1
2
3 Contrasting complexing capacity of dissolved organic matter produced
4 during the onset, development and decay of a simulated bloom of the
5 marine diatom *Skeletonema costatum*

6 J.I. Lorenzo^{1*}, M. Nieto-Cid², X.A. Álvarez-Salgado², P. Pérez¹, R. Beiras¹

7 ¹Universidad de Vigo, Facultad de Ciencias del Mar, Dept. de Ecología y Biología Animal,
8 Lagoas-Marcosende, 36200 Vigo, Spain

9 ² CSIC, Instituto de Investigaciones Mariñas, Dept. de Oceanografía, Eduardo Cabello 6, 36208
10 Vigo, Spain

11
12 * Corresponding author:

13 Tel. +34 986 81 4087

14 Fax. +34 986 81 2556

15 Email: lorenzo@uvigo.es

16
17 **Submitted to:** Limnology and Oceanography

18
19
20 **Version 4:** 14 August 2004

21
22
23 **Running head:** Complexing capacity of a phytoplankton bloom

1 **Acknowledgements**

2 The authors wish to thank J. Gago for his help with the DOC and fluorescence
3 measurements, and to E. Fernández for his advice and fruitful comments. Financial support
4 for this work came from the Spanish 'Ministerio de Educación y Ciencia' (MEyC), grants nº
5 REN2000-0880-C02-01 MAR and REN2003-00958 MAR and the 'Xunta de Galicia' grant nº
6 PGIDT01MAR40201PN.

1 **Abstract**

2 The capacity of natural dissolved organic matter (DOM) produced during the
3 onset, development and decay of a simulated bloom of the marine diatom
4 *Skeletonema costatum* to complex free copper has been followed for a 2 week period.
5 Total dissolved organic carbon (DOC) concentrations could not accurately predict the
6 complexing capacity of the culture, especially at the end of the bloom, and better
7 predictions were obtained when fluorescence measurements were considered.
8 During the onset and development (days 0 to 7) exuded dissolved free amino acids
9 (DFAA) efficiently complexed free copper (60–80% of the total complexing capacity
10 of the culture). During the decay (days 8 to 13), DFAA were replaced by humic
11 substances as the complexing agent for copper (60–100% of the total complexing
12 capacity). Humic substances were produced in situ as a by-product of the bacterial
13 degradation of phytogetic materials, essentially during the decay of the bloom.

1 **Introduction**

2 Marine dissolved organic matter (DOM) is the largest pool of reduced carbon of the
3 earth surface (Hedges 2002). It is composed of a myriad of different compounds, covering a
4 wide range of molecular weight, chemical structures and functions. From the environmental
5 point of view, marine DOM plays several relevant roles (Sunda 1995) : 1) it contributes to the
6 recycling , accumulation, and export of biogenic matter in ocean biogeochemical cycles; 2) it
7 is an electron donor in the photo reduction and solubilisation of iron and manganese; 3) it
8 contributes to the acidification of the atmosphere, as it is the case of dimethyl sulphide ; 4) it
9 buffers the pH of river streams; 5) it absorbs the incident solar radiation, at UV and visible
10 wavelengths ; 6) it reduces the gas exchange across the air-sea interface; 7) it forms trace
11 gases such as CO and COS after photochemical decomposition; and 8) it reduces the toxicity
12 of free metal ions by means of the formation of stable metal/organic complexes.

13 Most of the marine DOM is produced in situ from the accumulation and subsequent
14 degradation of the products of synthesis of marine phytoplankton (Biddanda and Benner
15 1997; Carlson 2002), and only 10% is originated in the continents (Druffel et al. 1992).
16 Mechanisms of DOM production include direct exudation by marine phytoplankton, cell
17 autolysis in response to nutrient stress conditions or senescence, and zooplankton mediated
18 liberation in connection with an inefficient grazing process (Nagata 2000). In addition,
19 photochemical and bacterial respiration processes give rise to a complex series of poorly
20 understood transformations experienced by marine DOM, globally known as humification
21 (Hedges 1988; Kieber et al. 1997).

22 Among the products liberated during the production of marine phytoplankton
23 photosyntates, essentially carbohydrates and amino acids (Myklestad 1995; Biddanda and
24 Benner 1997), only the latter have the capacity to complex free metal ions (thus being metal

ligands). Zooplankton mediated or auto lysis of phytoplankton leads to the liberation of the full content of the cells to the medium. Whereas DFAA or simple peptides are liberated during the exudation processes, proteins are mainly liberated during cell lysis. Consequently, the complexing capacity of amino acids per unit weight will be larger during exudation rather than during cell lysis processes because the number of free amino/carboxylic acid pairs is larger. 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). Copper complexation stability constants of most amino acids are very similar ($\log K_{Cu-L} \sim 7 - 8.5$), except for the case of histidine and cysteine, which are the most efficient complexing amino acids ($\log K_{Cu-L} = 11.1$ and 19.2 , respectively).

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). Humic substances are usually isolated by adsorption of acidified waters onto non-ionic XAD resins (Benner et al. 1992). In seawater, they usually constitute 5 – 15 % of the total DOM concentration in carbon units (Benner et al. 1992), although in estuaries and coastal waters they can reach concentrations as high as 1.5 ppm 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). Despite its essentiality, 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). Copper is a metal of environmental concern, because it reaches concentrations higher than the tolerance limits of certain organisms in many aquatic environments (WHO 1998).

