth and all incubations were maintained in identical conditions to facilitate comparisons. The initial concentration of algae was approximately 0.5×10^6 cells l^{-1}. *E. huxleyi* was also grown in natural seawater enriched only with 176 μM nitrate and 7.26 μM phosphate (control medium). Cell numbers were counted (Stein, 1973) using a microscope (Nikon, Eclipse E400). The inoculation with the microalgae in the media caused addition of 1000-fold diluted quantities of EDTA, Fe, Mn and Co from the f/2 medium used for the stock cultures. The stability constant for complexation of Cu by EDTA (log $K'_{CuEDTA} = 10.1$) is much less than that for the natural ligands (L) in the cultures (log $K'_{CuL} = \sim 12$), so the calculated percentage of Cu complexed with EDTA (12 nM final concentration) was $\leq 0.3\%$ and, therefore, insignificant. The stability constant for reaction of EDTA with Pb, Cd and Zn is lower than that for Cu, so very little complexation is expected of these metals at the low residual EDTA concentration. During algal growth (at day 2 and at day 7), partial replacement of Cu was carried out.

For comparison purposes, the set of experiments were repeated in culture media where synthetic thioacetamide (50 nM), cysteine (150 nM) or glutathione (100 nM) were added instead of natural exudates (see Fig. 1).

All experiments were carried out in triplicate.

2.4. Determination of the total trace metal contents in the seawater and algae

The total dissolved concentration ($[M]_d$) of the different metals was determined in the seawater and in the culture media after removing the algae by filtration (0.45-μm pore size filter) in a vacuum filtration system (Millipore) at 0.6 bar. Microscopic examination of the algae on the filter revealed that they did not suffer from rupture or break during filtration. Aliquots of the media were acidified to pH 2.2 and were UV-digested (Ultramed 1000-W mercury vapour lamp, from Osram) in PTFE-capped quartz silica tubes for 1 h. Acidification at a lower pH value (pH 1.5) and/or higher UV-irradiation time resulted in statistically identical results. Metal concentrations (except of Hg) were determined by CSV, after neutralisation of the pH with 6 M ammonia, or ASV (Pb and Cd). The voltammetric equipment has been described before (Leal et al., 1999). The determination of Cu, Zn, Mn, Ni and Co was carried out by CSV at pH 8.3 (0.01 M borate buffer) using, as the competitive ligand, 25 μM SA for Cu (Campos and van den Berg, 1994), 100 μM APDC for Zn (van den Berg, 1985) and 0.2 mM DMG for Ni and Co (Colombo and van den Berg, 1997). The determination of Fe was carried out also by CSV at pH 6.9 (0.01 M PIPES buffer) using 0.4 mM catechol as the competitive ligand (van den Berg and Huang, 1984). The determination of Mn was carried out by CSV at pH 8.3 (0.01 M borate buffer) but without any added competitive ligand (Vasconcelos and Leal, 2001a). The determination of Pb and Cd was carried out by ASV at pH 2.2. Deposition step: E_d (V) = − 1.1 (Cu), − 1.2 (Pb, Cd), − 1.3 (Zn), − 0.1 (Fe), − 1.7 (Mn), − 0.8 (Ni, Co); t_d (s) = 60–120. The scanning parameters were—frequency (Hz): 50 (Cu, Zn), 200 (Mn), 10 (Fe, Ni, Co); modulation amplitude (mV): 25 (Cu, Mn, Zn), 50 (Pb, Cd, Fe, Ni, Co); step potential (mV): 2.44 (Cu, Zn), 3.66 (Fe, Mn, Ni,

Co), 5 (Pb, Cd); modulation time (s): 0.06 (Pb, Cd), 0.01 (Fe, Ni, Co); interval time (s): 0.2 (Pb, Cd), 0.1 (Fe, Ni, Co); square wave (Cu, Zn, Mn), differential-pulse (Pb, Cd, Fe, Ni, Co). In all cases, a hanging mercury electrode (HMDE) was used. Hg was determined by mercury cold vapour (Philips PU 9360 X-PU 9200 X) after preconcentration with a Chelamine column (Vasconcelos and Leal, 1997). $[M]_d$ was also determined, using the same procedures, in a seawater reference material for trace metals (NASS-5) and the experimental values were found to not be statistically different from the certified values (*t*-test, $P > 0.05$).

Total metal concentrations in the algae ($[M]_{algae}$) (extracellular adsorption plus intracellular uptake) were determined after microwave-digestion (Milestone MLS-1200 Mega) of the filters with 1 ml of suprapure, concentrated, nitric acid (from Merck) in high-pressure Teflon vessels and then were diluted with water and neutralised with ammonia. The metal concentrations were then determined as in the culture media. The metal fixed per microalgal cell ($[M]_{cell}$) was calculated from the $[M]_{algae}$ and cell counts. Control filters were processed using the same procedure, but the metal contamination was found to be insignificant (< 1%). $[M]_{algae}$ approximately balanced the metal lost from the seawater (with an error < 5%), confirming that metal adsorption onto the polycarbonate culture bottles was negligible. Reference standard seaweed (CRM 279 *Ulva lactuca*) was used to check the accuracy of the digestion and analysis procedures. The metal contents found were not significantly different from expected values (*t*-test, $P > 0.05$).

2.5. Speciation of Cu, Pb, Cd and Zn in the cultures

The concentrations of complexing organic ligands (C_L) in the media and the respective conditional stability constants (K'_{ML}) were determined by titration of the filtered culture media with the respective metal ion. Pb and Cd were measured by ASV while Cu and Zn by CSV with ligand competition (Leal et al., 1999; Campos and van den Berg, 1994; van den Berg, 1985). For CSV determinations, the pH was fixed at 8.3 (0.01-M borate) and SA ligand (5 μM) (for Cu) or APDC ligand (100 μM) (for Zn) were added to 120 ml of sample solution. It was observed in a previous study (Leal and van den Berg, 1998) that during the CSV Cu-complexing ligand titrations (added Cu concentration in the range 0–50 nM), the detected concentration of two synthetic organic ligands (cysteine and glutathione) and the conditional stability constants of their Cu complexes, did not vary significantly when the SA concentration was varied in the range 2–10 μM.

In all cases, aliquots of 10 ml of sample solution were pipetted into 11 polystyrene vials also containing added metal in the range of 0–200 nM (or other, if required), in approximately equal increments. The solutions were equilibrated overnight (12–15 h) prior to the determinations. The operationally labile metal concentration ($[M]_{labile}$) was determined as was described for $[M]_d$, but without previous acidification and UV digestion, using a 60-s adsorption step (t_d) and deposition potentials (E_d) of −0.2 V (Cu), −0.85 V (Zn), −0.6 V (Pb) or −0.8 V (Cd), while stirring. These E_d values were used rather than the more negative ones used for $[M]_d$, so as to prevent dissociation of the CuL, ZnL, PbL and CdL complexes. The scanning parameters were the same ones used in the $[M]_d$ determinations (described above).

2.6. Identification and quantification of thiol compounds in the cultures

Specific thiol compounds were identified and quantified in the culture media using the method of Al-Farawati and van den Berg (Leal et al., 1999; Al-Farawati and van den Berg, 1997). The determinations were carried out by CSV at pH 8.3 (0.01 M borate buffer). Deposition step: $E_d = -0.25$ V; $t_d = 60$ s. The scanning parameters were: scan rate, 20 mV s^{-1}; modulation amplitude, 25 mV; and square wave frequency of 50 Hz. The procedure was repeated after the addition of thiol standard (cysteine, glutathione or thioacetamide) for calibration. The cysteine peak was located at −0.47 V, the glutathione peak at −0.58 V and the thioacetamide peak at −0.68 V.

High performance liquid chromatography (HPLC) coupled to electrochemical detection (Shea and MacCrehan, 1988) was used with the purpose of confirming the voltammetric results. However, the limit of detection of the technique was not sufficient to identify the thiol compounds in the culture media where they are present at nanomolar levels. Preconcentration of the samples (by slow evaporation) was also processed but the necessary levels could not be achieved. The high

salinity of the samples constitutes an additional difficulty for the sample preconcentration. Vairavamurthy and Mopper (1990) were able to measure low nanomolar concentrations of thiols (including glutathione) using a preconcentration step and a HPLC separation of fluorescently labelled products. However, the method they used might not be suitable for samples as small as those we dispose in the present study for the speciation of thiol compounds (less than 20 ml per sample).

2.7. Calculations

The total ligand concentration (C_L) and the conditional stability constant of its complexes for the different metals (K'_{ML}) were calculated using the following relationship (van den Berg, 1982)

$$[M]_{labile}/[ML] = [M]_{labile}/C_L + \alpha/(K'_{ML}C_L) \quad (1)$$

where α is the coefficient of the overall side-reactions of the metal ion in which labile compounds are produced and [ML] is calculated as follows:

$$[ML] = [M]_d - [M]_{labile} \quad (2)$$

Linearity of the $[M]_{labile}/[ML]$ vs. $[M]_{labile}$ plot was interpreted as indicating complexation of the metal by a single type of ligand.

In a CSV titration of a natural seawater sample where the inorganic metal, [M′] (aquocomplex plus complexes with inorganic ligands) is in equilibrium with metal complexed by organic ligands, L, and an organic ligand added for competition, AL, the mass balance for $[M]_d$, is:

$$[M]_d = [M'] + [MAL] + [ML] \quad (3)$$

$[M]_{labile}$ includes the inorganic metal (as this equilibrates with the metal added during calibration) as well as any metal released from organic complexes in competition with the added competing ligand:

$$[M]_{labile} = [MAL] + [M'] = [M^{n+}]\alpha \quad (4)$$

where

$$\alpha = \alpha_M + \alpha_{MAL} \quad (5)$$

where α_M is the inorganic side-reaction coefficient for the metal and α_{MAL} the coefficient for the reactions of the metal ion with the added competing ligand.

$$\alpha_M = [M']/[M^{n+}] = 1 + \sum \beta'_{MX_n}[X]^n \quad (6)$$

β'_{MX_n} is the overall conditional stability constant for the inorganic complex MX_n of M with the X (OH^-, HCO_3^-, CO_3^{2-}, PO_4^{3-}, SO_4^{2-}, F^-, Cl^- and Br^-) and [X] is the concentration of uncomplexed X. Since the anion concentrations in the equation are generally expressed as free concentrations, these concentrations ([X]) are themselves calculated in a manner that accounts for interactions with the major cations (Na^+, Mg^{2+}, Ca^{2+} and K^+) in seawater. Values of α_M for the different metals studied, valid for pH 8 seawater, 35‰ of salinity and 25 °C temperature (there is a lack of values for stability constants at 18 °C), were calculated using an ion-pairing model and metal stability constants from Turner et al. (1981). The contribution of inorganic Cu(I) is ignored as its concentration is much less than that of inorganic Cu(II), and cannot be calculated accurately because the redox potential of the system is not known. Values of α_M were as follows: 36 (Cu); 34 (Pb); 35 (Cd) and 2.1 (Zn), and must be considered approximations of the actual values at 18 °C (temperature used in the present study).

$$\alpha_{MAL} = \sum \beta'_{M(AL)n}[AL']^n \quad (7)$$

where [AL′] is the free concentration of AL. For the Cu determination, AL = SA and for the Zn determination, AL = APDC. The following values were used for the conditional stability constants: $\log K'_{CuSA} = 9.52$ and $\log \beta'_{Cu(SA)_2} = 14.89$ (Campos and van den Berg, 1994); and $\log K'_{ZnAPDC} = 4.4$ (van den Berg, 1985; Donat and Bruland, 1990). The concentration of AL added to the solution was much greater than that of the respective metal; hence, the total AL added concentration, C_{AL}, can be used instead of [AL′].

