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# Production of chromophoric dissolved organic matter by marine phytoplankton

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*Abstract*

Incubation experiments with axenic cultures of four common phytoplankton species of the genera *Chaetoceros*, *Skeletonema*, *Prorocentrum*, and *Micromonas* were performed to test for the production of fluorescent dissolved organic matter (FDOM) by marine phytoplankton. Our results prove that the four species exuded both fluorescent protein-like and marine humic–like materials in variable amounts, with more production by the diatoms *Chaetoceros* sp. and *Skeletonema costatum* and less by *Prorocentrum minimum*. Whereas the exudation of protein-like substances by healthy phytoplankton cells has been recognized, the in situ production of marine humic-like substances is still a matter of debate. Using axenic cultures, we demonstrate unequivocally that phytoplankton can directly contribute to the autochthonous production of colored humic-like substances in the ocean. Extrapolation of these findings to the field indicates that about 20% of the marine humic–like substances produced in the highly productive coastal upwelling system of the Ría de Vigo could originate from growing phytoplankton. Therefore, the exudation of FDOM by marine phytoplankton should be considered in future studies of the dynamics of colored DOM in marine systems.

Marine dissolved organic matter (DOM) represents the largest pool of reduced carbon in the Earth. Phytoplankton is one of the major sources of DOM, which is released to the water column by exudation, excretion, and cell lysis due to viral attack, grazing, and sloppy feeding (Myklestad 2000), and constitutes a substrate that supports heterotrophic bacterial growth (Azam et al. 1983). The chemical composition, origin, and fate of the different components of the DOM pool in aquatic systems are still poorly known (Hansell 2002).

Recent methodological advances have enabled identification of different fractions of DOM based on their optical properties in a way that is methodologically fast and simple and that might provide indications about the origin and degradability of the DOM pool (Coble 1996). A fraction of the DOM pool absorbs light at both ultraviolet (UV) and visible wavelengths, and this portion of the DOM pool is termed colored dissolved organic matter (CDOM; Coble 2007). A subfraction of CDOM can emit blue fluorescence when it is irradiated with UV light, and this subfraction is called fluorescent CDOM (FDOM; Coble 1996, 2007). It is possible to distinguish between two main groups of FDOM substances, depending on their excitation and emission (Ex/Em) wavelengths. One group fluoresces at wavelength pairs characteristic of the aromatic amino acids (Ex/Em 280 nm/350 nm) that correspond to the peak-T reported by Coble (1996). This group, known as protein-like substances, has been considered as a proxy for labile DOM (Yamashita and Tanoue 2003; Nieto-Cid et al. 2006). The other group, which emits radiation in the wavelength range of 380–420 nm when excited at 320 nm (peak-M, as reported by Coble [1996]), is termed marine humic–like substances, and it is considered to be photo-labile and bio-refractory (Chen and Bada 1992; Nieto-Cid et al. 2006). Changes in FDOM are a good indication of biological (Chen and Bada 1992; Nieto-Cid et al. 2006) and photochemical processes (Moran et al. 2000; Nieto-Cid et al. 2006) acting upon the bulk DOM pool.

The role of CDOM is key for ocean biogeochemical cycles. since it can control light penetration in the water column. A high concentration of CDOM can reduce harmful UV effects on phytoplankton, acting as a photoprotector, but it can also attenuate photosynthetic usable radiation, reducing primary production in regions where light is limiting (Arrigo and Brown 1996). On the contrary, at low concentrations of CDOM, sunlight can damage not only phytoplankton cells but also bacterioplankton physiology and deoxyribonucleic acid as well (Herndl et al. 1993). Another important role of CDOM involves its capacity for metal scavenging and the formation of complexes that can be beneficial to phytoplankton when metals present in the medium reach toxic concentrations (Midorikawa and Tanoue 1998).

Sources of CDOM include continental runoff that transports DOM, primarily from soils (Coble 2007); abiotic condensation and transformation of biopolymers (e.g., photo-oxidized polyunsaturated lipids released into the water column by plankton) (Kieber et al. 1997); and in situ biological production (Yentsch and Reichert 1961; Kramer and Herndl 2004). Within these autochthonous sources, CDOM can be produced as a by-product of DOM metabolism, mainly by bacteria. But its production by copepods, krill, and other planktonic organisms has also been demonstrated in recent studies (Steinberg et al. 2004; Ortega-Retuerta et al. 2009).