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. The inclusion of DOM in water quality criteria has also been recommended (Nor 1987, Allen and Hansen 1996). Therefore, to understand how DOM affects metal bioavailability has been a key issue during the last years. Several models have been developed to predict the biological effect of metals in the environment. 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 the 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). The aim of this work is to study the contrasting copper complexing capacity of natural marine DOM produced during the onset, development and decay of a simulated bloom of the marine diatom *Skeletonema costatum* on the basis of the changes observed in the proportions of amino acids and humic substances throughout the bloom.

Material and methods

The culture -- The marine diatom *S. costatum* was cultured in artificial seawater (Lorenzo et al. 2002) in two acid washed 4 L-polycarbonate bottles, named A and B, with the aim of producing natural DOM. The cultures were kept at 18°C under a 14 h light:10 h dark photoperiod (70 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$) 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 $\frac{1}{2}$ f/2 (Guillard 1975) without EDTA (ethylene diamine tetra-acetic acid). 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⁻¹. The pH of the medium was kept between 8.05 and 8.25 by adding diluted HCl when necessary. 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 hours. Samples were analysed using a Turner TD-700 fluorometer calibrated against pure Chlorophyll *a* (Sigma).

2) Cell density: it was assessed daily using Neubauer haemocytometer.

Filtration procedures -- About 300 ml of each culture were gently filtered (~ 150 mm Hg vacuum) to minimize cell lysis, at different time intervals through acid cleaned 0.22 µm Millipore polyethersulphone (PES) filters. Acid cleaned polycarbonate filter holders were used to avoid metal contamination. The filtrate was used for dissolved organic carbon (DOC) and fluorescence of dissolved organic matter (FDOM) analyses and copper titrations.

Dissolved organic carbon (DOC) -- The filtrate was collected into 10 ml precombusted (450°C, 12 h) glass ampoules. After acidification with H₃PO₄ 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 a mixture 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).

Fluorescence of dissolved organic matter (FDOM) -- Aliquots of the DOC filtrate were collected into 20 ml acid cleaned polycarbonate flasks and frozen to - 20°C until analysis. FDOM of

these samples was measured with a Perkin Elmer LS 55 Luminescence spectrometer. The instrument was equipped with a 20 kW xenon lamp 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: 320nm/410 nm) and T (aromatic amino acids; Ex/Em: 280nm/350 nm), specifically tryptophan (Coble et al. 1990, Mopper and Schultz 1993, Nieto-Cid et al. submitted). Four replicate measurements were performed for each Ex/Em wavelength. A four points standard curve was prepared daily with a mixed standard of quinine sulphate (QS) and tryptophan (Trp) in sulphuric acid 0.05 mol L⁻¹ (Nieto-Cid et al. submitted). 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.

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 N₂, copper was accumulated on a mercury drop of 0.52 mm² at -0.55 V for 15 seconds at the maximum stirring speed (3000 rpm) and 10 s of equilibration were let before the voltage scan. The conditions of the square-wave (SW) scan were an initial potential of -0.55 V, a SW amplitude of 25 mV, a 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, cell was not washed during each titration. All solutions were prepared in duplicate, and one of each solutions was only used for cleaning the cell, and the other one was measured afterwards.

Samples with increasing Cu additions were measured in order, starting from the culture filtrate with no Cu addition.

Copper titrations -- Between 14 and 17 samples of 16-mL culture filtrates were dosed in 20 ml polypropylene vials. Solutions were stored at -20 °C until analyses. Before analyses, increasing Cu additions (from 0 to 5 µmol L⁻¹) 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 % HNO₃ at least 24 h and rinsed 5 times with deionised water (MQ, Millipore) before use.

The peak height of each voltammogram was measured, and they were transformed to labile Cu concentrations ([Cu']) by dividing by the slope of the linear section of each titration (sensitivity) (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, this is only one type of ligand and a reaction stoichiometry of 1:1. Titration plots were fitted to the following equation obtained from the theoretical complexation model previously explained (see 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⁻¹), respectively, L is the ligand concentration (mol L⁻¹) and K' is the conditional stability constant of the Cu-L complexes. K' is not a thermodynamic stability constant, but depends on the composition of the medium. This 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'_{Cu2+} (Donat et al. 1994):

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

where α_{Cu2+} is the inorganic reaction side coefficient, which accounts for the inorganic copper speciation. For the artificial seawater used in the present study ($S = 35$ and $pH = 8.17$) the α_{Cu2+} obtained was 16.7 (Lorenzo 2003).

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 Rolf, 1995).

Statistical analyses were performed with STATISTICA version 6.0. (StatSoft Inc). The parameters of the complexation model in Cu titrations were estimated by means of non-linear square fitting procedures with Sigma Plot 2001 version 7.0 (SPSS Inc).

Results

Onset, development and decay of the cultured S. costatum bloom -- Figure 1 shows the time evolution of the culture. Similar patterns were observed in all measured variables in both cultures (A and B). Figure 1a shows a logarithmic increase in the number of cells during the initial 4 days. No lag time in the culture growth was observed. From day 4 to 6, the number of cells remained stationary, and after day 7 there was a continuous decrease in cell density.

1 Analogous results were observed in Chlorophyll a (Chl a) concentrations (not shown). Chl a
2 levels of 700–1000 $\mu\text{g L}^{-1}$ were achieved from days 4 to 7.

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

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

18 *Evolving copper binding capacity of DOM produced by the culture* — Figure 3a shows the
19 evolution of Cu titration plots in the culture over time. Some specific days were selected to
20 clearly show the shift in the plots to the right, due to the increase in Cu complexing capacity.
21 The complexation model previously described (eq. 1) accurately fitted the data, and r^2 was
22 between 0.997 and 0.9996, even when individual measurements and no mean values were
23 used for fitting purposes.