In ASV, $\alpha = \alpha_M$ since a competing ligand is not added prior to the titration and the eventually labile organic complexes can not be accounted. Therefore, the value of α and the calculated K'_{ML} will be possibly slightly lower than the real values.

C_L and K'_{ML} had been determined (by Eq. (1), both by CSV or ASV titrations) $[M^{n+}]$ in the seawater was

calculated by solving the following quadratic equation (Leal et al., 1999):

$$[M^{n+}]^2\alpha_M K'_{ML} + [M^{n+}](K'_{ML}C_L - K'_{ML}[M]_d + \alpha_M) - [M]_d = 0 \quad (8)$$

The inorganic metal concentration was then calculated using:

$$[M'] = [M^{n+}]\alpha_M \quad (9)$$

and the organic metal concentration using:

$$[ML] = [M]_d - [M'] \quad (10)$$

The [ML] estimated as described is an operational value, which depends on the time scale of the method used and, particularly, on the nature (strength as ligand) of the competing ligand in CSV and on the deposition potential in ASV. By ASV, the value of [ML] is probably underestimated since all the labile metal ($[M]_{labile}$) is accounted as inorganic metal, in spite of some organic metal that may also be dissociated. In such case, $[M^{n+}]$ will be overestimated.

3. Results and discussion

3.1. Organic compounds in the culture media

As can be seen in Table 1, the exudates of different origins enriched the medium with ligands, that is, the C_L values were higher in the media where micro- or macroalgae have been grown (B solutions in Fig. 1). The total concentrations decreased in the order of: *P. tricornutum* > *Enteromorpha* > *E. huxleyi* > *Porphyra* > Control. A C_L value of 72 nM observed in the control culture (natural coastal seawater) is relatively high when compared with values observed in oceanic waters, between 10 and 20 nM (Buckley and van den Berg, 1986; Coale and Bruland, 1988). However, values of C_L higher than 100 nM have been determined in coastal waters by different authors (van den Berg, 1984; Manping et al., 1990). In the present study, the results obtained in the cultures enriched with exudates were systematically compared with those obtained in the control cultures; the background level of C_L was not a very relevant parameter.

The conditional Cu complex stabilities, determined in titration curves up to 200 nM added $[Cu]_d$, were not markedly different in the various cultures: log K'_{CuL} ranged from 12.03 ± 0.01 (Control) to 12.49 ± 0.09 (*Porphyra*). Fig. 2 shows two examples of Cu-complexing ligand titrations: one in the control medium and another in the medium with *P. tricornutum* exudates. Only one class of ligand was found because Cu was only added up to 200 nM $[Cu]_d$, in order to determine the stronger ligands. Weaker ligands could probably be determined for higher metal to ligand concentration ratios.

In addition, by using CSV, we were able to identify compounds that behaved like glutathione in all media, including in the natural seawater (control medium) and cysteine in all but the medium enriched with *Porphyra*

Table 1
Ligand concentrations and respective conditional stability constants of the Cu complexes determined in natural seawater and in that enriched with exudates of different algae

Medium	Ligand concentrations (nM)[a]			Log K'_{CuL}[a]
	Total ligands (C_L)	Cysteine[b]	Glutathione[c]	
Seawater (control)	72 ± 3	11.4 ± 0.6	50 ± 2	12.03 ± 0.01
Seawater enriched with exudates of				
E. huxleyi	114 ± 3	27 ± 1	77 ± 4	12.34 ± 0.06
P. tricornutum	160 ± 5	152 ± 8	76 ± 3	12.18 ± 0.01
Porphyra	92 ± 4	–	82 ± 4	12.49 ± 0.09
Enteromorpha	127 ± 4	17.6 ± 0.8	105 ± 5	12.40 ± 0.08

[a] Mean value ± standard deviation ($n = 3$).
[b] Compounds that reduce at the cysteine potential in CSV.
[c] Compounds that reduce at the glutathione potential in CSV.

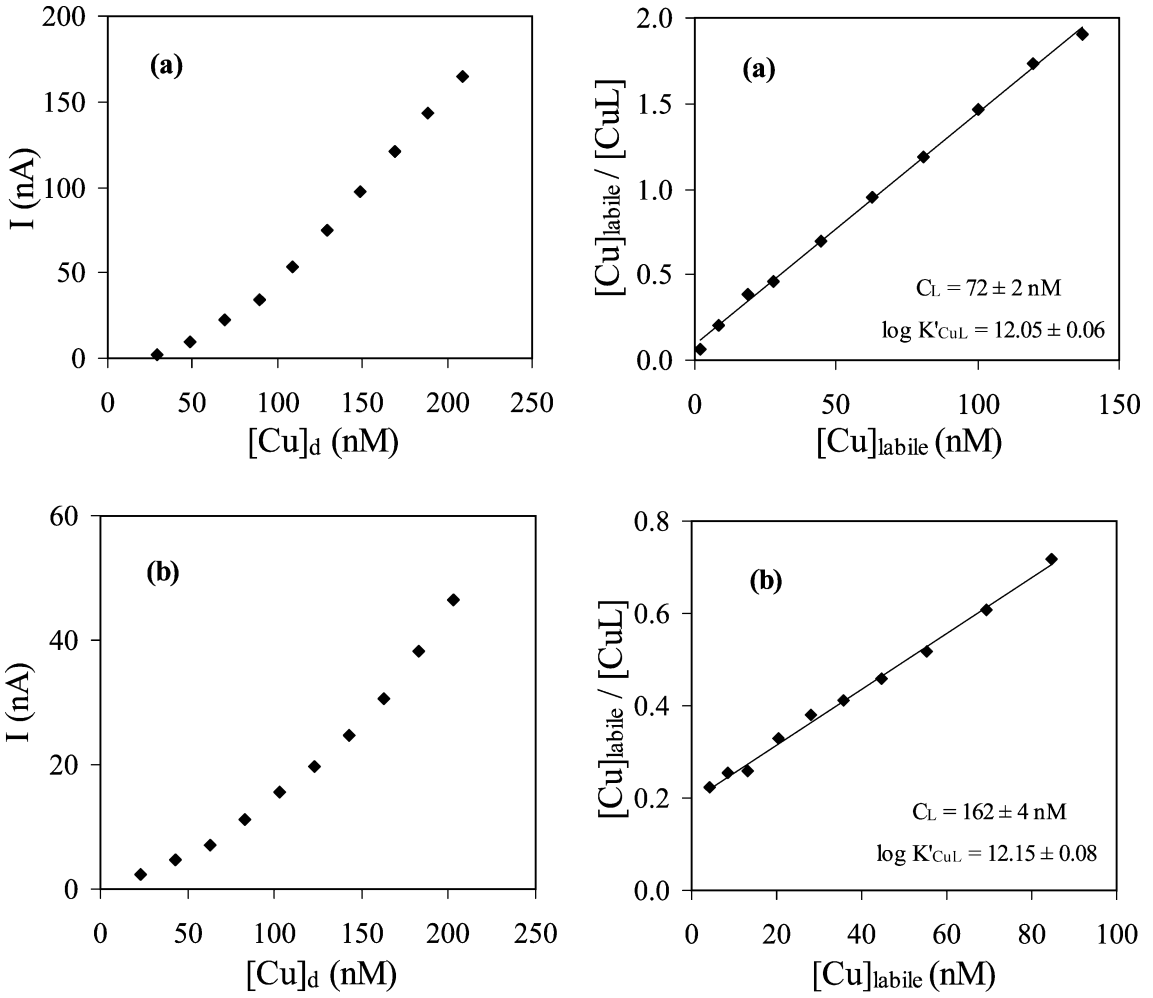


Fig. 2. Cu-complexing ligand titration curves obtained for the (a) control medium and (b) that enriched with *P. tricornutum* exudates, before *E. huxleyi* inoculation (day 0).

exudates (see Fig. 3 and Table 1). The cysteine peak was practically absent of the voltammograms for the medium enriched with *Porphyra* exudates (Fig. 3). It is possible that the cysteine-like ligands initially present in the seawater had been taken up by the *Porphyra*. This hypothesis was not experimentally tested. However, decrease of cysteine-like compounds in the medium was observed in the cultures of *E. huxleyi* enriched with *P. tricornutum* exudates (see below). The disappearance of cysteine might be due to bacterial activity, but this is unlikely because the presence of bacteria was not detected. Another hypothesis is that the cysteine-like compounds, free or bound by a metal ion, had been taken up by the cells. Free cysteine is known to occur in seawater (van den Berg et al., 1988). Compounds identified by CSV as glutathione have been also found before in seawater (Le Gall and van den Berg, 1993).

