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Contrasting complexing capacity of dissolved organic matter produced during the onset, development and decay of a simulated bloom of the marine diatom *Skeletonema costatum*

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Abstract

The capacity of natural dissolved organic matter (DOM) produced during the onset, development and decay of a simulated bloom of the marine diatom *Skeletonema costatum* to complex free copper has been followed for a 2 week period. Copper binding capacity of the culture was measured by anodic stripping voltammetry (ASV) with a hanging mercury drop electrode (HMDE). The concentration of dissolved organic carbon (DOC) and two fluorophores, M (humic-like, Ex/Em: 320 nm/410 nm) and T (protein-like, Ex/Em: 280 nm/350 nm), were followed during the course of the incubation. Models using DOC concentrations alone could not accurately predict the complexing capacity of the culture, especially at the end of the bloom, and better predictions were obtained when fluorescence measurements were considered. They were helpful in characterising two types of copper ligands produced in the culture. The first type, traced by the fluorescence of peak T, was related to labile DOC directly exuded by phytoplankton. The second type, traced by the fluorescence of peak M, was the refractory humic-like material presumably produced *in situ* as a by-product of the bacterial degradation of phytogenic materials. During the onset and development of the bloom (days 0 to 7), the fluorescence of peak T explains 60–80% of the total complexing capacity of the culture, suggesting that exuded "protein-like" compounds among other exuded complexing agents efficiently complexed free copper. On the contrary, during the decay (days 8 to 13), these ligands were replaced by humic substances as the complexing agent for copper.

Keywords: Dissolved organic matter; Phytoplankton culture; Ligands; Fluorescence; Voltammetry

1. Introduction

Marine dissolved organic matter (DOM) is the largest pool of reduced carbon on the Earth's surface (Hedges, 2002). It is composed of a myriad of different compounds, covering a wide range of molecular weights, chemical structures and functions. From the environmental point of view, marine DOM plays several relevant roles (Sunda, 1995), some of them being connected to the fate of metals in the environment: (1) it contributes to the recycling, accumulation, and export of biogenic matter in ocean biogeochemical cycles, (2) it is an electron donor in the photoreduction and solubilisation of iron and manganese and (3) it reduces the toxicity of free metal ions by the formation of stable metal/organic complexes.

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Most of the marine DOM is produced *in situ* from the accumulation and subsequent degradation of the products of synthesis of marine phytoplankton (Biddanda and Benner, 1997; Carlson, 2002), and only 10% originates on the continents (Druffel et al., 1992). Mechanisms of DOM production include direct exudation by marine phytoplankton, cell autolysis in response to nutrient stress conditions or senescence, and zooplankton mediated liberation in connection with an inefficient grazing process (Nagata, 2000). Among the products liberated during the production of marine phytoplankton are photosynthates, essentially carbohydrates and amino acids (Myklestad, 1995; Biddanda and Benner, 1997; Granum et al., 2002), mainly the latter have high capacity to complex free metal ions. In addition, photochemical and bacterial respiration processes give rise to a complex series of poorly understood transformations experienced by marine DOM, globally known as humification (Hedges, 1988; Kieber et al., 1997).

Metal complexation properties of amino acids have been described a long time ago and they have been collected and critically reviewed in several databases (Martell and Smith, 1974; IUPAC series: Kiss et al., 1991; Pettit and Powell, 1993; Berthon, 1995; Yamauchi and Odani, 1996). Most amino acids can act as bidentate metal binding ligands through their α-amino and α-carboxylate groups, and their stoichiometric stability constants are thus very similar to the one from glycine ($\log K_{Cu-L} \sim 8.1$), because their side chains are usually not involved in the metal binding process. These stability constants are low for effectively complex metal ions in natural systems because of their low concentrations, and specially in seawater, due to the competition with other cations and the high ionic strength. However, histidine and cysteine present strong donor groups in their side chains, imidazole and thiol groups, respectively, being much more efficient complexing amino acids. Moreover, proteins with high metal binding properties (e.g., metallothionein, zinc finger peptides, metalloproteins) are also rich in those amino acids (Farkas and Sóvágó, 2002).

Humic and fulvic acids, either of continental or *in situ* origin, are complex high molecular weight substances, with a capacity to complex metal ions that depends on the abundance of carboxylic acid groups per unit weight (Midorikawa and Tanoue, 1998). In seawater, they usually constitute 5–15% of the total DOM concentration in carbon units, ca. 8–25 μM C (Benner et al., 1992), although in estuaries and coastal waters they can reach concentrations as high as 125 μM C (Ishiwatari, 1992; Burney, 1994).

Copper is an essential element for living organisms, a natural component of a variety of mineral salts, and also an element used in many industrial applications and in water piping. Therefore, natural and anthropogenic inputs of copper to the environment are both relevant (Scheinberg, 1991). Although an essential element, copper is one of the most toxic metals for many organisms above certain levels, especially for aquatic invertebrate larvae (His et al., 1999) and phytoplankton species (Sunda and Lewis, 1978).

Increasing attention has been paid to the DOM pool in environmental studies due to its role in buffering the toxicity of metals for aquatic organisms (Campbell, 1995). The inclusion of DOM in water quality criteria has also been recommended (Nor, 1987; Allen and Hansen, 1996). Therefore, understanding how DOM affects metal bioavailability has been a key issue in recent years. Several models have been developed to predict the biological effect of metals in the environment. The biotic ligand model, BLM (Di Toro et al., 2001), combined the free ion activity model with the gill surface interaction model, and thus considers metal speciation in aquatic systems together with the effect of competing cations at the membrane surface of the organisms. Nevertheless, discrepancies have been observed between the predictions made in terms of dissolved organic carbon concentrations (DOC) and the observed bioavailability data, suggesting that further DOM characterisation is needed (Playle, 1998; Ryan et al., 2004). Clear differences in metal complexation properties have been also found in natural organic matter from different sources (Benedetti et al., 1996; Abbt-Braun and Frimmel, 1999) that cannot be explained in terms of their DOC content.

The aim of this work is to study the production and transformation of the DOM originated during the onset, development and decay of a simulated bloom of the marine diatom *Skeletonema costatum* and its implications for their copper binding properties. The fluorescence of DOM, in addition to traditional case variables such as DOC and chlorophyll *a*, is investigated to get a better characterisation of DOM in the culture.

2. Material and methods

2.1. The culture

The marine diatom *S. costatum* was cultured in artificial seawater (Lorenzo et al., 2002) in acid-washed 4-l polycarbonate bottles, with the aim of producing natural DOM. Two replicates, named A and B, were performed. The cultures were kept at 18 °C under a 14 h light:10 h dark photoperiod (70 μmol quanta/m^2 s) with bubbling filtered air (0.45 μm PTFE, polytetrafluoroethylene), and gentle magnetic stirring was provided when cell density increased. On the initial day, the artificial seawater was enriched with half of the nutrients of a standard f/2 media (Guillard, 1975)

without added EDTA (ethylenediaminetetraacetic acid). The initial concentrations of N and P were 700 μM and 18 μM, respectively. Both bottles were inoculated with a non-axenic culture of *S. costatum* in exponential growth phase to reach a final density of 10^4 cells/ml. The pH of the medium was kept between 8.05 and 8.25 by adding diluted HCl when necessary. All reagents used were analytical-grade quality, to minimize the metal content of the culturing media. The cultures were monitored along the experiment following the time course of two state variables:

(1) Chlorophyll *a* (Chl *a*) concentration: 20 ml of the culture were filtered through Millipore APFF glass-fibre prefilters for total Chl *a* determination. Samples were kept at −20 °C until analyses. Chl *a* was extracted in 90% acetone at −20 °C for ca. 24 h. Samples were analysed using a Turner TD-700 fluorometer calibrated against pure Chlorophyll *a* (Sigma).
(2) Cell density was assessed daily using a Neubauer haemocytometer.