24 Copper titration fitting parameters ($\pm$ 95 % confidential interval, CI) are shown in
25 Table 1. There was a good agreement between the parameters obtained in both cultures, as

1 previously observed with DOC measurements (Fig. 2b). There was a marked increase in
2 ligand concentration over time (see also Fig. 3b). The ligand increase showed an intermediate
3 trend to those observed for both fluorophores: an initial and sharp increase at the end of the
4 exponential growth (days 4–5) coinciding with FDOM_T and no decrease between days 5 and
5 8, as FDOM_M . Day 8 showed statistically higher conditional stability constants, coinciding
6 with the initiation of the catabolic phase of the culture. The ligand capacity at the initial day
7 of the culture was very low, which was also reflected in the low confidence of the obtained
8 K' value.

9
10 *DOM composition versus copper binding capacity* -- Figure 4a shows the ligand concentration
11 measured during the culture versus the DOC content in the filtrates. In the anabolic phase, a
12 significant correlation was observed between DOC concentration and the metal binding
13 capacity (ligand concentration) of the culture. Up to 70 % of the ligand variability was
14 explained by the DOC concentration. However, when the catabolic phase of the culture was
15 considered, the regression did not predict the ligand concentration. A sharp increase in
16 ligand concentration was observed between days 8 and 11 that cannot be explained by the
17 increase in DOC concentration. The opposite trend was observed on day 13, when a 10-fold
18 increase in DOC was produced, but just a small increase in ligand concentration was
19 observed.

20 The different fluorophores were used to predict ligand concentration in the cultures
21 (see Fig. 4b and 4c). FDOM_T was the best predictive variable, and 88 % of the ligand
22 concentration in the anabolic phase could be explained by the obtained regression. The
23 regression model with FDOM_M was also significant, but it did not improve the predictive
24 power of the model obtained with the DOC concentration. The significant correlation
25 observed between both predictive variables ($r^2 = 0.53$, $p = 0.011$, $n = 10$) is remarkable. None
26 of the regression models obtained with the fluorophores could 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. 4b and 4c).

A multiple regression model considering the independent contribution of both DOC components (FDOM_M and FDOM_T) to the ligand concentration was also tested. The following equation was obtained:

$$[L] = 0.05 (\pm 0.03) + 0.007 (\pm 0.001) \text{ FDOM}_T + 0.008 (\pm 0.002) \text{ FDOM}_M \quad (4)$$

where L is the ligand concentration ($\mu\text{mol Cu L}^{-1}$), and the standard deviation of predicted parameters are shown in brackets. The regression model could explain 96 % of the observed ligand variability in the culture (see black dots of Fig. 4d), and trends in the residues from the regression were not observed at any time of the anabolic phase. 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, the expected ligand concentrations (open dots in Fig. 4d) departed significantly from the expected values. Model predictions overestimate measured ligand concentrations during the late days of the cultures. Good fittings with the observed ligand concentrations were obtained lowering the active metal complexing FDOM_T to 15 % and 0 % in days 11 and 13 (grey dots in Fig. 4d and Table 2).

The parameters obtained from equation 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, dissolved free amino acids and small peptides directly exudated from phytoplankton made up most of the metal complexing capacity of the culture (between 60 and 80 %). However,

after day 6, humic substances contribution to the ligand concentrations started to be dominant.

Discussion

DOC production during the culture – An increase of DOC concentration was observed in both cultures, as a consequence of the liberation of DOM from phytoplankton cells either by direct exudation or cell lysis, especially at the end of the experiments. In this sense, two phases could be distinguished in the evolution of the cultures, named anabolic and catabolic. The anabolic phase coincided with the growing and stationary phases (days 0 to 7), and the catabolic phase coincided with the decay of the bloom (days 8 to 13), characterised by an increased cell lysis. Complete lysis of a bloom of $800 \mu\text{g L}^{-1}$ of Chla would produce a DOC concentration of 67–73 ppm C assuming a Chla/C ratio of 11–12 $\mu\text{g Chla/ mg C}$ (Sakshaug and Holm-Hansen 1977). DOC values observed at the end of present study were in the range of those estimated values (Fig. 1b), meaning that most of the cellular content was liberated as a result of cell rupture during the decay of the bloom. The DOC decrease during days 4 to 7 was due to bacterial consumption of labile DOM. High bacterial densities were observed after day 4 (retained in $0.22 \mu\text{m}$ filters), and a DOM incubation of a $5 \mu\text{m}$ filtered sample (removing phytoplankton but not bacteria) showed a DOC consumption higher than 70 % in 24 hours. This demonstrates the lability of the DOM exudates.

Although FDOM_T only represents the concentration of the aromatic amino acid tryptophan, it can be used as a proxy for the concentration of amino acids in the medium (Yamashita and Tanoue 2003). Production and consumption of dissolved free amino acids (DFAA) and small peptides followed the same pattern as labile DOC in both cultures. Similar patterns have been observed in both cultured and natural blooms by Hammer et al. (1983). In their study, accumulation of DFAA was observed at the end of the exponential growing phase, followed by a sharp decrease in the amino acid concentration due to

1 bacterial utilization. Phytoplankton blooms are so coupled to bacterial growth that changes
2 in amino acid concentration are difficult to follow in laboratory and field conditions. Several
3 authors have also pointed out the advantages of fluorescence measurements to study amino
4 acids in marine surface waters (Yamashita and Tanoue 2003) and sediment porewaters
5 (Coble 1996). Our results suggest that relevant information can also be obtained in culture
6 conditions from FDOM_T, allowing to follow easily the fast amino acid dynamics. At the end
7 of the cultures, FDOM_T was probably more related to proteins liberated from cell lysis than
8 to DFAA exudated from living cells.