The fraction of the organic compounds identified as either cysteine-like or glutathione-like varied with the origin of the exudates: the concentration of cysteine-like compounds was much higher in the medium with *P. tricornutum* exudates and the concentration of glutathione-like compounds was higher in that with *En-*

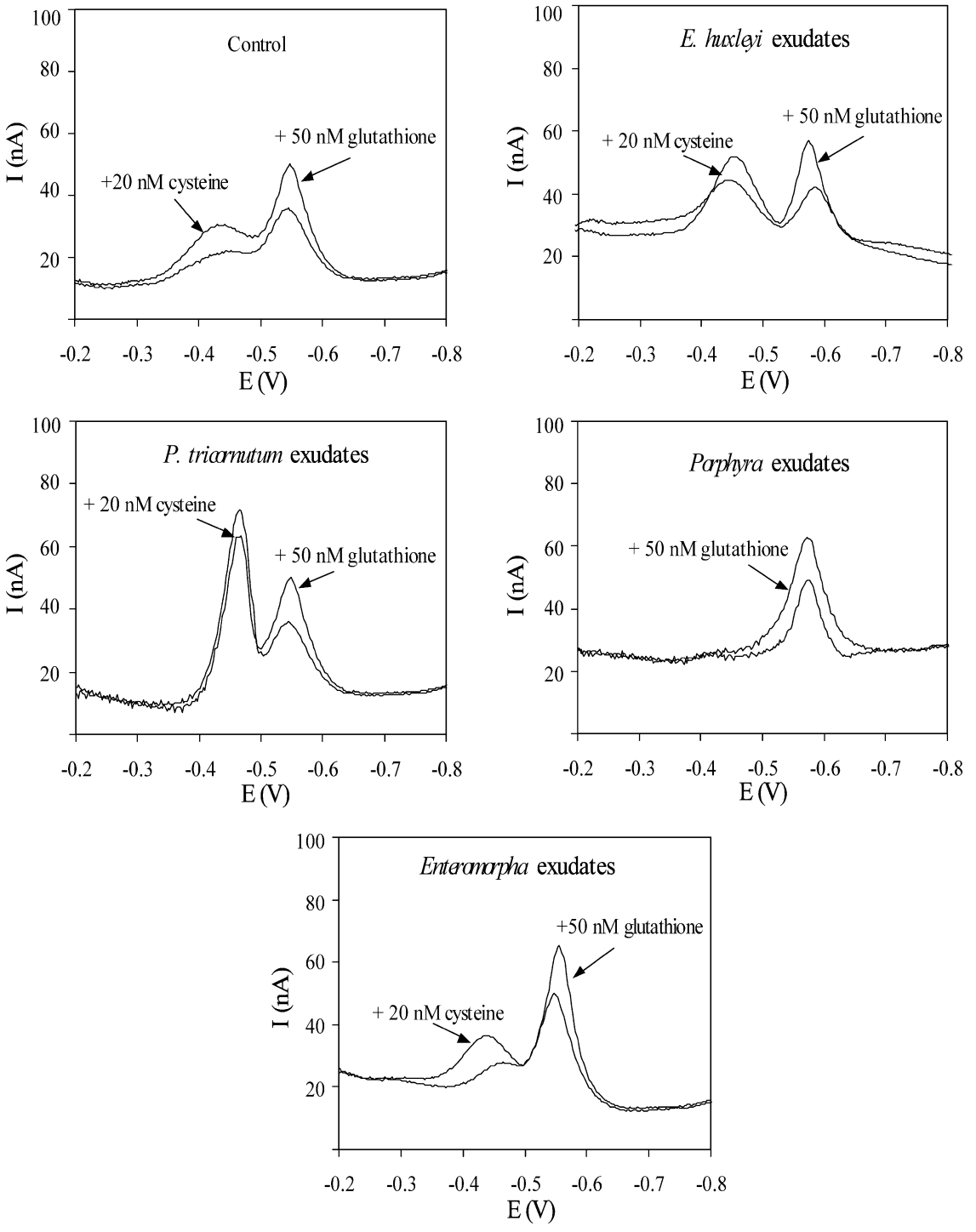


Fig. 3. Identification of thiol compounds in the different culture media, before *E. huxleyi* inoculation (day 0). Voltammograms obtained before and after the addition of specific thiol compounds.

teromorpha exudates. Therefore, the media enriched with exudates of the different origins differ both in the concentration and nature of the organic ligands that form strong complexes with Cu (and other metal ions).

In the medium with *P. tricornutum* exudates, the total concentration of Cu-complexing organic ligands (which was the highest of all media) was markedly lower than the sum of the concentrations of cysteine-like and glutathione-like compounds (Table 1). This may be because the stoichiometric ratios of Cu/thiol could be either 1:2 (Leal and van den Berg, 1998) or 1:1 (Le Gall and van den Berg, 1993), depending on the metal to ligand concentration ratio in the medium.

3.2. Biological response of *E. huxleyi* in media enriched with exudates of different algae

Table 2 shows that the initial total dissolved metal concentration ($[M]_d$) (measured immediately after the cells inoculation) was higher in the natural seawater and in that enriched with synthetic thioacetamide than in the media where the different algal species had been introduced to produce exudates. This was a result of metal uptake by the algae. The levels of total dissolved metal in the coastal seawater used were similar to those reported for polluted and industrialised European coastal areas (e.g., Belgian and Dutch coast, Mart et al., 1982; Northern Adriatic coast, Munda and Hudnik, 1991; central and southern North Sea coast, Tappin et al., 1995). Similar levels have been also found in other studies in the Oporto coast (Leal et al., 1997; Vasconcelos and Leal, 2001a), and are probably a result of the direct discharge of waste water in the Oporto shore, without any treatment. Cotté-Krief et al. (2000) also found similar metal levels in the coastal waters of southern Portugal.

Growth, Cu uptake and speciation in the media, total concentration and partial speciation of exudates released by *E. huxleyi* in natural seawater (control) and seawater enriched with exudates from the different algal species are shown in Fig. 4. An addition of 50 nM thioacetamide, instead of natural exudates, was included in the experimental set because thioacetamide-like compounds have been identified in older cultures of *E. huxleyi* in a previous study (Leal et al., 1999) carried out in different conditions (at 15 °C, seawater from open ocean water): ca. 20 nM after 10 days of growth and ca. 60 nM after 17 days of growth.

Based on the growth results of the control culture, we must assume that the primary cultures used to produce exudates of *E. huxleyi* (and also *P. tricornutum*, results not shown) were in the beginning of the stationary phase (transfers done after 7 days). As a result, it is likely that some cell death/lysis probably occurred and small quantities of this material was therefore in the cultures.

3.2.1. Growth

Final cell yield of *E. huxleyi* was similar in the media containing *E. huxleyi* and *Porphyra* exudates and did not markedly differ from that in the control medium (only a little lower for days 4 and 10). In the presence of *Porphyra* exudates, the growth rate was slightly higher. In contrast, the final cell yield was much greater in the presence of *Enteromorpha* exudates (although the growth rate increased only a little) and inhibition (decrease of both the growth rate and final cell yield) occurred in the presence of *P. tricornutum* exudates. The growth rate and final cell yield was also much diminished in the presence of 50 nM thioacetamide, confirming previous experiments at a lower thioacetamide concentration (25 nM) (Leal et al., 1999). The thioacetamide potentially causes a toxic effect to these microorganisms. The present results indicate that the compounds liberated by *E. huxleyi* in a previous study (Leal et al., 1999) (in spite of it has resulted in a voltammetric peak practically coincident with that of thioacetamide) probably was a thiolic species different from thioacetamide, considering that it is not expectable that an organism can produce compounds toxic to itself.

3.2.2. Trace metal contents

The final total dissolved metal concentrations in the distinct media and the respective uptake (considering both extra- and intracellular metal) by *E. huxleyi* cells are also shown in Table 2. For all the metals, the metal concentration measured per cell ($[M]_{cell}$) was much greater in the culture with *P. tricornutum* exudates, which displayed lack of growth, and it was also higher in the culture with thioacetamide, where a reduced growth occurred (see Fig. 4). In these cases, the cell number in the cultures after 10 days was much lower than in the other cultures, and the concentration of dissolved metal per cell was much higher. This may or may not be the main reason of the higher cellular metal

Table 2
Metal concentrations in the *E. huxleyi* cultures[a]

Medium		Cu	Pb	Cd	Zn	Fe	Mn	Ni	Co
Natural seawater (control)	Just after inoculation: $[M]_d$[b] (nM)	29 ± 2	0.34 ± 0.02	0.45 ± 0.02	16 ± 1	23 ± 1	8.6 ± 0.4	29 ± 2	0.24 ± 0.02
	After 10 days growth: $[M]_d$[b] (nM)	21 ± 1	0.020 ± 0.001	0.051 ± 0.003	4.9 ± 0.3	18 ± 1	1.6 ± 0.1	17.0 ± 0.8	0.16 ± 0.01
	Uptake[c] (%)	45	94	89	69	22	70	41	33
	$[M]_{cell}$ (10^{-18} mol cell^{-1})	42 ± 3	0.85 ± 0.06	1.1 ± 0.06	30 ± 2	13.0 ± 0.9	19 ± 1	32 ± 2	0.29 ± 0.02
	[Cells] (10^6 cell l^{-1}): 375 ± 18								
Seawater enriched with *E. huxleyi* exudates	Just after inoculation: $[M]_d$[b] (nM)	23 ± 1	0.15 ± 0.02	0.19 ± 0.03	6.5 ± 0.3	19 ± 0.8	2.4 ± 0.1	18 ± 0.7	0.22 ± 0.03
	After 10 days growth: $[M]_d$[b] (nM)	16.4 ± 0.8	0.008 ± 0.001	0.018 ± 0.002	1.2 ± 0.1	13.0 ± 0.6	0.52 ± 0.04	8.3 ± 0.3	0.12 ± 0.02
	Uptake[c] (%)	49	95	91	82	32	78	54	45
	$[M]_{cell}$ (10^{-18} mol cell^{-1})	35 ± 2	0.34 ± 0.02	0.42 ± 0.03	13.0 ± 0.9	15 ± 1	4.6 ± 0.3	24 ± 2	0.24 ± 0.02
	[Cells] (10^6 cell l^{-1}): 412 ± 21								
Seawater enriched with *P. tricornutum* exudates	Just after inoculation: $[M]_d$[b] (nM)	23 ± 1	0.12 ± 0.02	0.27 ± 0.01	11.0 ± 0.6	19 ± 1	2.1 ± 0.2	16 ± 1	0.21 ± 0.03
	After 10 days growth: $[M]_d$[b] (nM)	18.5 ± 0.8	0.081 ± 0.009	0.11 ± 0.01	7.3 ± 0.4	16 ± 1	1.3 ± 0.1	12.0 ± 0.5	0.15 ± 0.01
	Uptake[c] (%)	42	33	59	34	16	38	25	29
	$[M]_{cell}$ (10^{-18} mol cell^{-1})	505 ± 35	1.60 ± 0.07	6.4 ± 0.4	148 ± 10	120 ± 8	32 ± 2	160 ± 11	2.4 ± 0.2
	[Cells] (10^6 cell l^{-1}): 25.0 ± 0.9								
Seawater enriched with *Porphyra* exudates	Just after inoculation: $[M]_d$[b] (nM)	22 ± 1	0.072 ± 0.005	0.24 ± 0.02	14.0 ± 0.8	17 ± 1	7.4 ± 0.3	24 ± 2	0.23 ± 0.02
	After 10 days growth: $[M]_d$[b] (nM)	18.2 ± 0.9	0.021 ± 0.002	0.090 ± 0.008	9.5 ± 0.5	10.0 ± 0.7	1.50 ± 0.08	19 ± 1	0.14 ± 0.02
	Uptake[c] (%)	41	71	62	32	41	80	21	39
	$[M]_{cell}$ (10^{-18} mol cell^{-1})	32 ± 2	0.14 ± 0.01	0.40 ± 0.03	12.0 ± 0.8	19 ± 1	16 ± 1	13.0 ± 0.9	0.24 ± 0.02
	[Cells] (10^6 cell l^{-1}): 376 ± 19								
Seawater enriched with *Enteromorpha* exudates	Just after inoculation: $[M]_d$[b] (nM)	22 ± 1	0.023 ± 0.003	0.21 ± 0.01	15.0 ± 0.6	19.0 ± 0.5	7.9 ± 0.3	23 ± 2	0.22 ± 0.04
	After 10 days growth: $[M]_d$[b] (nM)	15.5 ± 0.8	0.012 ± 0.001	0.030 ± 0.002	9.7 ± 0.5	13.0 ± 0.8	2.0 ± 0.1	16.0 ± 0.9	0.18 ± 0.03
	Uptake[c] (%)	27	48	86	35	32	75	30	18
	$[M]_{cell}$ (10^{-18} mol cell^{-1})	24 ± 2	0.018 ± 0.001	0.29 ± 0.02	8.5 ± 0.6	9.6 ± 0.7	9.5 ± 0.7	11 ± 0.8	0.06 ± 0.01
	[Cells] (10^6 cell l^{-1}): 624 ± 31								
Seawater enriched with 50 nM thioacetamide	Just after inoculation: $[M]_d$[b] (nM)	28 ± 2	0.34 ± 0.02	0.45 ± 0.02	16.0 ± 0.8	23 ± 1	8.6 ± 0.4	29 ± 2	0.24 ± 0.02
	After 10 days growth: $[M]_d$[b] (nM)	21 ± 1	0.21 ± 0.03	0.25 ± 0.01	7.5 ± 0.2	20 ± 1	3.4 ± 0.1	21 ± 1	0.19 ± 0.01
	Uptake[c] (%)	44	38	44	53	13	60	28	21
	$[M]_{cell}$ (10^{-18} mol cell^{-1})	155 ± 10	1.3 ± 0.1	2.0 ± 0.1	83 ± 6	29 ± 2	51 ± 4	78 ± 5	0.49 ± 0.03
	[Cells] (10^6 cell l^{-1}): 102 ± 5								