2.2. Filtration procedures

About 300 ml of each culture were gently filtered (~150 mm Hg vacuum) to minimize cell lysis, through acid cleaned 0.22 μm Millipore PES (polyethersulphone) filters. Acid cleaned polycarbonate filter holders were used to avoid metal contamination. The filtrate was taken every 2 days during the culture and it was used for dissolved organic carbon (DOC) and fluorescence of dissolved organic matter (FDOM) analyses and copper titrations.

2.3. Dissolved organic carbon (DOC)

The filtrate was collected into 10 ml precombusted (450 °C, 12 h) glass ampoules. After acidification with H_3PO_4 to pH <2, the ampoules were heat-sealed and stored in the dark at 4 °C until analysis. DOC was measured with a Shimadzu TOC-5000 organic carbon analyser. The system was standardized with solutions of potassium hydrogen phthalate. The concentration of DOC was determined by subtracting the average peak area from the instrument blank area and dividing by the slope of the standard curve. The CV was ~1% and the accuracy of the measurements was successfully tested with the TOC reference materials provided by Prof. D. Hansell (University of Miami).

2.4. Fluorescence of dissolved organic matter (FDOM)

Aliquots of the DOC filtrate were collected before Cu addition into 20 ml acid cleaned polycarbonate flasks and frozen at −20 °C until analysis. FDOM of these samples was measured with a Perkin Elmer LS 55 Luminescence spectrometer. The instrument was equipped with a xenon discharge lamp, equivalent to 20 kW for 8 μs duration, and a 1 cm quartz fluorescence cell. Milli-Q water was used as a reference for fluorescence analysis, and the intensity of the Raman peak was checked regularly. Discrete excitation/emission pair measurements were performed at peaks M (marine humic substances, Ex/Em: 320 nm/410 nm) and T ("protein-like substances", Ex/Em: 280 nm/350 nm), specifically tryptophan (Coble, 1996; Nieto-Cid et al., 2005; Stedmon and Markager, 2005). Four replicate measurements were performed for each Ex/Em wavelength pairs. A four point standard curve was prepared daily with a mixed standard of quinine sulphate (QS) and tryptophan (Trp) in 0.05 mol/l sulphuric acid (concentrations from +0 to +150 ppb and from +0 to +100 ppb for QS and Trp, respectively; Nieto-Cid et al., 2005). The equivalent concentration of every peak was determined by subtracting the average peak height from the blank height, and dividing by the slope of the standard curve. Fluorescence units were expressed in ppb equivalents of QS (ppb eq QS) for $FDOM_M$ and ppb equivalents of Trp (ppb eq Trp) for $FDOM_T$. Note that ppb eq QS is identical to the quinine sulphate units (QSU) reported previously in other studies. The precision of the fluorescence measurements was ±0.1 ppb eq QS and ±0.6 ppb eq Trp, respectively.

In addition to these measurements, a trial was performed with the filtrate from the sixth day with different additions of Cu (ranging from 0 to 5 μM) to check the effect of Cu complexation in the different fluorophores. Twenty-milliliter samples were spiked with increasing Cu concentrations and allowed to equilibrate for 24 h in the dark. After that time, $FDOM_M$ and $FDOM_T$ were measured.

2.5. Cu measuring conditions

Square wave anodic stripping voltammetry (ASV) analyses were carried out with a hanging mercury drop electrode, a Ag/AgCl reference and a Pt-rod auxiliary electrode held in a Metrohm 663 VA polarographic stand coupled to an Eco-Chemie AutoLab PGSTAT10 potentiostat. Samples were placed in a 20 °C thermostatic glass cell. After solutions had been purged for 250 s with N_2, copper was accumulated on a mercury drop of 0.52 mm^2 at −0.55 V for 15 s at the maximum stirring speed (3000 rpm) and 10 s of equilibration were allowed before the voltage scan. The conditions of the square-wave (SW) scan were an initial potential of −0.55 V, an SW amplitude of 25 mV, an SW frequency of 25 Hz and a scan increment of 2 mV. Three voltammograms were

recorded for each solution. To minimize potential effects of adsorption of Cu to cell walls, cells were only washed with sample solution during each titration. Samples with increasing Cu additions were measured in order, starting from the culture filtrate with no Cu addition.

2.6. Copper titrations

Between 13 and 16 samples of 16 ml culture filtrates were dosed in 20 ml polypropylene vials. Solutions were stored at −20 °C until analyses. Before analyses,