The release of DOM by phytoplankton has been recognized as a major process in global biogeochemical cycles (Myklestad 2000). The nature of the dissolved organic carbon (DOC) derived from phytoplankton is highly complex, but major fractions include carbohydrates,

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followed by N-compounds, namely protein, polypeptides, and amino acids (Goldman et al. 1992; Myklestad 2000). However, the direct contribution of FDOM to the DOC released by phytoplankton is controversial. On the one hand, a few field studies (Carder et al. 1989; Twardowski and Donaghay 2001) and some culture experiments (Seritti et al. 1994) indicate that phytoplankton is a possible source of CDOM. However, other authors have reported the opposite, as they did not find a significant correlation between CDOM and phytoplankton biomass either in natural systems (Nelson et al. 1998) or in cultures (Rochelle-Newall and Fisher 2002).

To better understand the sources and sinks of different types of organic matter in the ocean we must determine unequivocally whether phytoplankton produces CDOM. In this study, we have quantified the buildup of FDOM and DOC in axenic cultures of four common marine phytoplankton species (two diatoms, *Chaetoceros* sp. and *Skeletonema costatum*; a dinoflagellate, *Prorocentrum minimum*; and a prasinophyte, *Micromonas pusilla*) in order to determine whether phytoplankton is a direct source of CDOM, particularly of fluorescent humic-like substances.

## Methods

*Phytoplankton cultures*—Four axenic species obtained from the Provasoli–Guillard National Center for Culture of Marine Phytoplankton (CCMP; https://ccmp.bigelow.org/) were cultured in axenic conditions. The strains used were the diatoms *Chaetoceros* sp. (CCMP199) and *S. costatum* (CCMP2092) (Greville) Cleve, the dinoflagellate *P. minimum* (CCMP1329) (Pavillard) J. Schiller, and the prasinophyte *M. pusilla* (CCMP1545) (R.W. Butcher) I. Manton and M. Parke.

An inoculum of each species was added to 2 liters of F/2 culture medium made with filtered and autoclaved coastal Mediterranean seawater. After gentle shaking, each mixture was distributed into three polystyrene bottles. Each bottle was filled with 600 mL and incubated at 20°C under artificial photosynthetic active radiation of 100 $\mu$mol photon $m^{-2}$ $s^{-1}$, using a 16 : 8-h light : dark cycle, until cell density increased about one order of magnitude.

Aliquots for phytoplankton counts, DOC concentration, and DOM fluorescence were taken at the beginning and end of the incubation period, which was 3 d for all microalgae except for *P. minimum*, which was incubated for 7 d. Controls of the F/2 medium were also taken before adding the inoculum. Since the F/2 medium was the same for the four cultures, the differences between the initial values of the variables studied reflected the variable composition of the aliquots of the four cultures added to the F/2 medium.

Algal cultures were initially axenic, as guaranteed by the CCMP. To determine phytoplankton cell abundance and to assure that the algal cultures were kept in axenic conditions, aliquots of 1 mL of each culture were fixed with 1% paraformaldehyde + 0.05% glutaraldehyde (final concentration), stained with 4,6 diamidino-2-phenylindole (10 $\mu$g $mL^{-1}$, final concentration), and counted with an Olympus BX61 epifluorescence microscope under blue and UV wavelength excitation at the beginning and at the end of the experiment.

At the beginning and at the end of the incubation, DOC and FDOM samples were filtered onto 0.2-$\mu$m Sterivex filters. Milli-Q water was filtered through this filtration system, and no significant changes were observed in DOC and FDOM analysis, proving that contamination did not occur during filtration. A control bottle containing only F/2 medium was incubated under the same conditions, and no significant differences were observed at the end of the experiment (details not shown).

Phytoplankton biomass, B, in pg C $cell^{-1}$, was estimated with the following conversion factors: $B = 0.216 \times V^{0.939}$ for *P. minimum* and *M. pusilla*, and $B = 0.288 \times V^{0.811}$ for diatoms (Menden-Deuer and Lessard 2000), where V is the cell volume in $\mu m^3$ and was calculated for the different species following the geometric models given by Sun and Liu (2003).