9 Different results were obtained for FDOM_M: a continuous increase at a constant
10 production rate was observed all along the culture. Thus, even when labile DOC was
11 consumed by heterotrophic bacteria (days 5 to 8), in situ produced humic substances
12 accumulated in the culture. Therefore, this study presents clear evidences of the production
13 of humic material during phytoplankton blooms, as suggested by other authors (Coble et al.
14 1990, Ferrari et al. 1996). We have obtained however no direct relationship between FDOM_M
15 and Chla content or algal growth, but probably humic substances are more associated with
16 the activity of heterotrophic bacteria during algal blooms (Coble 1996, Zou et al. 2004,
17 Yamashita and Tanoue 2004, Nieto-Cid submitted).

18
19 *Ligand production during the culture* – Clear production of metal binding material occurred
20 during the course of the culture. Ligand concentrations higher than 1 $\mu\text{mol Cu L}^{-1}$ were
21 measured at the end of the culture, showing that substances with metal binding properties
22 are produced during both the anabolic and catabolic phases of the simulated bloom. The
23 production of metal binding ligands in culture conditions has been observed since long time
24 ago (Swallow et al. 1978). Our results are also in agreement with the high complexing
25 capacity observed in coastal and estuarine waters related to phytoplankton productivity
26 (Moffet et al. 1990, Robinson and Brown 1991, González-Dávila 1995). Chla concentrations

1 during this study, as well as in other culture studies, are much higher than the measured on
2 natural blooms (nearly 2 orders of magnitude). Such differences appear also reflected in the
3 magnitude of the ligand concentrations observed.

4 Measurements performed at the initial day of the culture presented a high error of
5 estimation and, therefore, the conditional stability constant is not reliable. For accurately
6 describing the complexing capability at the initial day much higher deposition times and
7 lower Cu additions should be employed. However, for subsequent days, measured stability
8 constants were in the range of 6.7–7.5 expressed as $\log K'$. These values are very similar to
9 those reported by Robinson and Brown (1991), when measuring copper complexation during
10 a bloom of *Gymnodinium sanguineum* by ASV (6.6–7.8). Stability constants obtained in the
11 present study should be transformed into $\log K'_{\text{Cu}^{2+}}$ (7.9–8.7) for comparison purposes with
12 most of the values reported in the literature.

13 Complexation of copper in oceanic waters appears to be controlled by at least two
14 classes of organic ligands, and in estuarine waters three different classes have been described
15 (Donat 1994). These ligands are usually referred to as L_1 , L_2 and L_3 , and present decreasing
16 conditional stability constants with copper (Donat and Bruland 1995). L_1 is characterised by
17 stability constants in the order of $p K'_{\text{Cu}^{2+}} = 12\text{--}14$ and it is usually present in small
18 concentrations (1–40 nM). It seems to control the speciation of copper in most oceanic waters.
19 Several prokaryotic species have been proved to produce compounds able to bind copper
20 with that high strength: *Synechococcus* sp. (Moffet et al. 1990), *Vibrio* sp. (Gordon et al. 2000).
21 L_2 presents much lower stability constants: $p K'_{\text{Cu}^{2+}} = 8\text{--}10$, but it is usually present at much
22 higher concentrations in oceanic and coastal waters (6–150 nM). This kind of ligands is of
23 great importance in controlling metal speciation in coastal waters, especially in polluted
24 sites. It has been proved that many eukaryotic phytoplankton species are able to produce
25 metal binding compounds with similar stability constants to L_2 (Croot et al. 2000). L_3

1 presents lower stability constants than L_2 and it seems to be related to ligands of terrestrial
2 origin. Muller et al (2003) have shown the production of Cu-binding ligands by *Emiliana*
3 *huxleyi* cultured in mesocosms conditions and measured by DPASV. The three different type
4 of ligands were measured, but only L_2 with a $K'_{Cu^{2+}}$ range 9.6–10.5 showed an increase (~ 35
5 nM) related to the algal bloom.

6 The K' values measured in our work corresponded to the lower range of the usual
7 stability constants for L_2 . This result is in concordance with the hypothesis of Croot et al.
8 (2000): “organisms producing the strongest chelators are the most copper sensitive”. It is also
9 concluded in their study that “*Skeletonema sp.*, a relatively Cu tolerant species, does not
10 produce these binding compounds with the purpose of binding Cu, but they are a direct
11 release from the photosynthetic activity”.

12 The non-linear eq (1) cannot describe the presence of different ligands in the
13 titrations, however non evidences of misfits were observed. The copper complexing capacity
14 could be accurately described by the model, assuming only one ligand type and with a
15 simple 1:1 stoichiometry. However, it should be pointed out that a wide range of different
16 ligands or different binding sites in a single macromolecule can be present in natural
17 samples. For this reason, the $\log K'$ is an averaged value of the stability constant of natural
18 ligands inside the analytical window (González-Dávila et al. 2000). Obtained values of $\log K'$
19 and ligand concentrations are not independent from each other nor from the method used
20 for measuring metal speciation. Lower stability constants are usually obtained at higher
21 ligand concentrations and, for the same sample, the obtained values are also dependent on
22 the detection window of the technique. Taking into account these limitations, metal titrations
23 still provide relevant information about the metal buffering capacity within the range of the
24 copper additions used, and allow us to infer the effect of copper additions to the
25 environmental samples.