[a] The initial *E. huxleyi* cells concentration was about 0.5×10^6 cell l^{-1}.

[b] Mean values ± standard deviation ($n = 3$). In the control culture, $[Hg]_d$ was measured only after inoculation and it was 0.12 ± 0.01 nM.

[c] Percentage of the initially soluble metal that was bound up with the cells (extra- and intracellularly). For Cu, the external additions during growth was attended.

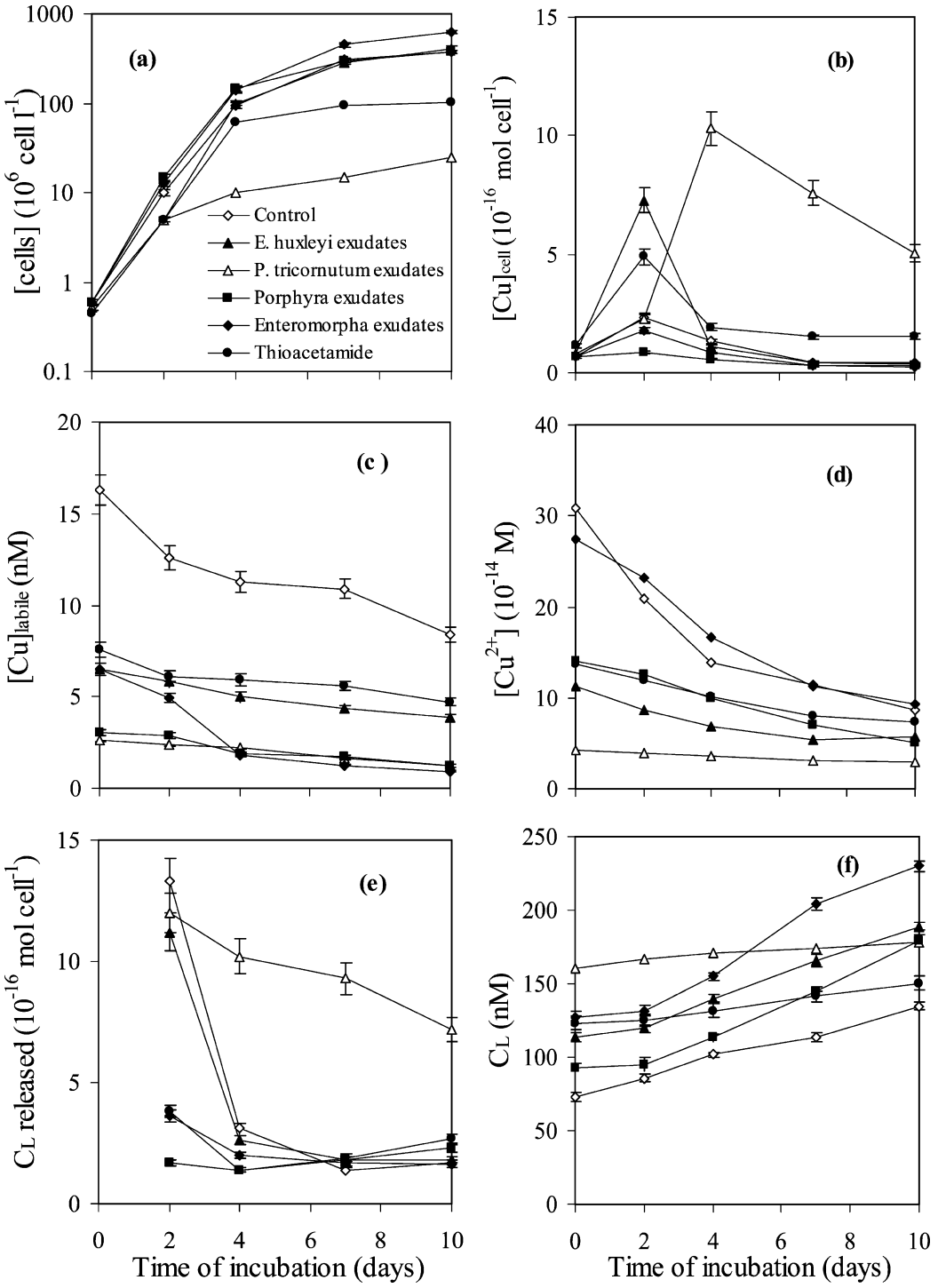


Fig. 4. (a) *E. huxleyi* growth, (b) Cu uptake, (c) labile Cu and (d) free Cu concentrations, (e) Cu-complexing ligands released (exudates) per cell and (f) total Cu-complexing ligands concentration in the different culture media. Standard deviations ($n=3$) are also given except for $[Cu^{2+}]$, which was estimated by speciation calculations. Growth rates (day $^{-1}$) (calculated for the exponential phase of growth): (◊) 1.27, (▲) 1.31, (△) 0.739, (■) 1.38, (♦) 1.37, and (●) 1.23.

levels because the concentration of organic ligands was also higher in the cultures richer in *P. tricornutum* exudates.

The influence of the exudates on the cellular metal uptake is apparent in Table 2. Cell yield of *E. huxleyi* was similar in the presence of exudates from *Porphyra* and those from *E. huxleyi* (slightly higher after 10 days growth in the last case). The initial levels of $[Ni]_d$ have also been similar in these two culture media. However, much more Ni was taken up in the presence of *E. huxleyi* exudates (where the initial concentration of organic ligands were slightly higher) than in the presence of the *Porphyra* exudates. In addition, in the culture enriched with *Enteromorpha* exudates, final cell yields were greater than in the presence of *E. huxleyi* exudates and that in the control medium (and also that with *Porphyra* exudates in most cases), the percentages of total uptake (i.e., total metal transferred to the cells) of Co, Ni, Pb and Cu were much lower. In comparison with the control, there was also lower uptake for Zn in the presence of *Porphyra* and *Enteromorpha* exudates, which were not consistent with the initial Zn concentrations and the number of cells. These data indicate that other factors besides metal speciation in the medium and the number of cells influence the metal uptake. Concentrations and composition of organic ligands seem to have direct influence on the metal uptake, through mechanisms that require investigation.

3.2.3. Cu speciation

Fig. 4 shows that the variation of $[Cu]_{cell}$ during growth was similar for all the media but that with *P. tricornutum* exudates. The cellular Cu levels increased at day 2 of the cultures (day 4 in the *P. tricornutum* exudates) and decreased again thereafter. The increase was greater in the presence of *P. tricornutum* exudates and thioacetamide possibly due to their much reduced growth rate (more Cu available per cell). Comparison shows that this high level of cellular Cu was not sufficiently high on its own to cause toxic effects to *E. huxleyi* (Leal et al., 1999). After 2 days of growth, when the cell number was still quite similar in the different cultures, the values of $[Cu]_{cell}$ were significantly different among the different culture media. The highest value, 7.3×10^{-16} mol cell^{-1}, occurred in the presence of *E. huxleyi* exudates, and the lowest, 0.89×10^{-16} mol cell^{-1}, in the presence of *Porphyra* exudates. We evaluated whether a major fraction of the Cu was effectively taken up, which demonstrated that 86–96% of the $[Cu]_{cell}$ was intracellular (Vasconcelos and Leal, 2001b). This means that the large differences between cultures seen here were not due to different amounts of Cu adsorption. Therefore, the present data indicate that the nature of the organic ligands, most of them exuded by algae, affect the trace metal uptake.

Fig. 4 shows that the $[Cu]_{labile}$ in the control culture medium was much higher than in all the other culture media. Two main facts contributed to these results: in the control, (i) the initial $[Cu]_d$ was the highest of all and (ii) the percentage of inert complexes ($[Cu]_d - [Cu]_{labile}$) was the lowest. The values of the free metal, $[Cu^{2+}]$, which depended on both the ligands concentration and the stability constant of the respective complexes, were five orders of magnitude lower than $[Cu]_{labile}$. Nevertheless, the medium where $[Cu]_{labile}$ was the lowest did not necessarily coincide with that where $[Cu^{2+}]$ was the lowest, indicating that the operational inertness of the organic complexes varied with their nature, probably by kinetic reasons. $[Cu']$ varied proportionally to $[Cu^{2+}]$, being 36 times higher.