*Determination of FDOM*—To quantify the production of FDOM, samples were measured immediately after collection following the method of Nieto-Cid et al. (2006). Single measurements and emission excitation matrices (EEMs) of the aliquots were performed with a LS 55 PerkinElmer Luminescence spectrometer, equipped with a xenon discharge lamp, equivalent to 20 kW for 8-$\mu$s duration. The detector was a red-sensitive R928 photomultiplier, and a photodiode worked as a reference detector. Slit widths were 10.0 nm for the excitation and emission wavelengths; scan speed was 250 nm $min^{-1}$. Measurements were performed at a constant room temperature of 20°C in a 1-cm quartz fluorescence cell. The Ex/Em wavelengths used for single measurements were those established by Coble (1996), thus: Ex/Em 280 nm/350 nm (peak-T) as indicator of protein-like substances and Ex/Em 320 nm/410 nm (peak-M) as indicator of marine humic–like substances. Following Coble (1996), fluorescence measurements were expressed in quinine sulfate units (QSU) (i.e., in $\mu$g eq QS $L^{-1}$) by calibrating the LS 55 PerkinElmer at Ex/Em 350 nm/450 nm against a quinine sulfate dihydrate (QS) standard made up in 0.05 mol $L^{-1}$ sulfuric acid.

EEMs were performed to track possible changes in the position of the protein-like and humic-like fluorescence peaks. These matrices were generated by combining 21 synchronous Ex/Em fluorescence spectra of the sample, obtained for excitation wavelengths from 250 to 400 nm, and an offset between the excitation and emission wavelengths of 50 nm for the first scan and 250 nm for the 21st scan. Rayleigh scatter does not need to be corrected when the EEMs are generated from synchronous spectra, and Raman scatter was corrected by subtracting the pure water (Milli-Q) EEM from the sample EEM.

*Determination of DOC*—Approximately 10 mL of water were collected in precombusted (450°C, 12 h) glass ampoules for DOC analysis. $H_3PO_4$ was added to acidify the sample to $pH < 2$, and the ampoules were heat-sealed and stored in the dark at 4°C until analysis. DOC was measured with a Shimadzu TOC-V organic carbon analyzer. The system was standardized daily with a

Table 1. Average cell abundances ($\bar{C}$), biomass ($\bar{B}$), and growth rates ($\mu_c$), calculated considering exponential algal growth. Apparent percentage of net photosynthetic extracellular release (APER) in each algal culture.

| Algal culture | Time (d) | $\bar{C}$ (cells mL$^{-1}$) | $\bar{B}$ ($\mu$g C L$^{-1}$) | $\mu_c$ (d$^{-1}$) | APER (%) |
|---|---|---|---|---|---|
| *Chaetoceros* sp. | 3 | 162±5.2×10$^3$ | 990±32 | 0.89±0.02 | 11.7±0.5 |
| *Skeletonema costatum* | 3 | 303±21×10$^3$ | 2272±158 | 0.87±0.03 | 10±2.0 |
| *Prorocentrum minimum* | 7 | 12.0±0.5×10$^3$ | 1254±136 | 0.45±0.02 | 18±1.0 |
| *Micromonas pusilla* | 3 | 3342±29×10$^3$ | 1975±13 | 0.59±0.05 | 12.8±0.8 |

potassium hydrogen phthalate standard. Each ampoule was injected three to five times, and the three replicates that yielded a standard deviation of $< 1\%$ were chosen to calculate the average DOC concentration of each sample. The performance of the analyzer was tested with the DOM reference materials provided by Prof. D. Hansell (University of Miami). We obtained a concentration of 45.2 ± 0.3 $\mu$mol C L$^{-1}$ for the deep ocean reference (Sargasso Sea deep water, 2600 m) minus blank reference materials, the day when the samples were analyzed. The nominal DOC value provided by the reference laboratory is 45 $\mu$mol C L$^{-1}$.

*Phytoplankton cell densities, growth rates, and net percentage of extracellular release*—Average of cell abundance ($\bar{C}$), biomass ($\bar{B}$), and growth rate ($\mu_C$) for the four cultures were calculated considering that the species were in exponential growth. Since the cultures were kept in axenic conditions throughout the growing period, it can be assumed that organic carbon dynamics depended only on phytoplankton activity. A proxy for the percentage of net photosynthetic extracellular release (PER) was calculated as APER = $\Delta$DOC/($\Delta$DOC + $\Delta$B) × 100, where APER is apparent PER and $\Delta$DOC and $\Delta$B are the net increase of DOC and phytoplankton biomass (both in $\mu$g C L$^{-1}$) through the incubation time, respectively.