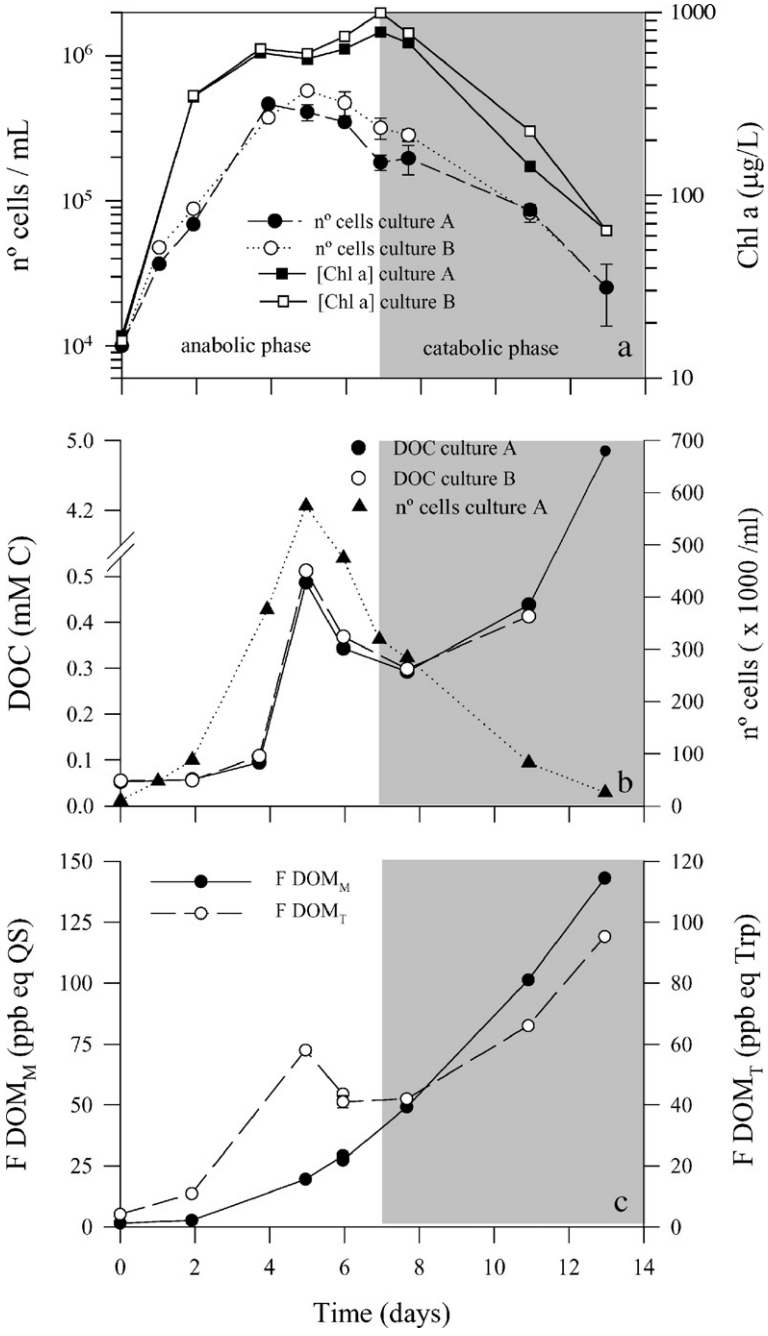

Fig. 1. Time evolution of the *Skeletonema costatum* culture and the dissolved organic matter concentration and composition. (a) Number of cells measured in cultures A and B. Error bars represent the 95% confidential intervals of the mean of three replicates. (b) DOC concentration in cultures A and B compared with cell numbers. (c) Fluorescence characterisation of the DOM pool: $FDOM_M$ is the fluorescence of humic substances in quinine sulphate units (ppb eq QS), and $FDOM_T$ is the fluorescence of the aromatic amino acids in tryptophan units (ppb eq Trp).

increasing Cu additions (from 0 to 5 μmol/l) were made to each set of samples from a diluted spectrophotometric Cu standard solution (1000 ppm, Panreac). Solutions were kept 24 h in the dark to allow the equilibration of the complexation reaction. All additions were prepared and analyses were performed on a laminar flow, class 100 filtered air, clean bench. All plastic lab ware was kept in 5% HNO_3 at least 24 h and rinsed 5 times with deionised water purified by ion exchange (resistivity ≥ 18.2 MΩ/cm; Milli-Q®) before use.

The peak height of each voltammogram was measured, and it was transformed into labile Cu concentrations ([Cu′]) dividing by the slope of the linear segment of each titration (Donat et al., 1994). Labile copper is the equivalent concentration of copper that contributes to the voltammetric signal to the same extent as the free metal ion (Muller et al., 2003). The titration curves were explained assuming the simplest complexation model; that is, only one type of ligand and a reaction stoichiometry of 1:1. Titration plots were fitted to Eq. (1), obtained from the theoretical complexation model previously explained (Lorenzo et al., 2002):

$$Cu' = \frac{-a + \sqrt{a^2 + 4Cu_T/K'}}{2} \quad (1)$$

where $a = (-Cu_T + L + 1/K')$, and Cu_T and Cu′ are the total and labile copper concentrations (mol/l), respectively, L is the total ligand concentration (mol/l) and K' is the conditional stability constant of the Cu–L complexes, valid for the conditions of the experimental medium. This

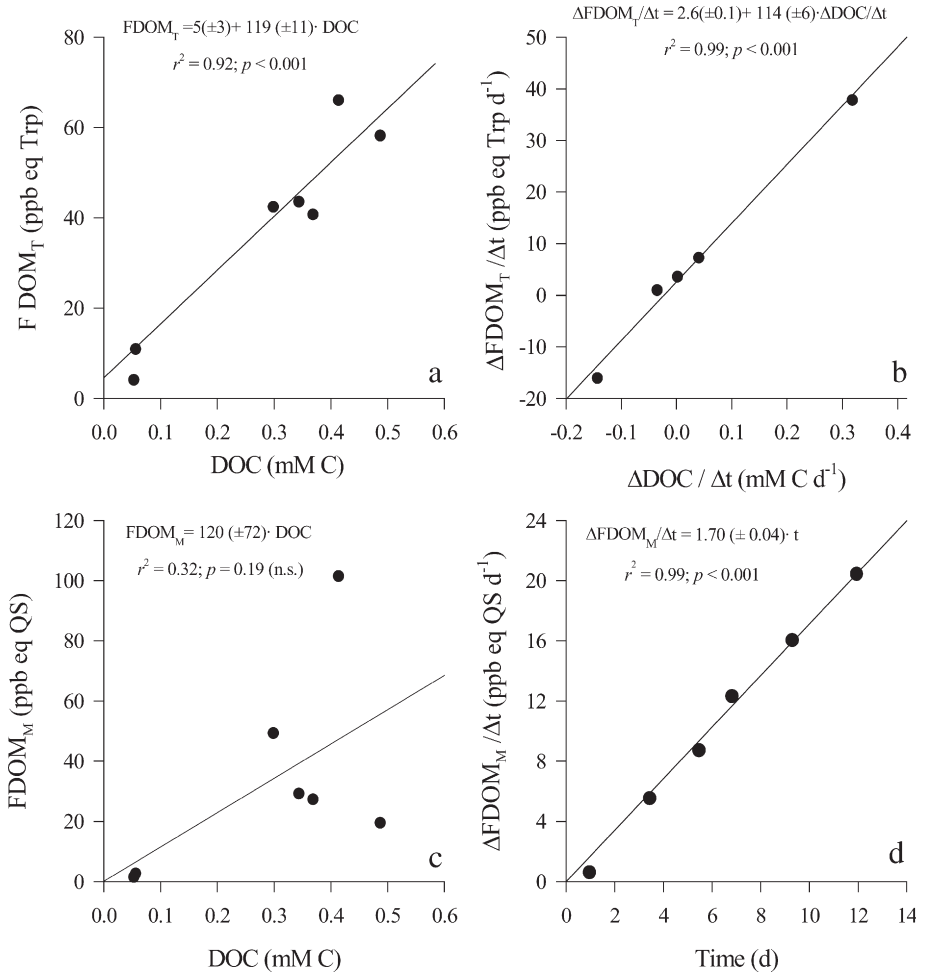

Fig. 2. Evolution of the measured fluorophores during the culture. (a) Comparison of $FDOM_T$ and DOC; (b) comparison of net production rates of $FDOM_T$ and DOC along the culture; (c) comparison of $FDOM_M$ and DOC; and (d) $FDOM_T$ net production rate versus time. Regression lines were fitted to model II in (a) and (b), and to model I in (c) and (d), respectively. The regression equations (± standard error), the correlation coefficients, and the significance of the relationships are also presented.

K' is referred to labile copper concentrations, not to free copper ion concentrations. It is necessary to consider inorganic copper speciation in the medium for converting K' to $K'_{Cu\ 2+}$ (Donat et al., 1994).

$$K'_{Cu2+} = K' \cdot \alpha_{Cu2+} \quad (2)$$

where $\alpha_{Cu\ 2+}$ is the inorganic reaction side coefficient, which accounts for the inorganic copper speciation. A value of 36 was assumed (Leal et al., 1999) for calculations.