3.2.4. Exudates

The way in which the ligand production by *E. huxleyi* was affected by the addition of exudates and thioacetamide is shown in Fig. 4. Maximal exudate release per cell occurred after 2 days growth, in mid-exponential phase of the cultures, indicating that the release was active (cells presumably would have intact cell membranes). The amount of Cu organic ligands released per cell was the highest for the culture without added organic compounds (13×10^{-16} mol cell^{-1}, control culture) and the lowest for the culture with *Porphyra* exudates (1.7×10^{-16} mol cell^{-1}). These results indicate that the nature of the organic species present in the medium may affect exudate production. After 4, 7 and 10 days growth, the exudates released per cell were similar in all cultures (at $2–3 \times 10^{-16}$ mol cell^{-1}) except for *P. tricornutum* exudate cultures, where the cellular ligand release remained much higher (decreased from 12×10^{-16} at the 2nd day to 7.2×10^{-16} mol cell^{-1} at the 10th day of growth). Initially (before inoculation), the maximal concentration of organic ligands was found in the medium enriched with *P. tricornutum* exudates (Table 1). Therefore, it could be hypothesised that the bioavailable

concentration of some essential trace metals was insufficient (see for instance, the $[Cu^{2+}]$ in Fig. 4) limiting the growth. A strong release of exudates would be a cellular strategy to improve the bioavailable metal. However, this hypothesis is not consistent with the highest cellular Cu levels observed in these cultures (see Fig. 4). It can be speculated that some *P. tricornutum* exudates free or bound with trace metals could be taken up by *E. huxleyi*, resulting in a toxic effect. The higher cellular release of exudates could be a resistance mechanism against the apparent toxicity of the *P. tricornutum* exudates. The cultures with 50 nM thioacetamide also released a slightly higher amount of exudates than most of the other cultures after 7 days growth, except those with the *P. tricornutum* exudates, probably for a similar reason.

Among the exudates, the thiol compounds were identified and quantified by CSV in the different *E. huxleyi* cultures using a method independent to that used for C_L determination, and the results are compared in Fig. 5. In the cultures with added *E. huxleyi* or *Porphyra* exudates, where the initial concentrations of glutathione-like compounds were similar (77 nM *E. huxleyi* and 82 nM *Porphyra*) and the initial concentration of cysteine-like compounds was relatively low (27 nM for *E. huxleyi* exudates and undetected for *Porphyra* exudates), the amount of compounds like glutathione and cysteine released during growth was similar to that in the control culture (which initially had 50 nM glutathione-like and 11 nM cysteine-like). In the presence of *Enteromorpha* exudates, which was initially richer in glutathione-like compounds (105 nM) and contained 18 nM of cysteine-like compounds, the concentration of glutathione-like compounds strongly increased during the growth of *E. huxleyi*, attaining the highest levels observed in the present study. In contrast, the concentration of cysteine-like compounds was almost constant during the growth. In the presence of *P. tricornutum* exudates (which inhibited *E. huxleyi* growth), the initial concentration of cysteine-like compounds was comparatively very high (152 nM) and it decreased very strongly with the age of the *E. huxleyi* cultures, whereas the glutathione-like concentration increased as usual. Therefore, glutathione-like compounds in some way stimulated the growth of the tested algae, whereas cysteine-like compounds did not. The decrease of the cysteine-like compound observed in the media with *P. tricornutum* exudates cannot be attributed to adsorption onto the container walls because it was observed that the cysteine concentration remained practically constant in time when *P. tricornutum* exudates and 150 nM cysteine were added separately to sterilised seawater, which had been conditioned like the algal cultures for 10 days. Bacterial activity was minimal (or even absent) as discussed above. All the media were sterilised and the presence of bacteria in the *E. huxleyi* cultures would result in interference in the other media enriched with exudates not only in that enriched with *P. tricornutum* exudates. Therefore, the present results suggest that the cysteine-like compounds were taken up by the algae, free or bound by a metal ion. Disappearing of cysteine-like compounds from the medium with *Porphyra* has also been observed, as mentioned above.

It deserves to be mentioned that preliminary studies using *P. tricornutum* as a test organism indicated that glutathione-like ligands did not stimulate the growth of *P. tricornutum*, in contrast to results presented for *E. huxleyi* (results not shown).

The presented data suggest that organic exudates can influence the growth rate and final cell yields of *E. huxleyi* batch cultures. They also may influence metal accumulation in both exponential and stationary phases as either a direct function of metal–ligand interaction or differences in the cell yield. Therefore, it can be speculated that a bloom of a species of algae, which will produce large amounts of specific exudates, may favour or inhibit the local growth of other algal species and, in an extreme situation, change the biodiversity.

3.3. Effects of synthetic cysteine and glutathione on growth of E. huxleyi

The results obtained in the first set of experiments (above discussed) suggest that compounds with a CSV behaviour identical to cysteine and glutathione are major components of the exudates of the algae, and that the concentration and composition of the exudates condition the biological response of *E. huxleyi*. To find experimental support for an eventual role of the thiol compounds on the results discussed above, 150 nM synthetic cysteine (value similar to that found in the medium with *P. tricornutum* exudates) and 100 nM synthetic glutathione (value similar to that found in the medium with *Enteromorpha*

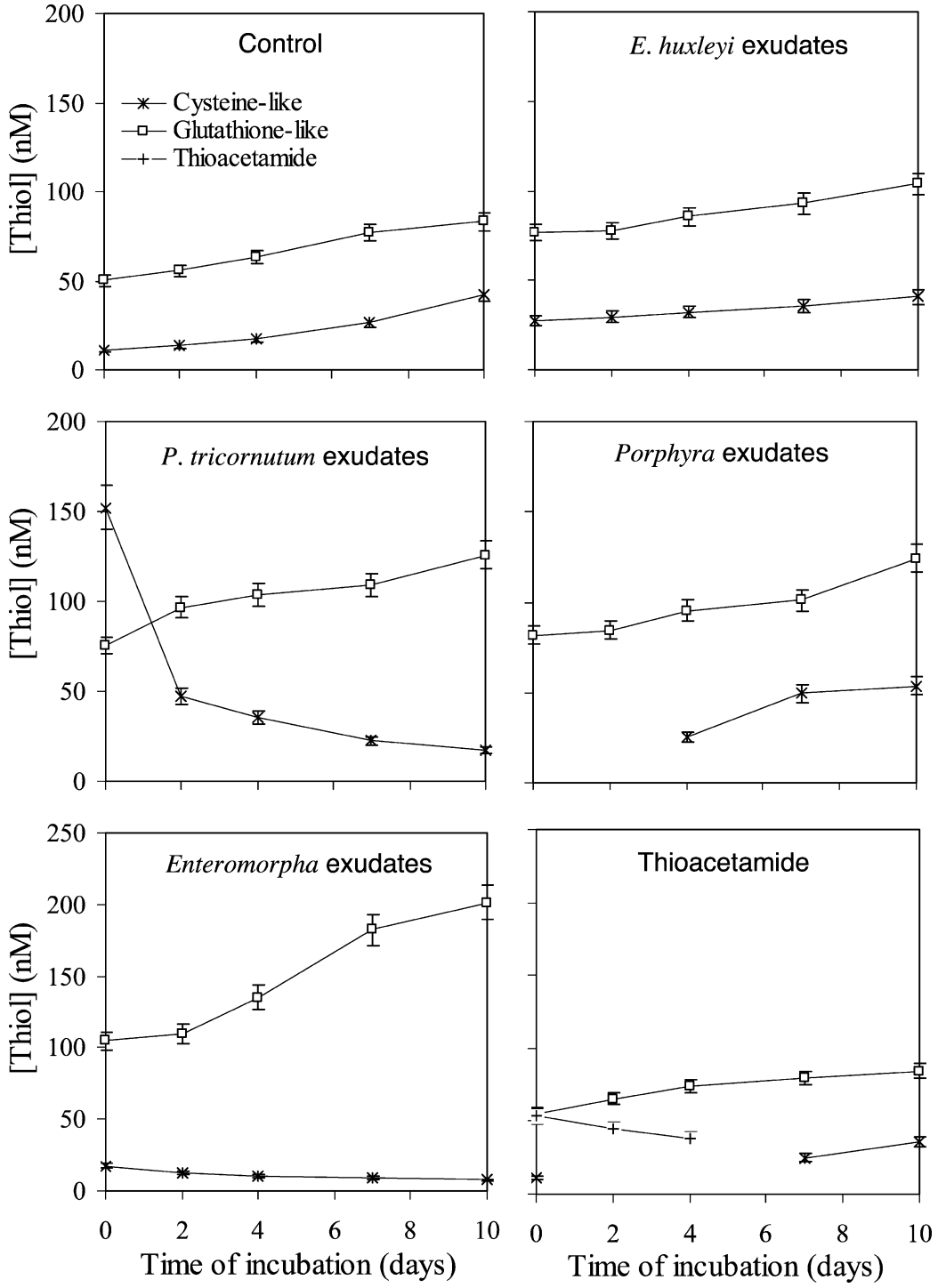


Fig. 5. Variations with the concentrations of the thiol compounds (quantified by CSV in the cultures) during the growth of *E. huxleyi*. Standard deviations ($n = 3$) are also given.