*Statistical tools*—The paired Student's *t*-test was used to check for significant differences in the measured variables between the initial and final incubation times (Sokal and Rohlf 1984). Regression model II was applied to calculate linear fitting parameters.

## Results

*Phytoplankton cell densities and growth rates*—Average cell abundance in the different cultures varied between 12 × 10$^3$ cells mL$^{-1}$ for *P. minimum* and 33 × 10$^5$ cells mL$^{-1}$ for *M. pusilla,* and biomass ranged from 990 $\mu$g C L$^{-1}$ for *Chaetoceros* sp. to 2272 $\mu$g C L$^{-1}$ for *S. costatum*. *Chaetoceros* sp. showed the highest exponential growth rates, at 0.89 d$^{-1}$, and *P. minimum* had the lowest growth rate, at 0.45 d$^{-1}$ (Table 1). These numbers are within the ranges reported in the literature (Rose and Caron 2007).

*Production of DOC*—DOC increased significantly ($p < 0.01$) in all cultures. *P. minimum*, incubated for 7 days, showed the largest bulk DOC rise: 0.85 mg C L$^{-1}$. Total DOC production in the other three cultures, which were incubated for 3 d, varied from 0.35 to 0.64 mg C L$^{-1}$ (Fig. 1a). APER values ranged between 10% and 18% (Table 1), and biomass-specific DOC production rates ranged from 86 × 10$^{-3}$ to 116 × 10$^{-3}$ $\mu$g C $\mu$g C$^{-1}$ d$^{-1}$ (Table 2). When we normalized to cell density instead of biomass, *P. minimum* presented the highest DOC production rates and *M. pusilla* the lowest. Both diatoms *Chaetoceros* sp. and *S. costatum* presented similar values of cell-specific DOC production rates (71.1 × 10$^{-8}$ and 70.1 × 10$^{-8}$ $\mu$g C cell$^{-1}$ d$^{-1}$, respectively), two orders of magnitude lower than *P. minimum* (10.2 × 10$^{-6}$ $\mu$g C cell$^{-1}$ d$^{-1}$). Turnover rates of DOC, calculated assuming that they were proportional to cell growth rate and cells were in exponential growth, ranged from 0.04 to 0.07 d$^{-1}$ (Table 3).

*Production of FDOM*—Fluorescence EEMs of the CDOM produced by each culture during the incubation time are shown in Fig. 2. These EEMs were obtained by subtracting the fluorescence intensities at the beginning of the culture from the end values. In all cultures, EEMs presented marked fluorescence peaks in the protein-like and humic-like areas. In the protein-like area, the most intense peak presented a maximum at Ex/Em 275 nm/358 nm for *Chaetoceros* sp., *P. minimum*, and *S. costatum* cultures that was slightly displaced toward longer emission wavelengths compared to that established by Coble (1996) at Ex/Em 275 nm/340 nm (peak-T). The *S. costatum* culture presented the highest fluorescence at peak-T, whereas *M. pusilla* showed the lowest value, and it was centered at an emission wavelength of 345 nm. In the humic-like area all cultures showed a conspicuous peak within the range of the marine humic–like substances defined by Coble (1996) at Ex/Em 312 nm/380-420 nm (peak-M). The position of the fluorescence maxima in this area differed slightly, but not significantly, for each culture with excitation maxima, displaced to shorter wavelength relative to peak-M (Coble 1996). *Chaetoceros* sp. and *P. minimum* showed their maxima at Ex/Em 310 nm/399 nm and 316 nm/397 nm, with an intensity of 1.36 QSU and 1.19 QSU, respectively. *S. costatum* and *M. pusilla* presented this maximum at Ex/Em 306 nm/396 nm, and their fluorescence intensities were the most pronounced with 3.89 QSU and 2.03 QSU, respectively.

The EEM of *M. pusilla* also showed three peaks of lower fluorescence intensity at Ex/Em 348 nm/436 nm, 354 nm/447 nm, and 271 nm/432 nm. Finally, both diatoms showed a third peak that *M. pusilla* and *P. minimum* did not produce: *Chaetoceros* sp. at Ex/Em 280 nm/381 nm (1.56 QSU) and *S. costatum* at Ex/Em 289 nm/391 nm (3.73 QSU).