2.7. Statistical analysis

The best-fit between any couple of variables (X, Y) was obtained minimizing the function:

$$\sum_i \left[(X_i - \hat{X}_i)^{w_X} \times (Y_i - \hat{Y}_i)^{w_Y}\right]^2 \quad (3)$$

where w_X and w_Y are weights for X and Y respectively, with $w_X, w_Y \geq 0$ and $w_X + w_Y = 1$. Any regression model can be expressed as one of two extreme cases: (a) model I, which should be applied when $w_X = 0$, $w_Y = 1$ and (b) model II when $w_X = w_Y = 0.5$ (Sokal and Rohlf, 1995).

Statistical analyses were performed with STATISTICA version 6.0 (StatSoft). The parameters of the complexation model in Cu titrations were estimated by means of non-linear fitting procedures with Sigma Plot 2001 version 7.0 (SPSS), which uses iterative processes for minimizing the sum of the squares of the residuals by means of the Marquardt-Levenberg algorithm.

3. Results

3.1. Onset, development and decay of the cultured S. costatum bloom

Fig. 1 shows the time evolution of the culture. Similar patterns were observed in both flasks (A and B) for the different measurements. Fig. 1a shows the logarithmic increase in the number of cells and chlorophyll *a* (Chl *a*) concentration during the initial 4 days. No lag time was observed in the culture growth. From days 4 to 6, the number of cells remained stationary, and after day 8 there was a continuous decrease in cell density. The maximum Chl *a* concentration (700–1000 μg/l) was achieved at day 7, and after that, a continuous decrease was also observed for Chl *a* levels.

Fig. 1b shows the high correlation between DOC accumulation in the culture and cell density during days 0 to 7 (the anabolic phase of the bloom). There was a continuous increase in the DOC content of the culture from the initial day, but this increase was much higher from days 4 to 5. After day 5, a clear DOC decrease was observed. A large discrepancy was observed after day 8 (named the catabolic phase of the bloom), when cell density decreased whereas the DOC content increased, especially at the end of the culture (note the change of scale). High bacterial densities were observed after day 4

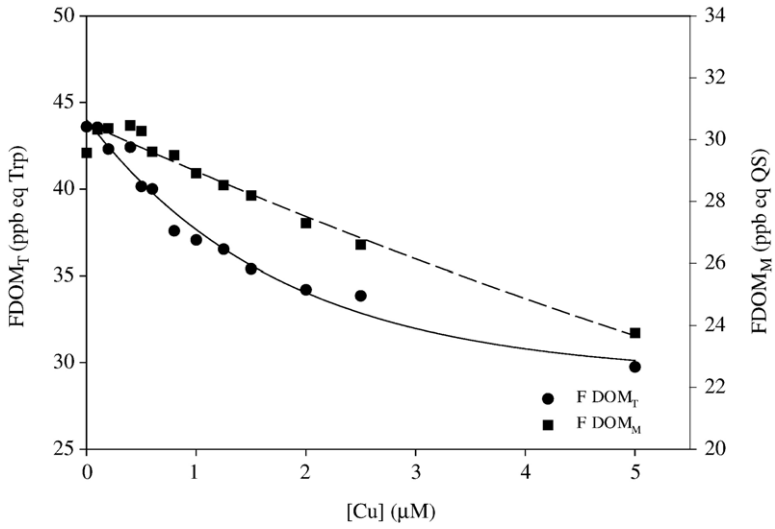

Fig. 3. Fluorescence quenching in the presence of copper. Filtered samples from the sixth day of the culture were spiked with increasing Cu concentrations and fluorescence of "humic-like" substances ($FDOM_M$) and of "protein-like" substances ($FDOM_T$) was measured after 24 h. Fittings of observed data to exponential decay equations are also presented.

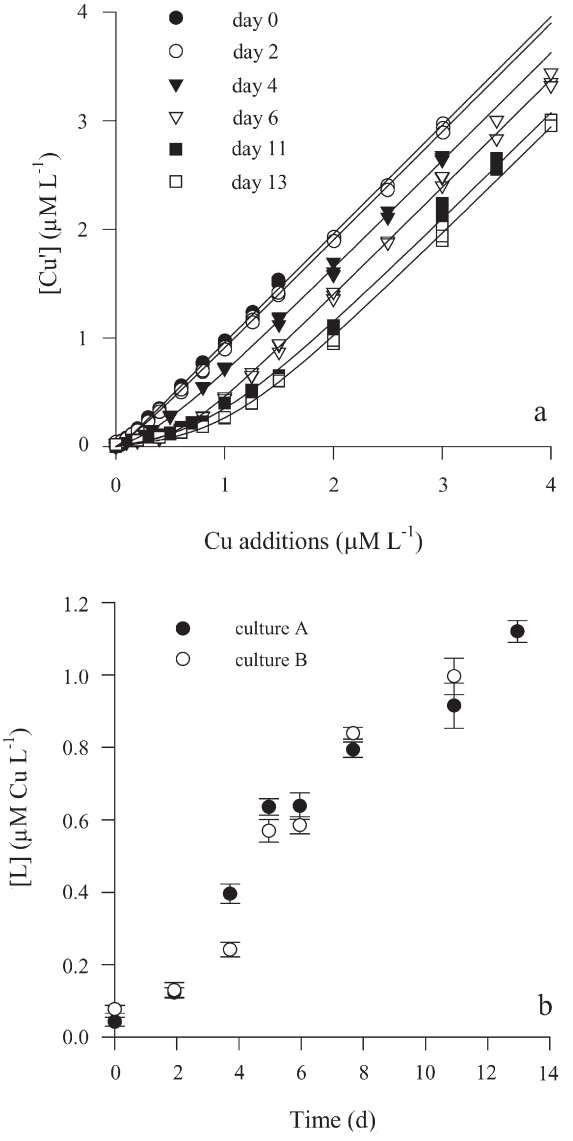

Fig. 4. (a) Time evolution of Cu titrations of the filtered culture. Solid lines represent the best non-linear squares fittings of the data to Eq. (1). (b) Time evolution of metal ligand concentration in the culture. Error bars represent the 95% confidential intervals obtained from the standard errors of the fittings.

(retained in 0.22 μm filters), and one sample was incubated to test bacterial consumption of the DOC in the culture. One aliquot of the culture in day 5 was filtered through a 5 μm pore size GF/F filter, which removes phytoplankton but not bacteria, and incubated for 24 h in the same culture conditions. A DOC decrease from 0.50 to 0.14 mM C was observed.

Fig. 1c shows the evolution of the two fluorophores measured during the culture. $FDOM_T$ reproduced the same temporal pattern than the DOC concentration (Fig. 2a). A high correlation was observed between both variables, but the correlation was even higher when production/degradation rates of $FDOM_T$ and DOC were compared up to day 11 (Fig. 2b). $FDOM_M$ showed a completely different pattern. A monotonic increase was observed in the culture and $FDOM_M$ was not significantly correlated with DOC (Fig. 2c). The same result was observed when comparing $FDOM_M$ and DOC production rates. However, $FDOM_M$ production rates were highly correlated with time (Fig. 2d).