Regarding the measuring technique, cathodic stripping voltammetric methods, which are used combined with an exchange reaction with ligands of well-known stability constants, allow to choose the strength of the ligands to be determined (González-Dávila 1995). In contrast, the detection window of ASV methods is only related to the range of copper additions performed. Thus, it is not possible to measure all the complexing ligands present in a mixture, but only those within the range of the Cu additions. ASV-stability constants slightly underestimate $\log K'$ values due to partial lability of copper-ligand complexes (Robinson and Brown 1991). To minimize this effect, the stirring speed during the deposition phase in the present study was chosen to be maximum. Despite these limitations, ASV is the most useful method for ligand determination when uncomplexed ligands are present (Gordon et al. 2000), and it has been proved to correlate with the observed toxicity for aquatic organisms (Deaver and Rodgers 1996, Lorenzo et al. 2002).

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 day 5 to 8, ligand concentrations showed a significant increase during the same period (compare Fig. 1b with Fig. 3b). In the same way, it was observed a clear decrease in the slope of the regression during the catabolic phase of the culture (see open dots in Fig. 4a).

Differences in the DOM composition were observed between the two phases of the culture (see Fig. 1c), and these differences could be easily followed by spectrofluorimetric measurements. The observed change in DOM composition might influence the metal

binding capacity of the culture, a fact not considered when only DOC concentration measurements are studied.

Current bioavailability models (BLM) use metal speciation calculations to predict the biological effect of metals to aquatic organisms. One of the major difficulties deals with modelling the complexation of metal cations by organic matter. For this reason, BLM is implemented with WHAM (Windermere humic aqueous model) to consider the effect of DOM on metal speciation (Di Toro et al. 2001). BLM assumes that a specific percentage of DOM is humic acids (HA) and that the remainder is entirely constituted by fulvic acids (Ryan et al. 2004). Thus, DOC measurements and the expected percentage of HA are the only incoming data to predict metal complexation by organic matter, and metal-humic complexes are the only organic complexes considered. It has been proved that BLM gives sufficiently accurate predictions in many circumstances, but discrepancies have also been found in the presence of different sources of natural organic matter (Playle 1998, Ryan et al. 2004). Differences in metal complexation properties have been found in natural organic matter from different sources (Ortego and Benson 1992, Benedetti et al. 1996, Abbt-Braun and Frimmel 1999).

Ligand concentration in the culture followed an intermediate trend to those observed by the two fluorophores: $FDOM_T$ and $FDOM_M$ (compare Fig. 1c and Fig. 3b). $FDOM_T$ has a sharp rise from day 2 to 5, and after day 8, $FDOM_M$ dramatically increased. Ligand concentrations showed the same pattern. Significant correlation was observed between both fluorophores and ligand concentrations during the anabolic phase of the culture (Fig. 4b and 4c). $FDOM_T$ described better the ligand concentration during the anabolic phase, 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 ligand evolution on the culture. A new model was built up considering the contribution of both fluorophores, and this model could explain the 96 % of the data variability during the anabolic phase. From this model it

1 was shown that the contribution of the amino-acid like ligands (DFAA and small peptides
2 exuded by the algae) are dominant during the initial days of the culture, but after day 6,
3 humic substances has a relevant contribution to ligand concentration (see Table 2).

4 The increase in $FDOM_T$ suggests a significant release of amino acids, and some of
5 them (e.g. histidine and cysteine) could be responsible for the measured ligand
6 concentration. Our results are in agreement with those reported by Leal et al. (1999). They
7 measured an increase in glutathione and other thiol compounds by cathodic stripping
8 voltammetry along the course of a *Emiliana huxleyi* culture. Those cysteine-like and
9 glutathione-like compounds were also found by Vasconcelos et al. (2002) with other three
10 eukaryotic alga species. Those results do not preclude from a significant contribution of
11 histidine-like compounds that could be also correlated. Amino acids and small peptides are
12 extremely labile, and their residence times in the environment are expected to be small.
13 However, continuous inputs can be measured on natural conditions due to phytoplankton
14 growth in the photic layer and resuspension processes in near surface sediment (Nieto-Cid et
15 al. submitted). The amino acid-related contribution to the ligand concentration is not
16 considered by BLM. This contribution is probably negligible when humic substances
17 concentrations are considerable, but certainly not at low DOC levels.

18 Many authors (Turner et al. 1987, Lorenzo et al. 2002) have reported the metal
19 complexing abilities of humic substances, and it has also been shown the contribution of
20 humic substances to the natural dissolved ligand pool (Midorikawa and Tanoue 1998, Shank
21 et al. 2004). Humic and fulvic acids (HA and FA) show small differences in their metal
22 complexation properties, but HA present higher fluorescence than FA. In our study,
23 measured $FDOM_M$ is probably related to fulvic-like substances. However, in natural waters
24 the contribution of both HA and FA should be distinguished in order to predict metal
25 speciation, and the higher absorbance properties of HA could be employed for this purpose
26 (Midorikawa and Tanoue 1998, Hautala et al. 2000).

None of the previous models (see Fig. 4) could predict ligand concentration in the catabolic phase of the culture. The sharp increase in DOC content during the catabolic phase was previously related to cell lysis and liberation of cell contents to the medium. This fact would produce a change in the composition of the measured FDOM_T, shifting from amino acid and small peptides liberated by direct phytoplankton exudation to large structural proteins. Metal binding capacity of these proteins is expected to be considerably lower. Thus, a new parameter was added to the model to consider the relative abundance of those structural proteins. Observed results could be successfully explained when non-metal binding proteins were considered (see Fig. 4d and Table 2). Midorikawa and Tanoue (1998) have reported that large proteins are not responsible of metal binding properties of natural DOM. Yamashita and Tanoue (2003) have proposed the use of both tyrosine-like and tryptophan-like fluorophores to discriminate amino acids and small peptides from large proteins fluorescence.