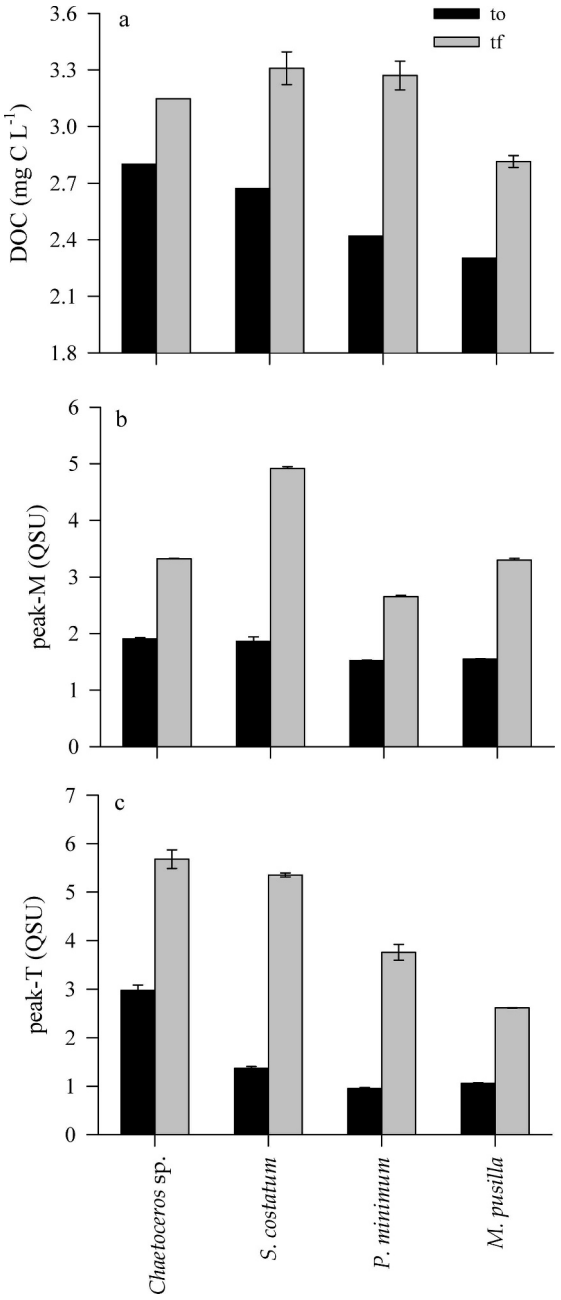


Fig. 1. Initial and final concentrations of (a) DOC (mg C $L^{-1}$), (b) peak-M (QSU), and (c) peak-T (QSU). Dark bars correspond to concentration at initial time ($t_0$) and gray bars to concentration at final time ($t_f$).

A significant ($p < 0.01$) net increase of FDOM (peak-M and peak-T) was observed in all cultures during the experiment (Fig. 1b,c). *S. costatum* exhibited the most pronounced increase: 3.05 ± 0.08 QSU for the humic-like substances. In the case of the protein-like substances, *S. costatum* also presented the highest increase (3.98 ± 0.06 QSU), whereas *M. pusilla* presented the lowest increase, 1.56 ± 0.01 QSU. The biomass-specific FDOM production rates were the highest for *Chaetoceros* sp. (Table 2) in both protein-like and humic-like peaks. *P. minimum* presented the lowest increase of humic-like substances and *M. pusilla* the lowest rates of protein-like substance production. For the case of the cell-specific FDOM production rates (data not shown), *P. minimum* presented the highest and *M. pusilla* the lowest values in both fluorescent peaks. Diatoms showed similar cell-specific production rates of protein-like and humic-like substances. The dimensionless Δpeak-T : Δpeak-M production ratio was calculated: it was maximum for *P. minimum* (2.49 ± 0.03) and minimum for *M. pusilla* (0.89 ± 0.03), whereas the diatoms presented intermediate values of 1.91 ± 0.05 for *Chaetoceros* sp. and 1.31 ± 0.03 for *S. costatum*.

Turnover rates of peak-T and peak-M were also calculated for all species (Table 3). *S. costatum* presented the highest values for both the protein-like and humic-like fluorescence, whereas *P. minimum* presented the lowest turnover rates.

Phytoplankton biomass was significantly correlated with the fluorescence of peak-M ($R^2 = 0.78$, $p < 0.0001$; Fig. 3a) when data for all species were included. Peak-T and DOC were significantly correlated with phytoplankton biomass (Fig. 3b,c) but with lower $R^2$ (0.36 and 0.44 for peak-T and DOC, respectively).