A significant decrease in fluorescence was observed when Cu additions were made to samples containing both fluorophores (Fig. 3). $FDOM_T$ was more affected by Cu additions than $FDOM_M$, and a 32% versus a 20% fluorescence decrease were measured, respectively.

3.2. Evolving copper binding capacity of DOM produced by the culture

Fig. 4a shows the evolution of Cu titration plots in the culture over time. Some specific days were selected to clearly show the shift in the plots to the right, due to the increase in Cu complexing capacity. The complexation model previously described (Eq. (1)) accurately fitted the data, and the obtained r^2 ranged between 0.997 and 0.9996, even when individual measurements and no mean values where used for fitting purposes.

Copper titration fitting parameters (±95% confidential interval, CI) are shown in Table 1. There was a good agreement between the parameters obtained in both cultures, as previously observed with DOC measurements (Fig. 2b). There was a marked increase in ligand concentration over time (see also Fig. 4b). The ligand increase showed an intermediate trend to those observed for both fluorophores: an initial and sharp increase at the end of the exponential growth (days 4–5) coinciding with $FDOM_T$ and no decrease between days 5 and 8, as

Table 1
Complexing capacity (L±95% CI, μmol Cu/l) and conditional stability constant ($\log K'_{Cu\ 2+}$±95% CI) in cultures A and B at different time intervals; *n*, number of copper additions in each titration

Time (days)	L_A (μmol Cu/l)	$\log K'_{Cu\ 2+}$	n	L_B (μmol Cu/l)	$\log K'_{Cu\ 2+}$	*n*
0	0.04±0.01	9.5±2	12	0.08±0.01	9.4±0.9	14
2	0.12±0.01	8.4±0.2	15	0.13±0.02	8.6±0.5	15
4	0.40±0.03	8.2±0.2	12	0.24±0.02	8.4±0.2	15
5	0.64±0.02	8.60±0.02	15	0.57±0.03	8.2±0.1	13
6	0.64±0.04	8.64±0.04	16	0.58±0.02	8.5±0.1	16
8	0.79±0.02	9.0±0.2	14	0.84±0.02	8.82±0.09	15
11	0.92±0.06	8.3±0.2	14	1.00±0.05	8.3±0.1	14
13	1.12±0.03	8.41±0.09	12			

$FDOM_M$. Day 8 showed statistically higher conditional stability constants, coinciding with the initiation of the catabolic phase of the culture. The ligand capacity at the initial day of the culture was very low, which was also reflected in the low confidence of the obtained K' value.

The sample from day 5 incubated for bacterial consumption was also titrated with Cu. The ligand concentration in the incubated sample decreased from 0.64±0.02 to 0.38±0.04 μM, i.e. a 40% decrease. This result contrasts with the 72% decrease of DOC concentration in the same sample.

3.3. DOM composition versus Cu binding capacity

Fig. 5a shows the ligand concentration measured during the culture versus the DOC content in the filtrates. In the anabolic phase, a significant correlation was observed between the DOC concentration and the metal binding capacity (ligand concentration) of the culture. Up to 70% of the ligand variability could be explained by the DOC concentration. However, when the catabolic phase of the culture was considered, the regression did not predict the ligand concentration. A sharp increase in ligand concentration was observed between days 8 and 11 coinciding with a clear decrease in DOC. The opposite trend was observed on day 13, when a 10-fold increase in DOC was produced, but just a small increase in ligand concentration was observed.

The two fluorophores were used to predict the ligand concentration in the cultures (see Fig. 5b and c). $FDOM_T$ was the best predictive variable: 88% of the variability of the ligand concentration in the anabolic

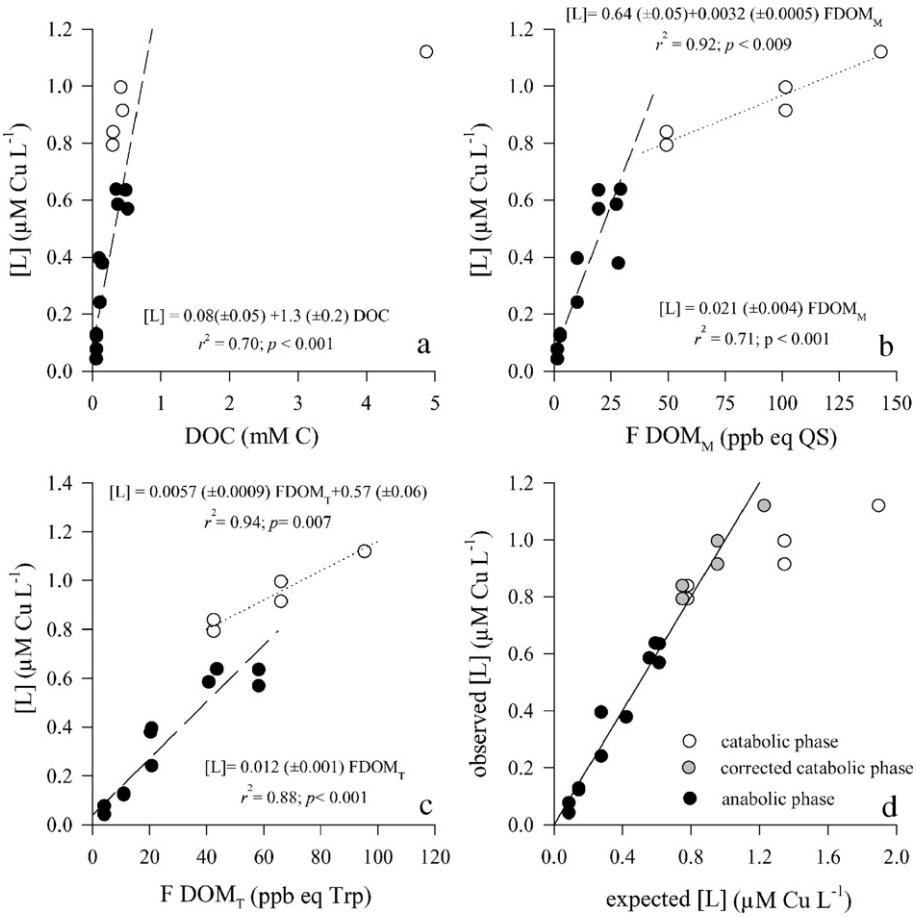

Fig. 5. Relationships between the concentration of ligand in the culture and different measurements of the DOM content in the medium. (a–c) Ligand concentrations expressed as a function of the DOC, $FDOM_T$ and $FDOM_M$ of the culture, respectively. (d) Observed versus expected ligand concentrations obtained from the multiple model II regression. The multiple regression (see text, Eq. (4)) was calculated with data from the anabolic phase. White and grey dots represent data from the catabolic phase fitted to the previous regression before and after $FDOM_T$ corrections. Black dots and long dashed lines correspond to data and fittings obtained in the anabolic phase of the culture. Open dots and dotted lines correspond to data and fittings from the catabolic phase. The regression equations (±standard error), the correlation coefficients and the significance of the model II relationships are also presented.

phase can be explained by $FDOM_T$. The regression model with $FDOM_M$ was also significant, but it did not improve the predictive power of the model obtained with the DOC concentration.