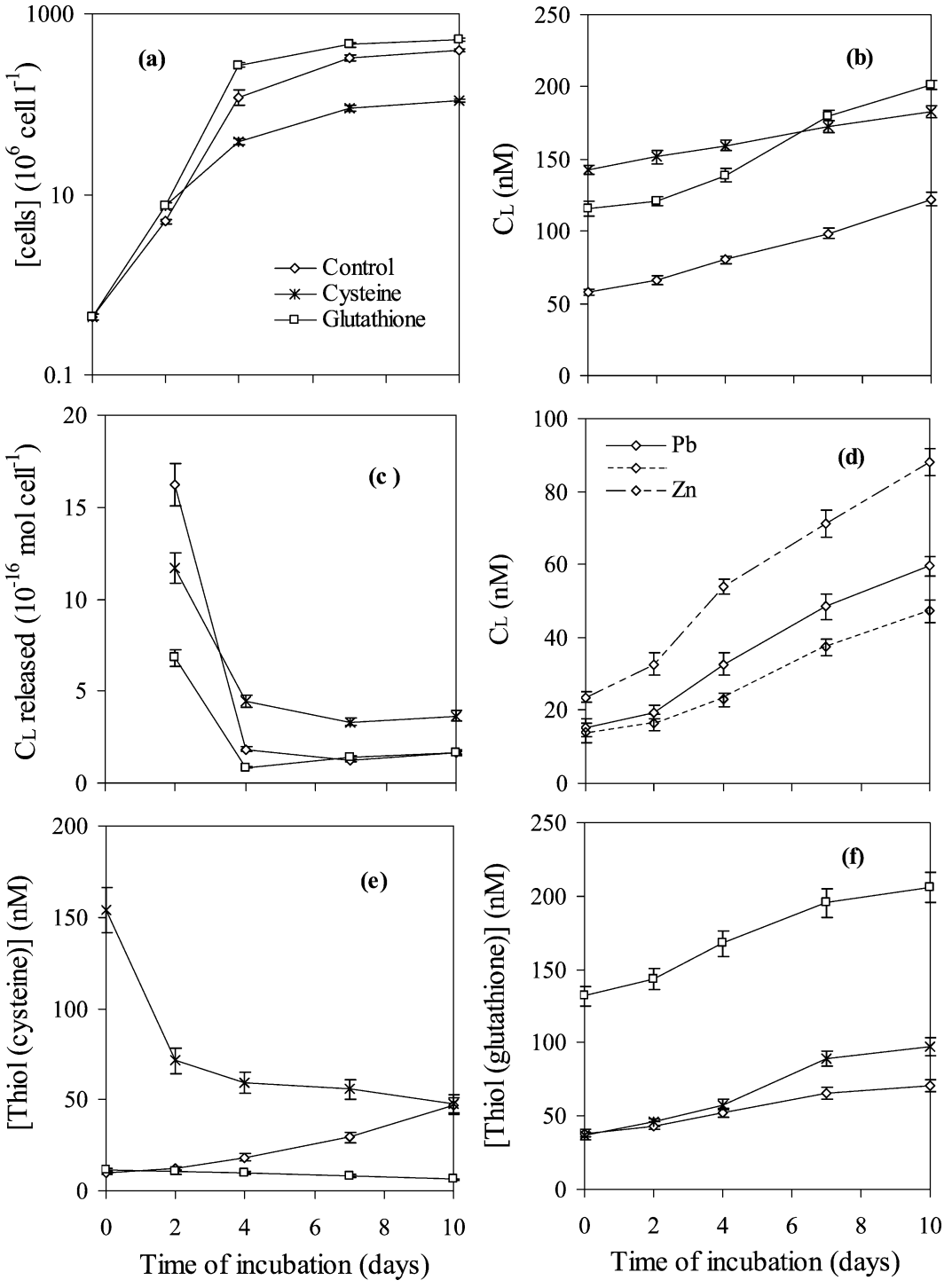


Fig. 6. (a) *E. huxleyi* growth, (b) total Cu-complexing ligands concentration and (c) Cu-complexing ligands released (exudates) per cell, (d) total Pb-, Cd- and Zn-complexing ligands in the control medium and (e) concentration of cysteine-like and (f) glutathione-like thiol compounds produced in natural seawater and in that enriched with 150 nM cysteine or 100 nM glutathione. Standard deviations ($n = 3$) are also given.

exudates) were added separately to seawater and a new set of experiments were carried out in identical experimental conditions to those used before. The seawater used in these set of experiments (II in Fig. 1) was collected on a date different from that used for the first study (I in Fig. 1) and, therefore, its trace metal composition was slightly different, as follows: $[M]_d$ (nM) = 9.5 Cu, 1.1 Pb, 1.2 Cd, 0.15 Hg, 15 Zn, 74 Fe, 14 Mn, 12 Ni, and 0.74 Co (in the first set of experiments they were: 29 Cu, 0.34 Pb, 0.45 Cd, 0.12 Hg, 16 Zn, 11 Fe, 7.7 Mn, 29 Ni, and 0.19 Co). In this case, the concentration of Cu, Pb, Cd and Zn organic ligands were determined as well as the respective conditional stability constants, K'_{CuL}, K'_{PbL}, K'_{CdL} and K'_{ZnL} (see below). Based on these data, speciation calculations were carried out for these four metals.

3.3.1. Growth of E. huxleyi

Fig. 6 shows that 100 nM glutathione improved the *E. huxleyi* growth whereas 150 nM cysteine reduced growth. The results are consistent with the results obtained in the cultures with added exudates from different algae: an improvement of the final *E. huxleyi* yield and a slight increase of the growth rate in the presence of *Enteromorpha* exudates (the richest in glutathione-like compounds) and a marked decrease of final cell yield and growth rate in the presence of *P. tricornutum* exudates (the richest in cysteine-like compounds).

Interestingly, the concentration of cysteine decreased during algal growth, as in the culture with *P. tricornutum* exudates, and the concentration of glutathione increased strongly during growth, as in the culture with *Enteromorpha* exudates (compare Fig. 5 with Fig. 6). Thioacetamide in the cultures with added thioacetamide (Fig. 5) was also found to decrease. These results could be due to either thiol uptake or to the decomposition of those compounds. To test the latter hypothesis, *P. tricornutum* exudates, 150 nM cysteine, 100 nM glutathione and 50 nM thioacetamide were added separately to sterilised seawater, which was conditioned like the algal cultures for 10 days. Analyses showed that the thiol concentrations were practically constant, indicating that they were chemically stable in the seawater cultures for this period. Therefore, the decrease was caused via uptake by the microorganisms.

3.3.2. Trace metal uptake

In the second set of experiments, the cellular metal was monitored during the algal growth for all the trace metals under study. The uptake followed a similar trend for all the metals, similar to that observed for Cu in most cultures with added exudates, except that with *P. tricornutum* exudates (Fig. 4). Greatest uptake occurred on the second day of growth, when the cells were in mid-exponential phase.

The cellular concentrations of the different trace metals after 10 days growth are shown in Fig. 7 as a function of the respective initial total dissolved concentration in the medium. Addition of cysteine (but not of glutathione) caused the apparent metal uptake to increase. Similar results were observed in the cultures with added *P. tricornutum* exudates (very rich in cysteine-like compounds) and with added thioacetamide, where some inhibition of the growth was also observed. As mentioned above, the available data are insufficient for allowing to conclude whether this is only a consequence of the lower cell growth (which results in more metal available per cell) or whether cysteine favoured the metal uptake, as would be the case if cysteine complexes were important.

Fig. 7 and Table 3 also show that for all the metals studied and for the different media, the uptake ($[M]_{cell}$) measured during stationary phase of the cultures was a linear function of the respective initial

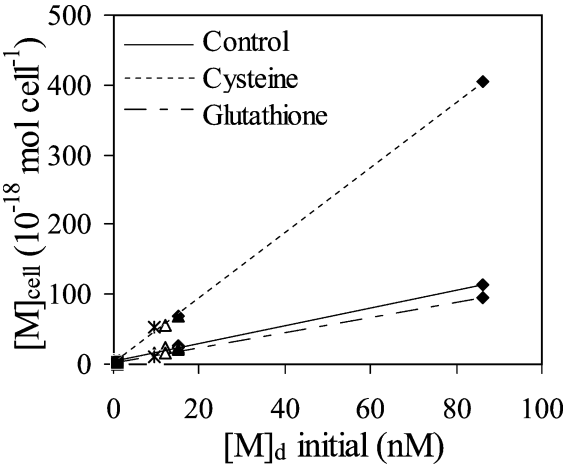


Fig. 7. Cellular metal concentrations ($[M]_{cell}$) as a function of the initial $[M]_d$ for three cultures of *E. huxleyi* at day 10 of growth. (*) Cu, (□) Pb, (■) Cd, (◊) Zn, (♦) Fe, (▲) Mn, (△) Ni, and (●) Co.

Table 3
Cellular metal ($[M]_{cell}$, 10^{-18} mol cell^{-1}) to initial dissolved metal ($[M]_d$, nM) concentration ratios for the cultures of *E. huxleyi* after 10 days of growth

Metals		Cu	Pb	Cd	Zn	Fe	Mn	Ni	Co
	$[M]_d$[a] (nM)	$[M]_{cell}$ (10^{-18} mol cell^{-1}) **$[M]_{cell}/[M]_d$ (10^{-9} cell^{-1} l^{-1})**							
Cu	9.5 ± 0.6	16 ± 1[b]; 53 ± 3[c]; 11 ± 1[d] **1.6[b]; 5.6[c]; 1.2[d]**							
Pb	1.1 ± 0.1		1.9 ± 0.1[b]; 5.5 ± 0.4[c]; 1.6 ± 0.1[d] **1.7[b]; 5.0[c]; 1.4[d]**						
Cd	1.2 ± 0.1			2.1 ± 0.2[b]; 5.5 ± 0.3[c]; 1.5 ± 0.2[d] **1.8[b]; 4.6[c]; 1.2[d]**					
Zn	15 ± 1				25 ± 2[b]; 68 ± 3[c]; 23 ± 1[d] **1.7[b]; 4.5[c]; 1.5[d]**				
Fe	86 ± 3					113 ± 6[b]; 405 ± 15[b]; 95 ± 4[b] **1.3[b]; 4.7[c]; 1.1[d]**			
Mn	15 ± 2						24 ± 2[b]; 69 ± 4[c]; 21 ± 2[d] **1.6[b]; 4.6[c]; 1.4[d]**		
Ni	12 ± 1							23 ± 1[b]; 55 ± 2[c]; 17 ± 1[d] **1.9[b]; 4.6[c]; 1.4[d]**	
Co	0.79 ± 0.05								1.2 ± 0.2[b]; 3.4 ± 0.2[c]; 0.93 ± 0.06[d] **1.5[b]; 4.3[c]; 1.2[d]**

[a] Determined just after inoculation.
[b] In natural seawater (control).
[c] In seawater enriched with 150 nM cysteine.
[d] In seawater enriched with 100 nM glutathione.