In summary, the evolution of the DOM pool in a simulated bloom of *S. costatum* was not successfully explained by DOC measurements alone. Spectrofluorimetric methods could trace better the evolution of DOM during the culture. Clear evidences of humic substance production were found, probably related to microbial respiration processes. A continuous increase in metal complexing ligand concentration was measured along the culture. Both, FDOM_T (protein-related) and FDOM_M (humic-related) were necessary to explain the evolution of the copper ligand concentration during the culture. Spectrofluorimetric measurements appeared as an useful tool for estimating the different contributions to the ligand pool, and 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 to accurately distinguish between HA and FA, and between DFAA and

- 1 small peptides contributing to the ligand pool and large proteins without metal binding
- 2 properties.

References

- Abbt-Braun, G. and F. Frimmel 1999. Basic characterization of Norwegian NOM samples - Similarities and differences. *Environment International* 25: 161-180.
- Allen, H.E. and D.J. Hansen 1996. The importance of trace metal speciation to water quality criteria. *Water Environment Research* 68: 42-54.
- Benedetti, M., W. Van Riemsdijk, L. Koopal, D. Kinniburgh, D. Gooddy, and C. Milne 1996. Metal ion binding by natural organic matter: from the model to the field. *Geochimica et Cosmochimica Acta* 60: 2503-2513.
- Benner, R., J. D. Pakulski, M. McCarthy, J. I. Hedges, and P. G. Hatcher 1992. Bulk chemical characteristics of dissolved Organic matter in the ocean. *Science* 255: 1561-1564.
- Biddanda, B., and R. Benner. 1997. Carbon, nitrogen, and carbohydrate fluxes during the production of particulate and dissolved organic matter by marine phytoplankton. *Limnology and Oceanography* 42: 506-518.
- Burney, C.M. 1994. Seasonal and diel changes in particulate and dissolved organic matter, p. 97-124. *In* [ed.], R.S. Wotton *The biology of particles in aquatic systems*. CRC Press Inc.
- Carlson, C. A. 2002. Production and removal processes, p. 91-151. *In* D. A. Hansell and C. A. Carlson [eds.], *Biogeochemistry of Marine Dissolved Organic Matter*. Academic Press.
- Coble, P. G. 1996. Characterization of marine and terrestrial DOM in seawater using excitation-emission matrix spectroscopy. *Marine Chemistry* 51: 325-346.
- Coble, P. G., S. A. Green, N. V. Blough, and R. B. Gagosian 1990. Characterization of dissolved organic matter in the Black Sea by fluorescence spectroscopy. *Nature* 348: 432-435.
- Croot, P.L., J.W. Moffett, and L.E. Brand 2000. Production of extracellular Cu complexing ligands by eucaryotic phytoplankton in response to Cu stress. *Limnology and Oceanography* 45: 619-627.

- 1 Deaver, E. and J.H. Rodgers 1996. Measuring bioavailable copper using anodic stripping
2 voltammetry. *Environmental Toxicology and Chemistry* 15: 1925-1930.
- 3 Di Toro, D.M., H.E. Allen, H.L. Bergman, J.S. Meyer, P.R. Paquin, and R.C. Santore 2001.
4 Biotic ligand model of the acute toxicity of metals. I. Technical basis. *Environmental*
5 *Toxicology and Chemistry* 20: 2383-2396.
- 6 Donat, J.R., and K.W. Bruland 1995. Trace elements in the Oceans, p. 247-281. *In* [eds.], B.
7 Salbu and E. Steinnes Trace elements in natural waters. CRC Press.
- 8 Donat, J.R., K.A. Lao, and K.W. Bruland 1994. Speciation of dissolved copper and nickel in
9 South San Francisco Bay: a multhi-method approach. *Analytica Chimica Acta* 284: 547-
10 571.
- 11 Druffel, E. R. M., P. M. Williams, J. E. Bauer, and J. R. Ertel. 1992. Cycling of dissolved and
12 Particulate Organic Matter in the Open Ocean. *Journal of Geophysical Research* 97:
13 15639-15659.
- 14 Ferrari, G.M., M.D. Dowell, S. Grossi, and C. Targa 1996. Relationship between the optical
15 properties of chromophoric dissolved organic matter and total concentration of
16 dissolved organic carbon in the southern Baltic Sea region. *Marine Chemistry* 55: 299-
17 316.
- 18 González-Dávila, M. 1995. The role of phytoplankton cells on the control of heavy metal
19 concentration in seawater. *Marine Chemistry* 48: 215-236.
- 20 González-Dávila, M., J. Santana-Casiano, and L. Laglera 2000. Copper adsorption in diatom
21 cultures. *Marine Chemistry* 70: 161-170.
- 22 Gordon, A.S., J.R. Donat, R.A. Kango, B.J. Dyer, and L.M. Stuart 2000. Dissolved copper-
23 complexing ligands in cultures of marine bacteria and estuarine water. *Marine*
24 *Chemistry* 70: 149-160.