None of these three regression models tested in the anabolic phase can be successfully applied to the catabolic phase of the cultures, and clear discrepancies with the predicted values for the ligand concentrations were observed already for the measured values on day 8 (open circles in Fig. 5a, b and c).

A multiple regression model considering the contribution of both fluorophores to the ligand concentration was also tested in the anabolic phase of the cultures. The following equation was obtained:

$$[L] = 0.05(\pm 0.03) + 0.007(\pm 0.001)FDOM_T + 0.008(\pm 0.002)FDOM_M \quad (4)$$

where L is the ligand concentration (μmol Cu/l), and the standard error of predicted parameters are shown in brackets ($n=11$). The regression model explains 96% of the observed ligand variability in the culture (see black dots of Fig. 4d). A significant contribution of both $FDOM_T$ and $FDOM_M$ to the ligand concentration was observed ($p<0.001$ and $p=0.005$, respectively).

When the same model was applied to the catabolic phase of the culture, good predictions were obtained for day 8. None of the previous models considering only DOC, $FDOM_T$ or $FDOM_M$ could predict ligand concentrations at this day. However, expected ligand concentrations (open dots in Fig. 4d) for the last days of the culture (days 11 and 13) departed significantly from measured values. Model predictions overestimate measured ligand concentrations, coinciding with the clear decay of the culture. Probably, $FDOM_T$ at this period is not related to labile exuded DOC by algae but to DOC liberated during cell lysis of the dead culture. Therefore, a new parameter was introduced to Eq. (4) to account for a possible decrease in ligand capacity of measured $FDOM_T$ at this period:

$$[L] = 0.05 + 0.007(1-\alpha)FDOM_T + 0.008FDOM_M \quad (5)$$

where α is the % of $FDOM_T$ without metal binding properties. Good fittings with the observed ligand concentrations were obtained considering that 85% and 100% of $FDOM_T$ had no metal complexing properties in days 11 and 13, respectively (grey dots in Fig. 4d; Table 2).

The parameters obtained from Eq. (4) are not directly comparable, because both fluorophores are not expressed in the same units. However, it is possible to calculate the contribution of both fractions to the ligand concentration by considering the measured $FDOM_T$ and $FDOM_M$ at any time (see Table 2). During the initial phase of the culture, "protein-like substances" together with other labile DOC compounds (traced by $FDOM_T$) directly exuded from phytoplankton living cells made up most of the metal complexing capacity of the culture (between 60% and 80%). However, after day 6, humic substance contribution to the ligand concentrations started to be dominant.

Table 2
Percentage of the measured ligand concentration explained by the $FDOM_T$ and $FDOM_M$ fluorophores (±standard deviation) during the course of the *Skeletonema costatum* culture according to Eq. (4)

Time(days)	$\%L_{FDOM_M}$	$\%L_{FDOM_T}$	% Non-complexing $FDOM_T$
0	28±1	72±1	0
2	22±2	78±5	0
4	37±13	63±23	0
5	28±2	72±6	0
6	44±1	56±1	0
8	58±2	42±2	0
11	92±6	8±1	85
13	100	0	100

The estimated percentage of $FDOM_T$ without metal binding properties according to Eq. (5) in the anabolic phase is also shown in the fourth column.

4. Discussion

4.1. DOC production during the culture

Two phases have been distinguished in the evolution of the culture, named anabolic and catabolic. The anabolic phase coincided with the growing and stationary phases (days 0 to 7), characterised by the exudation of DOM by *S. costatum* living cells. The catabolic phase coincided with the decay of the bloom (days 8 to 13), probably caused by P nutrient limitation, characterised by increased cell lysis.

DOC production by *S. costatum* has been described previously in field (Ignatiades, 1973) and in culture (Ignatiades and Fogg, 1973) conditions. The amount and composition of DOC released by marine diatoms has been proven to be dependent on the physiological status of the culture (Chen and Wagersky, 1996a; Terzić and Ahel, 1998; Granum et al., 2002). Whereas low molecular weight materials are released in the exudation processes during the growing phases of cultures, high molecular weight materials are dominant in the senescent phases (Chen and Wagersky, 1996b).

The DOC decrease during days 4 to 7 was due to bacterial consumption of labile DOM. High bacterial densities were observed in the culture and DOC consumption rates higher than 72%/day were measured. The lability of exuded DOC has been shown in diatom and other phytoplankton cultures before (Chen and Wagersky, 1996b).

At the end of the culture a sharp increase of DOC was observed, coinciding with the decrease in cell numbers and Chl *a* concentrations. Complete lysis of a bloom of 800 μg/l of Chl *a* would produce a DOC concentration of 67–73 ppm C assuming a Chl *a*/C ratio of 11–12 μg Chl *a*/mg C (Sakshaug and Holm-Hansen, 1977). The DOC concentration observed at the end of present study was in the range of this estimate (Fig. 1b). Therefore, most of the cellular content was liberated as a result of cell rupture during the decay of the bloom.

Although $FDOM_T$ is specifically related to tryptophan, it can be used as a proxy for the concentration of amino acids or "protein-like" substances in general (Coble, 1996; Yamashita and Tanoue, 2003; Stedmon and Markager, 2005). A clear increase in $FDOM_T$ was observed at the end of the exponential growth phase, followed by a decrease along the stationary phase (Fig. 1c). A similar pattern of dissolved free amino acid (DFAA) dynamics was observed in cultured and natural blooms by Hammer et al. (1983). In their study, accumulation of DFAA was observed at the end of the exponential growth phase, followed by a sharp decrease due to bacterial utilisation. $FDOM_T$ and DOC during the anabolic phase of the bloom were extremely correlated, and production and consumption of labile DOC was coupled closely to $FDOM_T$ (see Fig. 2b). Although under natural conditions, with low concentrations of DOM and in the presence of different kinds of fluorescent material, the use of fluorescence to measure concentrations of DOM is still controversial (Mayer et al., 1999), some recent works have shown the potential of $FDOM_T$ to trace labile DOM (Nieto-Cid et al., 2005; Stedmon and Markager, 2005). Our results suggest that $FDOM_T$ allows to easily follow labile DOC dynamics in culture conditions. During the anabolic phase of the culture, when exudation of DFAA and small peptides dominate, $FDOM_T$ can be used to follow the time course of the concentration of "protein-like" ligands and any other Cu ligand present in the labile DOC pool. At the end of the culture, when cell lysis was occurring, $FDOM_T$ would be rather related to structural proteins included in organelles or cell fragments considered as dissolved (less than 0.2 μm pore size, in this case) than to small molecules exuded from living cells.