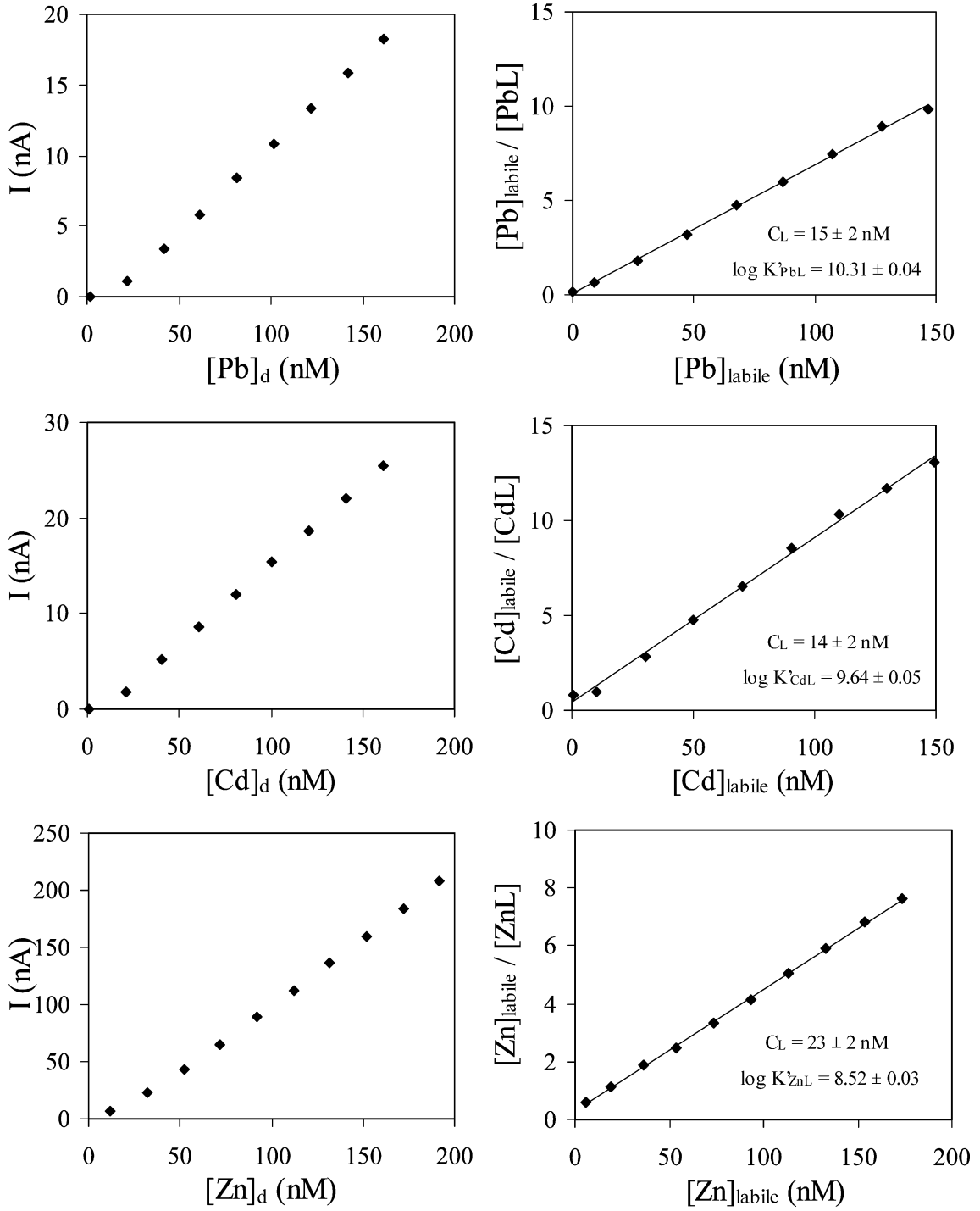


Fig. 8. Pb-, Cd- and Zn-complexing ligand titration curves obtained in the control medium, before the growth of *E. huxleyi* (day 0).

external total metal concentration ($[M]_d$), all the metals obeying the same function, in each specific medium, independently of their natures. For instance, for the control medium, the equation $[M]_{cell}=(1.29 \pm 0.04)\ [M]_d+(3 \pm 1)$ ($R^2=0.994$, $n=8$) fits data for Cu, Pb, Cd, Zn, Fe, Mn, Ni and Co. This characteristic could be explored in the future for use of *E. huxleyi* as a bioindicator of trace metals in the natural system.

3.3.3. Cu, Pb, Cd, and Zn speciation

Fig. 8 shows examples of metal complexing ligand titrations in the control medium before the inoculation of the algae (day 0 of growth). For Cu, the curves were identical to those shown in Fig. 2. The determined concentrations of ligands with capability to bind Cu, Zn, Pb and Cd in the control medium were initially 58, 24, 15 and 14 nM, respectively, and increased during the culture growth: after 10 days, they were (in nM): 122 Cu, 89 Zn, 59 Pb, and 47 Cd. Therefore, the liberation of organic compounds with capability to bind Zn, 65 nM, and Cu, 64 nM, seemed to be more extensive than that with capability to bind Pb, 44 nM, and Cd, 33 nM.

The determined values of K'_{ML} for the different metals did not vary significantly during the *E. huxleyi* growth and were, by a decreasing order of magnitude: $\log K'_{ML}=12.27\pm0.07$, Cu>$10.0\pm0.3$, Pb>$9.62\pm0.04$, Cd>$8.6\pm0.1$, Zn. Values of similar magnitude have been found before in sea and estuarine waters (Muller, 1996; Kozelka and Bruland, 1998; van den Berg, 1985). Thus, exudates released by *E. huxleyi* have similar metal complexing strength to the ligands present in seawater, suggesting that a major fraction of the ligands in seawater may be derived from algae (and possibly bacteria).

Inorganic/organic metal speciation was calculated and it was found that more than 99.9% of Cu was organically bound, Zn was 72% organically bound at day 0 and 94% at day 10, Pb 82% at day 0 and 95% at day 10 and Cd 61% at day 0 and 85% at day 10. These results confirm the importance of natural organic ligands on the speciation of these trace metals in oceanic waters (Buckley and van den Berg, 1986; van den Berg, 1985; Capodaglio et al., 1990; Bruland, 1992).

A direct relationship between the initial total dissolved concentration of each metal ($[M]_d$ (nM): 15 Zn, 9.5 Cu, 1.2 Cd, and 1.1 Pb) and the amount (nM) of released metal complexing ligands (65 Zn, 64 Cu, 33 Cd, and 44 Pb) were not found in the present study. Considering only the fraction of each metal not organically bound, a similar conclusion can be taken. Therefore, the present data did not allow to conclude if either the released ligands are specific of a metal or if they were released as an answer to a specific metal.

3.4. Role of the released thiols on the total trace metal ligands

The concentrations of the combined thiol compounds identified in the different cultures of the first set of experiments (I in Fig. 1) were directly related to that of the total Cu ligand concentration present in the media (see Fig. 9): $[Thiols]_t=(0.87 \pm 0.06)C_L+(2 \pm 8)$, $R^2=0.896$, $t_R=14.6$ and $t_{(P=0.05)}=2.04$. Statistically significant correlations were also found when the data for each medium were treated separately, except for the culture with *P. tricornutum* exudates. The similar magnitude and the covariation of the total organic ligand and total thiol concentrations suggest that these thiol

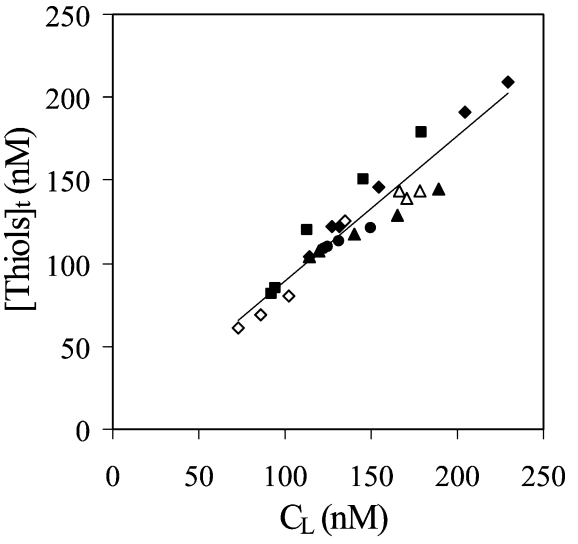


Fig. 9. Relationship between the thiol concentrations ($[Thiols]_t$) and the concentrations of Cu-complexing ligands (C_L) observed in the *E. huxleyi* cultures. Seawater (◊) natural, and enriched with (▲) *E. huxleyi* exudates, (△) *P. tricornutum* exudates, (■) *Porphyra* exudates, (♦) *Enteromorpha* exudates and (●) 50 nM of thioacetamide.

compounds may be major contributors to the organic Cu binding ligands released by *E. huxleyi* and other eukaryotic algae. In the present case, the measured thiols account with about 87% of the total Cu organic ligands.

In addition, the conditional stability constants for Cu(II)–cysteine and Cu(II)–glutathione complexes in seawater (determined for synthetic ligands) were found to be (log value) 12.68 and 12.44, respectively (Leal and van den Berg, 1998). These values are very similar to log K'_{CuL} values obtained in the present work. These results are consistent with thiol compounds, like cysteine and glutathione, being probably the major part of the Cu-complexing organic ligands released by *E. huxleyi.*

As it was observed for Cu in experiment I and also in experiment II, the concentrations of thiol compounds increased with increases in the determined total ligand concentrations for the different metals. For the control culture (the only tested for this purpose), the equations were: $[\text{Thiols}]_t=(1.07 \pm 0.09)C_L(\text{Zn})+20 \pm 5$, $R^2=0.981$; $[\text{Thiols}]_t=(1.52 \pm 0.08)C_L(\text{Pb})+24 \pm 3$, $R^2=0.993$; $[\text{Thiols}]_t=(2.01 \pm 0.08)C_L(\text{Cd})+22 \pm 2$, $R^2=0.995$; $n=5$ in all cases. For Cu, the parameters of the equation $[\text{Thiols}]_t=f(C_L)$ were statistically identical to those observed in experiment I. Interestingly, the values of the slope were close to 1 for Zn and for Cu and much higher for Pb (1.5) and particularly for Cd (2.0). The values of the slopes observed for Cu and Zn are compatible with the predominance of thiol compounds among the Cu and Zn organic ligands since a stoichiometry of 1:1 is considered. The higher slopes obtained for Pb and Cd will be compatible with predominance of thiol ligands only admitting a stoichiometry of 1:2 (M/L). The fact that Pb and Cd concentrations are one order of magnitude lower than those of Zn and Cu supports this hypothesis since the excess of organic ligands in the media are higher for Pb and Cd. Nevertheless, this interpretation is speculative; more research is being required before coming to a conclusion on this topic.

4. Conclusions

The present work was pioneer in demonstrating that exudates produced by a specific eukaryotic algal species, in the case of the diatom *P. tricornutum*, are able to inhibit the growth rate and final cell yield of other eukaryotic algae, the coccolithophore *E. huxleyi* in batch cultures. In contrast, exudates produced by the green macroalgae *Enteromorpha* improved the final cell yield and slightly the growth rate of *E. huxleyi*.