- 1 Guillard, R.R.L. 1975. Culture of phytoplankton for feeding marine invertebrates, p. 29-60. *In*
2 [eds.], W. Smith and M. Chanley Culture of Marine Invertebrate Animals. Plenum
3 Press.
- 4 Hammer, K.D., K. Eberlein, G. Kattner, and U.H. Brockmann 1983. Fluctuations of dissolved
5 amino acids: a comparison of natural and enclosed phytoplankton populations in the
6 North Sea, p. 559-572. *In* [eds.], J. Sündermann and W. Lenz North Sea Dynamics.
7 Springer-Verlag.
- 8 Hedges, J. I. 1988. Polymerization of Humic Substances in Natural Environments, p. 45-58. *In*
9 F. H. Frimmel and R. F. Christman [eds.], Humic substances and their role in the
10 environment. John Wiley & Sons Limited.
- 11 Hedges, J. I. 2002. Why dissolved organic matter?, p. 1-33. *In* D. A. Hansell and C. A. Carlson
12 [eds.], Biogeochemistry of Marine Dissolved Organic Matter. Academic Press.
- 13 His, E., R. Beiras, and M.N.L. Seaman 1999. The assessment of marine pollution - Bioassays
14 with bivalve embryos and larvae, p. 3-178. *In* [eds.], A.J. Southward, P.A. Tyler, and
15 C.M. Young Advances in Marine Biology. Academic Press.
- 16 Ishiwatari, R. 1992. Macromolecular material (humic substance) in the water column and
17 sediments. Marine Chemistry 39: 151-166.
- 18 Kieber, R. J., L. H. Hydro, and P. J. Seaton 1997. Photooxidation of triglycerides and fatty
19 acids in seawater: implication toward the formation of marine humic substances.
20 Limnology and Oceanography 42: 1454-1462.
- 21 Leal, M.F.C., M.T.S.D. Vasconcelos, and C.M.G. van den Berg 1999. Copper-induced release
22 of complexing ligands similar to thiols by *Emiliania huxleyi* in seawater cultures.
23 Limnology and Oceanography 44: 1750-1762.
- 24 Lorenzo, J.I., O. Nieto, and R. Beiras 2002. Effect of humic acids on speciation and toxicity of
25 copper to *Paracentrotus lividus* larvae in seawater. Aquatic Toxicology 58: 27-41.

- 1 Lorenzo, J.I. 2003. Metal speciation and bioavailability in the presence of organic matter
- 2 studied using two marine biological models: the sea-urchin embryo (*P. lividus*) and the
- 3 mussel (*M. edulis*). Ph.D. thesis. Univ. of Vigo.
- 4 Martell, A.E., and R.M. Smith 1974. Critical Stability Constants. Vol. 1. Amino acids. Plenum
- 5 Press.
- 6 Midorikawa, T., and E. Tanoue 1998. Molecular masses and chromophoric properties of
- 7 dissolved organic ligands for copper (II) in oceanic water. Marine Chemistry 62: 219-
- 8 239.
- 9 Moffett, J.W., R. Zika, and L.E. Brand 1990. Distribution and potential sources and sinks of
- 10 copper chelators in the Sargasso Sea. Deep-Sea Research 37: 27-36.
- 11 Mopper, K., and C. A. Schultz 1993. Fluorescence as a possible tool for studying the nature
- 12 and water column distribution of DOC components. Marine Chemistry 41: 229-238.
- 13 Muller, F., S. Jacquet, and W. Wilson 2003. Biological factors regulating the chemical
- 14 speciation of Cu, Zn, and Mn under different nutrient regimes in a marine mesocosm
- 15 experiment. Limnology and Oceanography 48: 2289-3202.
- 16 Mykkestad, S. M. 1995. Release of extracellular products by phytoplankton with special
- 17 emphasis on polysaccharides. Science of the Total Environment 165: 155-164.
- 18 Nagata, T. 2000. Production mechanisms of dissolved organic matter, p. 121-152. In D. L.
- 19 Kirchman [ed.], Microbial Ecology of the Oceans. Wiley-Liss.
- 20 Nieto-Cid, M., X. A. Álvarez-Salgado, J. Gago, F. F. Pérez. DOM fluorescence, a tracer for
- 21 biogeochemical processes in a coastal upwelling system (Ría de Vigo, NW Iberian
- 22 Peninsula). Marine Ecology Progress Series submitted.
- 23 Nor, Y.M. 1987. Ecotoxicity of copper to aquatic biota: A Review. Environmental Research 43:
- 24 274-282.
- 25 Ortego, L.S., and W.H. Benson 1992. Effects of dissolved humic material on the toxicity of
- 26 selected pyrethroid insecticides. Environmental Toxicology and Chemistry 11: 261-265.

- 1 Playle, R.C. 1998. Modelling metal interactions at fish gills. *The Science of the Total*
2 *Environment* 219: 147-163.
- 3 Robinson, M. and L. Brown 1991. Copper complexation during a bloom of *Gymnodinium*
4 *sanguineum* Hirasaka (Dinophyceae) measured by ASV. *Marine Chemistry* 33: 105-118.
- 5 Ryan, A., E. Van Genderen, J. Tomasso, and S. Klaine 2004. Influence of natural organic
6 matter source on copper toxicity to larval fathead minnows (*Pimephales promelas*):
7 implications for the biotic ligand model. *Environmental Toxicology and Chemistry* 23:
8 1567-1574.
- 9 Sakshaug, E. and O. Holm-Hansen 1977. Chemical composition of *Skeletonema costatum*
10 (Grev.) Cleve and *Pavlova* (Monochrysis) *lutheri* (Droop) Green as a function of nitrate-,
11 phosphate-, and iron-limited growth. *Journal of Experimental Marine Biology and*
12 *Ecology* 29: 1-34.
- 13 Scheinberg, H. 1991. Copper, p. 893-908. *In* [ed.], E. Merian Metals and their compounds in
14 the environment. VCH.
- 15 Shank, G., S. Skrabal, R. Whitehead, and R. Kieber 2004. Strong copper complexation in an
16 organic-rich estuary: the importance of allochthonous dissolved organic matter. *Marine*
17 *Chemistry* 88: 21-39.
- 18 Sokal, R.R. and F.J. Rohlf. 1995. *Biometry*, 3 ed. W.H. Freeman and Company.
- 19 Sunda, W. G. 1995. The influence of nonliving organic matter on the availability and cycling
20 of plant nutrients in seawater, p. 191-207. *In* R. G. Zepp and C. Sonntag [eds.], *Role of*
21 *nonliving organic matter in the Earth's carbon cycle*. John Wiley and Sons Ltd.
- 22 Sunda, W.G. and J.A.M. Lewis 1978. Effect of complexation by natural organic ligands on the
23 toxicity of copper to a unicellular alga, *Monochrysis lutheri*. *Limnology and*
24 *Oceanography* 23: 870-876.