Different results were obtained for $FDOM_M$: a continuous increase, at a constant production rate, was observed all along the culture. Other fluorophore characteristics of humic substances were also measured (A: Ex/Em: 250 nm/435 nm, and C: Ex/Em: 340 nm/440 nm), and they followed the same trend as fluorophore M (correlation coefficients of 0.997 in both cases). Thus, even when labile DOC was partially consumed by heterotrophic bacteria (days 5 to 8), *in situ* produced humic substances accumulated in the culture. Therefore, this study has found evidence of fast production of humic material during phytoplankton blooms, as suggested previously by other authors (Ferrari et al., 1996; Nieto-Cid et al., 2006). We have obtained however no direct relationship between $FDOM_M$ and Chl *a* content or algal growth, probably because humic substances are more associated with the activity of heterotrophic bacteria during algal blooms (Ogawa et al., 2001; Yamashita and Tanoue, 2004; Nieto-Cid et al., 2006). Thus, $FDOM_M$ can

Table 3
Comparison of ligand concentrations and stability constants of copper ligands produced by phytoplankton cultures in the present study and in the literature

Type of ligand	[L] (nM)	Log$K'_{Cu\ 2+}$	Type of sample	[Cu] (nM)	Method	Reference
L_1	20–120	12.3–13.3	*Synechococcus* sp. (cyanobacteria)	10–103	CSV	Moffett and Brand (1996)
L_1	5–83	11.8 12.2	*Synechococcus* sp.	38	CSV	Croot et al. (2000)
L_1	2000	>12	*Synechococcus* sp.	4000	ASV	Gordon et al. (2000)
L_1	96–1400	>12	*Vibrio alginoliticus* (heterotrophic bacteria)	100–1700	CSV	Gordon et al. (2000)
L_2	100–300	7.8–9.0	*Gymnodinium sanguineum* (dinoflagellate)	<16	ASV	Robinson and Brown (1991)
L_2	26	9.6–10.5	*Emiliana huxleyi* (coccolithophore)	11–14	ASV	Muller et al. (2003)[a]
L_2	40–80	10.6	*Thalassiosira weissfoglii* (diatom)	4–40	CSV	Croot et al. (2000)
L_2	38–207	9.1–10.1	*Ditylum brightwellii* (diatom)	10–126	ASV	Gerringa et al. (1995b)
L_2	20–42	11.6–12.3	*Skeletonema. costatum* (diatom)	4–40	CSV	Croot et al. (2000)
L_2	80–1080	7.9–8.7	*S. costatum*	20	ASV	This study

Only stripping voltammetry techniques are presented for comparison.
Stability constant values can vary ±0.2 units due to differences in inorganic side reaction coefficients.
Copper concentrations in the cultures or samples are also presented.
[a] It is not a laboratory culture but a mesocosms experiment.

be used to follow a completely different compartment of DOC, much more refractory, and not directly exuded by phytoplankton.

4.2. Ligand production during the culture

Production of metal binding material occurred during the course of the culture. Ligand concentrations higher than 1 μM (in Cu equivalents) were measured at the end of the culture, showing that substances with metal binding properties are produced during both the anabolic and catabolic phases of the simulated bloom. The production of metal binding ligands under culture conditions has been observed previously (Swallow et al., 1978; Zou and Wangersky, 1989; Table 3). Chl *a* concentrations during this study, as well as in other culture studies, are much higher than those measured during a natural bloom (nearly 2 orders of magnitude). Such differences appear also reflected in the magnitude of the ligand concentrations observed.

Measurements performed at the initial day of the culture presented a high error of estimation and, therefore, the conditional stability constant is not reliable. For accurately describing the complexing capability at the initial day much higher deposition times and lower Cu additions should be employed. However, for subsequent days, the presented model (Eq. (1)) fitted accurately the observed titration data. Usually, linearisation methods are employed for fitting the data (Van den Berg, 1982; Ružić, 1982), but more recent literature criticise these methods because they modify enormously the distribution of residues (Apte et al., 1988; Gerringa et al., 1995a; Garnier et al., 2004). Meaured stability constants were in the range of 6.7–7.5 expressed as $\log K'_{Cu'}$. These values are very similar to those reported by Robinson and Brown (1991), when measuring copper complexation during a bloom of *Gymnodinium sanguineum* by ASV (6.6–7.8). Stability constants obtained in the present study should be transformed into $\log K'_{Cu\ 2+}$ (8.2–9.0) for comparison with most of the values reported in the literature (see Table 3).

Complexation of Cu in oceanic waters appears to be controlled by at least two classes of organic ligands, and in estuarine waters three different classes have been described (Donat et al., 1994). These ligands are usually referred to as L_1, L_2 and L_3, and present decreasing conditional stability constants with Cu (Donat and Bruland, 1995). L_1 is characterised by stability constants in the order of $pK'_{Cu\ 2+}$=12–14 and it is usually present in small concentrations (1–40 nM). It seems to control the speciation of copper in oceanic waters. Several prokaryotic species have been proved to produce compounds which are able to bind copper with high strength: *Synechococcus* sp. (Moffett et al., 1990) and *Vibrio* sp. (Gordon et al., 2000). L_2 presents much lower stability constants ($pK'_{Cu\ 2+}$=8–11), but it is usually present at higher concentrations in oceanic and coastal waters (6–150 nM). This ligand class is of great importance in controlling metal speciation in coastal waters, especially in polluted sites. It has been shown that many eukaryotic phytoplankton species are able to produce metal binding compounds with similar stability constants to L_2 (Croot et al., 2000). L_3 presents lower stability constants than L_2 and it seems to be related to ligands of terrestrial origin. However, some recent works have also challenged this classification (Town and Filella, 2000).

The K' values measured in our work corresponded to the lower range of the usual stability constants for L_2. This result is in accordance with the hypothesis of Croot et al. (2000): "organisms producing the strongest chelators are the most copper sensitive". It is also concluded in their study that "*Skeletonema* sp., a relatively Cu tolerant species, does not produce these binding compounds with the purpose of binding Cu, but they are a direct release from the photosynthetic activity".

Eq. (1) cannot describe the presence of different ligands in the titrations. The model, assuming only one ligand type and a simple 1:1 stoichiometry, could accurately describe Cu complexation in the performed titrations. However, it should be pointed out that a wide range of different ligands or different binding sites in a single macromolecule could be present in natural DOM. For this reason, the $\log K'$ presented here is just an average value of the stability constants of all the ligands present in the medium covered by the detection window of the ASV technique (González-Dávila et al., 2000). $\log K'$ values and ligand concentrations are neither independent from each other nor from the method used for measuring metal speciation. Lower stability constants are usually obtained at higher ligand concentrations and, for the same sample, the obtained values are also dependent on the detection window of the technique.