The findings reported here for oceanic algae have some similarities with exudates of certain terrestrial flora, which are able to inhibit the growth of other floral species.

The improvement of *E. huxleyi* final yield seems to be related with the presence in the culture medium of relatively high concentrations of glutathione-like compounds (i.e. chemical species identified by CSV as glutathione), which were produced by *Enteromorpha*. The *E. huxleyi* growth inhibition seems to result to the relatively high concentrations of cysteine-like compounds produced by *P. tricornutum*. In fact, additions of synthetic cysteine and glutathione to the *E. huxleyi* cultures originated changes in the growth similar to those caused by the mentioned exudates.

The nature and concentration of the organic compounds present in the culture medium also influenced, directly or indirectly, trace metal uptake and the concentration and composition of the exudates produced by *E. huxleyi*. These are important achievements but the concerned mechanisms are still unknown and deserve further investigation.

The implications of these findings on algal biodiversity in the surface oceans are extremely important. It can be speculated that a bloom of a species of algae that produces large amounts of specific exudates may favour or inhibit the local growth of other algal species and, in an extreme situation, change the local biodiversity.

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References

Al-Farawati, R., van den Berg, C.M.G., 1997. The determination of sulfide in seawater by flow-analysis with voltammetric detection. Mar. Chem. 57, 277–286.

Azenha, M.A.G.O., Vasconcelos, M.T.S.D., Cabral, J.P.S., 1995. Organic ligands reduce copper toxicity in *Pseudomonas syringae*. Environ. Toxicol. Chem. 14, 369–373.

Biersmith, A., Benner, R., 1998. Carbohydrates in phytoplankton and freshly produced dissolved organic matter. Mar. Chem. 63, 131–144.

Bruland, K.W., 1992. Complexation of cadmium by natural organic ligands in the central North Pacific. Limnol. Oceanogr. 37 (5), 1008.

Buckley, P.J.M., van den Berg, C.M.G., 1986. Copper complexation profiles in the Atlantic Ocean. Mar. Chem. 19, 281–296.

Campos, M.L.A.M., van den Berg, C.M.G., 1994. Determination of copper complexation in sea water by cathodic stripping voltammetry and ligand competition with salicylaldoxime. Anal. Chim. Acta 284, 481–496.

Capodaglio, G., Coale, K.H., Bruland, K.W., 1990. Lead speciation in surface waters of the Eastern North Pacific. Mar. Chem. 29, 221.

Carvalho, R.A., Benfield, M.C., Santschi, P.H., 1999. Comparative bioaccumulation studies of colloidally complexed and free-ionic heavy metals in juvenile brown shrimp *Penaus aztecus* (Crustacea: Decapoda: Penaeidae). Limnol. Oceanogr. 44 (2), 403–414.

Coale, K.H., Bruland, K.W., 1988. Copper complexation in the Northeast Pacific. Limnol. Oceanogr. 33, 1084–1101.

Colombo, C., van den Berg, C.M.G., 1997. Simultaneous determination of several trace metals in seawater using cathodic stripping voltammetry with mixed ligands. Anal. Chim. Acta 337, 29–40.

Cotté-Krief, M.-H., Guieu, C., Thomas, A.J., Martin, J.-M., 2000. Sources of Cd, Cu, Ni and Zn in Portuguese coastal waters. Mar. Chem. 71 (3–4), 199–214.

Croot, P.L., Karlson, B., van Elteren, J.T., Kroon, J.J., 1999. Uptake of 64Cu-Oxine by marine phytoplankton. Environ. Sci. Technol. 33, 3615–3621.

Donat, J.R., Bruland, K.W., 1990. A comparison of two voltammetric techniques for determining zinc speciation in Northeast Pacific waters. Mar. Chem. 28, 301–323.

Hering, J.G., Morel, F.M.M., 1990. Kinetics of trace metal complexation: role of alkaline-earth metals. Environ. Sci. Technol. 24, 242–252.

Kaplan, D., Christiaen, D., Malis-Arad, S., 1987. Binding of heavy metals by algal carbohydrates. Algal Biotechnology. Elsevier, London, pp. 179–187.

Karman, C.C., Jak, R.G., 1997. Evaluation of the applicability of risk assessment methodologies for essential elements. TNO-MEP-R97/306. TNO-report. TNO Institute of Environmental Sciences, Energy Research and Process Innovation, The Netherlands.

Kozelka, P.B., Bruland, K.W., 1998. Chemical speciation of dissolved Cu, Zn, Cd, Pb in Narragansett Bay, Rhode Island. Mar. Chem. 60, 267–282.

Leal, M.F.C., van den Berg, C.M.G., 1998. Evidence for strong copper(I) complexation by organic ligands in seawater. Aquat. Geochem. 4, 49–75.

Leal, M.F.C., Vasconcelos, M.T.S.D., Sousa-Pinto, I., Cabral, J.P.S., 1997. Biomonitoring with benthic macroalgae and direct assay of toxic metals in seawater of the Oporto coast (Northwest Portugal). Mar. Pollut. Bull. 34 (12), 10006–11015.

Leal, M.F.C., Vasconcelos, M.T.S.D., van den Berg, C.M.G., 1999. Copper induced release of complexing ligands similar to thiols by *Emiliania huxleyi* in seawater cultures. Limnol. Oceanogr. 44 (7), 1750–1762.

Lee, J.G., Roberts, B., Morel, F.M.M., 1995. Cadmium: a nutrient for the marine diatom *Thalassiosira weisflogii*. Limnol. Oceanogr. 40 (6), 1056–1063.

Le Gall, A., van den Berg, C.M.G., 1993. Cathodic stripping voltammetry of glutathione in natural waters. Analyst 118, 1411–1415.

Manping, Z., Bosh, G., Shengbin, Z., Liansheng, L., 1990. Heavy metal complexation capacity of the South China seawater. Chin. J. Oceanol. Limnol. 8, 158–166.

Mart, L., Rutzel, H., Klahre, P., Sipos, L., Platzek, U., Valenta, P., Nurnberg, H.W., 1982. Comparative studies on the distribution of heavy metals in the oceans and coastal waters. Sci. Total Environ. 26, 1–17.

Moffett, J.W., Brand, L.E., Zika, R.G., 1990. Distribution and potential sources and sinks of copper chelators in the Sargasso Sea. Deep-Sea Res. 37, 27–36.

Muller, F.L.L., 1996. Interactions of copper, lead and cadmium with the dissolved, colloidal and particulate components of estuarine and coastal waters. Mar. Chem. 52, 245–268.

Munda, I.M., Hudnik, V., 1991. Trace metal content in some seaweeds from the Northern Adriatic. Bot. Mar. 34, 241–249.

Phinney, J.T., Bruland, K.W., 1994. Uptake of lipophilic organic Cu, Cd, and Pb complexes in the Coastal diatom *Thalassiosira weissflogii*. Environ. Sci. Technol. 28 (11), 1781–1790.

Shea, D., MacCrehan, W.A., 1988. Determination of hydrophilic thiols in sediment porewater using ion-pair liquid cromatography coupled to electrochemical detection. Anal. Chem. 60, 1449–1454.

Simkiss, K., Taylor, M.G., 1989. Metal fluxes across the membranes of aquatic organisms. Vertbr. Aquat. Sci. 1, 173–188.

Stein, J.R., 1973. Culture methods and growth measurements, isolation and purification (section I). Handbook of Phycological Methods. University Press, Cambridge.

Sunda, W.G., 1989. Trace metal interactions with marine phytoplankton. Biol. Oceanogr. 6, 411–442.

Sunda, W.G., Huntsman, S.A., 1996. Antagonisms between cadmium and zinc toxicity and manganese limitation in a coastal diatom. Limnol. Oceanogr. 41 (3), 373–387.

Sunda, W.G., Huntsman, S.A., 1998. Interactions among Cu^{2+}, Zn^{2+}, and Mn^{2+} in controlling cellular Mn, Zn, and growth rate in the coastal alga *Chlamydomonas*. Limnol. Oceanogr. 43 (6), 1055–1064.

Tappin, A.D., Millward, G.E., Statham, P.J., Burton, J.D., Morris, A.W., 1995. Trace metals in the central and southern North Sea. Estuarine, Coastal Shelf Sci. 41, 275–323.

Tubbing, D.M.J., Admiraal, W., Cleven, R.F.M.J., Iqbal, M., van de Meent, D., Verweij, W., 1994. The contribution of complexed

copper to the metabolic inhibition of algae and bacteria in synthetic media and river water. Water Res. 28, 37–44.

Turner, D.R., Whitfield, M., Dickson, A.G., 1981. The equilibrium speciation of dissolved components in freshwater and seawater at 25 °C and 1 atm pressure. Geochim. Cosmochim. Acta 45, 855–882.

Vairavamurthy, A., Mopper, K., 1990. Field method for determination of traces of thiols in natural waters. Anal. Chim. Acta 236, 363–370.

van den Berg, C.M.G., 1982. Determination of copper complexation with natural organic ligands in seawater by equilibration with MnO_2: I. Theory. Mar. Chem. 11, 307–322.

van den Berg, C.M.G., 1984. Organic and inorganic speciation of copper in the Irish Sea. Mar. Chem. 14, 201–212.

van den Berg, C.M.G., 1985. Determination of the zinc complexing capacity in seawater by cathodic stripping voltammetry of zinc-APDC complex ions. Mar. Chem. 16, 121–130.

van den Berg, C.M.G., Huang, Z.Q., 1984. Determination of iron in seawater using cathodic stripping voltammetry preceded by adsorptive collection with the hanging mercury drop electrode. J. Electroanal. Chem. 177, 269–280.

van den Berg, C.M.G., Househam, B.C., Riley, J.P., 1988. Determination of cystine and cysteine in seawater using cathodic stripping voltammetry in the presence of Cu(I). J. Electroanal. Chem. 239, 137–148.

Vasconcelos, M.T.S.D., Leal, M.F.C., 1997. Speciation of Cu, Pb, Cd and Hg in waters in the Oporto coast in Portugal, using pre-concentration in a chelamine resin column. Anal. Chim. Acta 353, 189–198.

Vasconcelos, M.T.S.D., Leal, M.F.C., 2001a. Seasonal variability in the kinetics of Cu, Pb, Cd and Hg accumulation by macroalgae. Mar. Chem. 74 (1), 65–85.

Vasconcelos, M.T.S.D., Leal, M.F.C., 2001b. Adsorption and uptake of Cu by *Emiliania huxleyi* in natural seawater. Environ. Sci. Technol. 35, 508–515.