1 Swallow, K., J.C. Westall, D.M. McKnight, N. Morel, and F.M.M. Morel 1978. Potentiometric
2 determination of copper complexation by phytoplankton exudates. *Limnology and*
3 *Oceanography* 23: 538-542.

4 Vasconcelos, M.T.S.D., M.F.C. Leal, and C.M.G. van den Berg 2002. Influence of the nature of
5 the exudates released by different marine algae on the growth, trace metal uptake and
6 exudation of *Emiliania huxleyi* in natural seawater. *Marine Chemistry* 77: 187-210.

7 WHO. 1998. Environmental Health Criteria, N° 200 : Copper. 1-360.

8 Xue, H., A. Oestreich, D. Kistler, and L. Sigg 1996. Free cupric ion concentrations and Cu
9 complexation in selected Swiss lakes and rivers. *Aquatic Sciences* 58: 69-86.

10 Yamashita, Y., and E. Tanoue. 2003 (¿ó 2004?). Chemical characterization of protein-like
11 fluorophores in DOM in relation to aromatic amino acids. *Marine Chemistry* 82: 255-
12 271.

13 Zou, L., X.-C. Wang, J. Callahan, R. Culp, R. Chen, M. Altabet, and M.-Y. Sun 2004. Bacterial
14 roles in the formation of high-molecular-weight dissolved organic matter in estuarine
15 and coastal waters: evidence from lipids and the compound-specific isotopic ratios.
16 *Limnology and Oceanography* 49: 297-302.

Table 1. Complexing capacity ($L \pm CI$ 95%, $\mu\text{M Cu}$) and conditional stability constant ($\log K' \pm CI$ 95%) in cultures A and B at different time intervals; n, number of Cu^{2+} additions in each titration.

Time (days)	L_A ($\mu\text{M Cu}$)	$\log K'_{\text{Cu}'}$	n	L_B ($\mu\text{M Cu}$)	$\log K'_{\text{Cu}'}$	n
0	0.04 ± 0.01	8 ± 2	12	0.08 ± 0.01	7.9 ± 0.9	14
2	0.12 ± 0.01	6.9 ± 0.2	15	0.13 ± 0.02	7.1 ± 0.5	15
4	0.40 ± 0.03	6.7 ± 0.2	12	0.24 ± 0.02	6.9 ± 0.2	15
5	0.64 ± 0.02	7.08 ± 0.02	15	0.57 ± 0.03	6.6 ± 0.1	13
6	0.64 ± 0.04	7.12 ± 0.04	16	0.58 ± 0.02	7.0 ± 0.1	16
8	0.79 ± 0.02	7.5 ± 0.2	14	0.84 ± 0.02	7.30 ± 0.09	15
11	0.92 ± 0.06	6.8 ± 0.2	14	1.00 ± 0.05	6.8 ± 0.1	14
13	1.12 ± 0.03	6.89 ± 0.09	12			

Table 2. Percentage of the measured ligand concentration explained by the FDOM_T (L_{Prot}) and FDOM_M (L_{HA}) fluorophores ($\pm$ standard deviation) during the course of the *Skeletonema costatum* culture according to equation (4). The estimated percentage of the FDOM_T corresponding to DFAA and small peptides with metal binding properties is also shown in the second column.

Time (days)	% Active FDOM_T	% L_{Prot}	% L_{HA}
0	100	72 $\pm$ 1	28 $\pm$ 1
2	100	78 $\pm$ 5	22 $\pm$ 2
4	100	63 $\pm$ 23	37 $\pm$ 13
5	100	72 $\pm$ 6	28 $\pm$ 2
6	100	56 $\pm$ 1	44 $\pm$ 1
8	100	42 $\pm$ 2	58 $\pm$ 2
11	15	8 $\pm$ 1	92 $\pm$ 6
13	0	0	100

Figure captions

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 number measurements. (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).

Fig. 2. Evolution of the measured fluorophores during the culture. (a) Comparison of FDOM_T and the DOC content of the culture. (b) FDOM_T production rate versus the DOC production rate. (c) Comparison of FDOM_M and the DOC content of the culture. (d) FDOM_T production rate versus time. Lines correspond to the model II linear regressions fitting obtained data in (a) and (b), and to the model I linear regression in (c) and (d), respectively. The regression equations ($\pm$ standard error), the correlation coefficients, and the significance of the relationships are also presented.

Fig. 3. (a) Time evolution of Cu titrations of the filtered culture. Solid lines represent the best non-linear squares fittings of obtained data to the equation [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.

Fig. 4. Relationships between the amount of ligand in the culture and different measurements of the DOM content in the medium.. (a), (b), (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, equation (4)) was calculated with data from the anabolic phase. White

1 and grey dots represent data from the catabolic phase fitted to the previous regression before
2 and after FDOM_T corrections. Black dots and long dashed lines correspond to data and
3 fittings obtained in the anabolic phase of the culture. Open dots and dotted lines correspond
4 to data and fittings from the catabolic phase. The regression equations ($\pm$ standard error), the
5 correlation coefficients and the significance of the model II relationships are also presented.

Lorenzo et al., Figure 1

Lorenzo et al., Figure 2

Lorenzo et al., Figure 4