Regarding the measuring technique, cathodic striping voltammetric (CSV) is nowadays more often used to measure metal ligands in natural waters than ASV. Changing the type and concentration of the competing ligand is easy to vary the detection window of the method, which allows to choose the strength of the ligands to be determined (Van den Berg et al., 1990; González-Dávila, 1995). However, copper ligands in the culture were too weak and present in too high concentrations to be easily determined by CSV. In contrast, the detection window of ASV is framed by the detection limit of the measurements and by the lability of the complexes being measured (Van den Berg et al., 1990). This detection window can be

slightly changed modifying some measuring conditions such as stirring rate or deposition potential (Omanović et al., 1996), but certainly the choice is much restricted than in CSV. ASV does not allow an accurate estimation of the stability constants when the ligand reaction side coefficient in the sample is high. Thus, it is not possible to measure accurately the stability constants of all the complexing ligands present in a mixture, but only those which are initially free and within the range of the Cu additions performed in the titration (e.g., for $[L] \approx 10^{-6}$ M, $K'_{Cu'} \approx 10^{6-8}$ can be accurately determined). Moreover, ASV-stability constants slightly underestimate logK' values due to the partial lability of copper–ligand complexes (Robinson and Brown, 1991). To minimize this effect, the stirring speed during the deposition phase was chosen to be maximum in the present study. Despite these limitations, ASV is a useful method for determining ligand concentrations when uncomplexed ligands are present (Gordon et al., 2000). In addition, it has been proved that ASV methods accurately predict the observed Cu toxicity for aquatic organisms in the presence of humic substances (Ma et al., 1999; Lorenzo et al., 2002, 2006) and in the presence of the ligands obtained in this study. Therefore, metal titrations performed in this work provide relevant information about the total metal buffering capacity within the range of the copper additions used, although the exact logK' and the presence of different type of ligands cannot be accurately described. Thus, only ligand concentrations data will be further discussed in the present work.

4.3. Evaluating the different contributions of DOM to ligand concentration during the culture

The ligand concentration in the culture showed a significant correlation with the DOC concentration in the anabolic phase of the bloom (see Fig. 4a). However, increasing discrepancies were observed when the culture reached the stationary and decay phases. Despite a clear decrease in DOC from days 5 to 8, ligand concentrations showed a significant increase during the same period (compare Fig. 1b with Fig. 3b). The bacteria incubation experiment showed a fast consumption of the DOC exuded in the anabolic phase of the culture. However, this consumption was almost twice the observed decrease in ligand concentration, pointing that at least part of the ligand is related to the non-labile fraction of DOC. In addition, a clear decrease in the slope of the ligand versus DOC regression during the catabolic phase of the culture was observed (see open dots in Fig. 4a).

Differences in the DOM composition were observed between the two phases of the culture (see Fig. 1c), as followed by fluorescence measurements. Changes in the DOM composition might influence the metal binding capacity of the culture, a fact not accounted when only DOC concentrations are measured.

Both fluorophores, $FDOM_M$ and $FDOM_T$, showed a significant decrease in their fluorescence with copper additions (see Fig. 3). Fluorescence quenching in the presence of paramagnetic metals, such as copper, has been proposed as an alternative method for determining metal complexing capacities of DOM (Saar and Weber, 1980, Ryan and Weber, 1982). The 20% decrease in humic-like fluorescence was similar to the decrease reported by other authors at analogous fulvic acid concentrations (Cabaniss and Shuman, 1988). $FDOM_T$ fluorescence showed higher quenching than that measured for "humic-like" substances produced in the culture. It is thus clear that both measured fluorophores present metal binding properties. However, not all the metal binding groups in DOM show fluorescence, and thus quenching fluorescence measures accurately the binding capacity of DOM only "if the material in the sample that fluoresce is representative of the bulk of the material" (Ryan and Weber, 1982; Cabaniss and Shuman, 1986, 1988). This assumption does not hold if fluorescent and not fluorescent binding groups have different metal binding affinities. Fluorophores are not proposed here as the only ligands present, since other non-fluorescent ligands may be present and correlate with them along the culture. Nevertheless, fluorescence measurements will be used as a proxy of two main groups of substances in the culture: labile exuded DOM ($FDOM_T$) and a more refractory group composed by humic-like substances ($FDOM_M$).

$FDOM_T$ described better the ligand concentration during the anabolic phase of the culture, but the decrease in its concentration after day 5 was not followed by a decrease in ligand concentration. After that day, $FDOM_M$ explained better the evolution of the ligand concentration. A linear regression model was built up considering the contribution of both fluorophores (Eq. (4)), and both contributions were found to be highly significant. This model was able to explain 96% of the variability of the ligand concentration during the anabolic phase of the culture. It also indicates (Table 2) that $FDOM_T$ (tracer of labile DOC exudates) accounts for the dominant ligand during the initial days of the culture, but after day 6, the dominance dramatically change to $FDOM_M$ (marine humic substances, by-product of the bacterial degradation of labile DOM).

The increase in $FDOM_T$ suggests a significant release of DFAA and small peptides, together with other labile DOC, seeming that they could be responsible for the measured ligand concentration in the culture. Because of their high stability constants to complex metal ions,

histidine and cysteine are probably the main amino acids involved in this labile ligand pool, although any other complexing and labile DOC cannot be excluded from present data. Our results are in agreement with those reported by Leal et al. (1999). They measured an increase in glutathione and other thiol compounds by cathodic stripping voltammetry along the course of an *Emiliana huxleyi* culture. Those cysteine-like and glutathione-like compounds were also found by Vasconcelos et al. (2002) with other three eukaryotic alga species. Those results do not preclude from a significant contribution of histidine-like compounds.

Many authors have reported the metal complexing abilities of humic substances (Lorenzo et al., 2002, 2006), and the contribution of humic substances to the natural dissolved ligand pool has also been shown (Midorikawa and Tanoue, 1998).

None of the models used in this work can predict the ligand concentration in the catabolic phase of the culture. The sharp increase in DOC content during the catabolic phase has been related to cell lysis and liberation of the cell contents to the medium. As discussed previously (Section 4.1), this would imply a dramatic shift in DOC composition, from exuded small labile compounds to large cell fragments and organulles. This could also be related to a change from DFAA and small peptides to large structural proteins, some of them embedded in cell fragments, in the $FDOM_T$ measurements. Metal binding capacity of DOC from cell lysis appeared to be relatively much lower than that exhibited by the labile DOC directly exuded in the anabolic phase of the culture. Thus, a new parameter was added to the model to consider this change in metal binding capacity in $FDOM_T$. The observed results could be successfully explained when the presence of non-metal binding proteins are considered (see Fig. 4d and Table 2). In this sense, Midorikawa and Tanoue (1998) have reported that large proteins are not responsible of metal binding properties of natural DOM.

5. Conclusions

The time evolution of the DOM pool in a simulated bloom of *S. costatum* was not successfully explained by DOC measurements alone. Fluorimetric methods could trace better the DOC changes during the culture. Evidences have been found in the production, at daily time scales, of DFAA, small peptides and other low molecular weight DOC by exudation of living cells, and humic substances as a by-product of microbial respiration processes.

A continuous increase in metal complexing ligand concentration was measured along the culture. Two different sources appeared necessary to explain the evolution of those ligands: one related to labile DOC directly exuded by phytoplankton (easily traced by $FDOM_T$), and other refractory source related to bacterial degradation of labile DOC (easily traced by $FDOM_M$).

Fluorescence measurements appear as a useful tool for estimating the different contributions to the ligand pool in culture conditions. They could be easily included in actual bioavailability models for assessing the speciation and biological effects of metals in the environment. However, further studies should be developed for accurately distinguishing between HA and FA fluorescence in coastal waters, and between labile DOC directly exuded by living algae and other substances coming from senescence phytoplankton blooms, probably showing lower metal binding properties.

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