## Discussion

Since all cultures were kept axenic during the course of the experiment, carbon dynamics were based exclusively on phytoplankton activity. We calculated a proxy to PER, APER, substituting ΔPOC (particulate organic carbon) by ΔBiomass, assuming that the production of transparent exopolymer particles and DOC uptake did not occur during the course of the experiment. It is likely that these processes were negligible during the exponential phase of the cultures, but we have no direct evidence to prove it. The observed buildup of DOC concurs with the results of many studies showing significant DOC excretion by phytoplankton (Bjørnsen 1988). Net APER values obtained in this

Table 2. Net increase of the concentration of dissolved organic carbon (DOC) and the fluorescence of peak-T and peak-M normalized to average biomass ($\bar{B}$) and incubation time ($t$).*

| Species | $\Delta DOC/\bar{B}\ t$ ($\mu$g C $\mu$g C$^{-1}$ d$^{-1}$) | $\Delta$peak-T$/\bar{B}\ t$ ($\mu$g eq QS $\mu$g C$^{-1}$ d$^{-1}$) | $\Delta$peak-M$/\bar{B}\ t$ ($\mu$g eq QS $\mu$g C$^{-1}$ d$^{-1}$) |
|---|---|---|---|
| *Chaetoceros* sp. | $116\pm11\times10^{-3}$ | $9.1\pm0.8\times10^{-4}$ | $4.8\pm0.1\times10^{-3}$ |
| *Skeletonema costatum* | $93\pm13\times10^{-3}$ | $5.8\pm0.1\times10^{-3}$ | $4.5\pm0.1\times10^{-3}$ |
| *Prorocentrum minimum* | $97\pm9\times10^{-3}$ | $3.2\pm0.2\times10^{-3}$ | $1.3\pm0.03\times10^{-3}$ |
| *Micromonas pusilla* | $86\pm5\times10^{-3}$ | $2.6\pm0.02\times10^{-3}$ | $3.0\pm0.1\times10^{-3}$ |

* QS, quinine sulfate dihydrate.

Table 3. Turnover rates of the concentration of dissolved organic carbon (DOC) and the fluorescence of peak-T and peak-M for each culture, calculated as $\mu_{DOC} = \ln[DOC(t_f)/DOC(t_0)]/t$; $\mu_T = \ln[\text{peak-T}(t_f)/\text{peak-T}(t_0)]/t$; and $\mu_M = \ln[\text{peak-M}(t_f)/\text{peak-M}(t_0)]/t$.*

| Species | $\mu_{DOC}$ ($d^{-1}$) | $\mu_T$ ($d^{-1}$) | $\mu_M$ ($d^{-1}$) |
|---|---|---|---|
| *Chaetoceros* sp. | $3.9\pm0.3\times10^{-2}$ | $22\pm1\times10^{-2}$ | $18.5\pm0.1\times10^{-2}$ |
| *Skeletonema costatum* | $7.1\pm0.9\times10^{-2}$ | $45.4\pm0.3\times10^{-2}$ | $32.3\pm0.2\times10^{-2}$ |
| *Prorocentrum minimum* | $4.3\pm0.3\times10^{-2}$ | $19.6\pm0.6\times10^{-2}$ | $7.9\pm0.1\times10^{-2}$ |
| *Micromonas pusilla* | $6.7\pm0.4\times10^{-2}$ | $30.16\pm0.06\times10^{-2}$ | $25.2\pm0.3\times10^{-2}$ |

* $t_f$, final time; $t_0$, initial time; $t$, time.

work were in the range of PER values found by other authors using the $^{14}C$ incorporation method. Mague et al. (1980) obtained a PER of 8% during the exponential growth phase of *S. costatum*, which is very close to the APER of 10% found in our study for the same species. According to Lancelot and Billen (1985), PER values are higher for microflagellates, with an inverse relationship to nutrient concentration. In our experiments with excess nutrients we obtained APER values of 13% for *Micromonas*, which is at the lower end of the PER range (10–60%) found by Lancelot and Billen (1985). Fieldwork carried out in the North Sea by Lancelot (1983) showed that within the same range of inorganic nitrogen concentrations, flagellates presented higher PER values than diatoms. The same author obtained low PER values (< 10%) when diatom species dominated the bloom, regardless of nutrient conditions. We also found this trend in our experimental work: the cultures of *M. pusilla* and *P. minimum* presented higher APER values (13% and 18%, respectively) than the diatoms (< 12%).

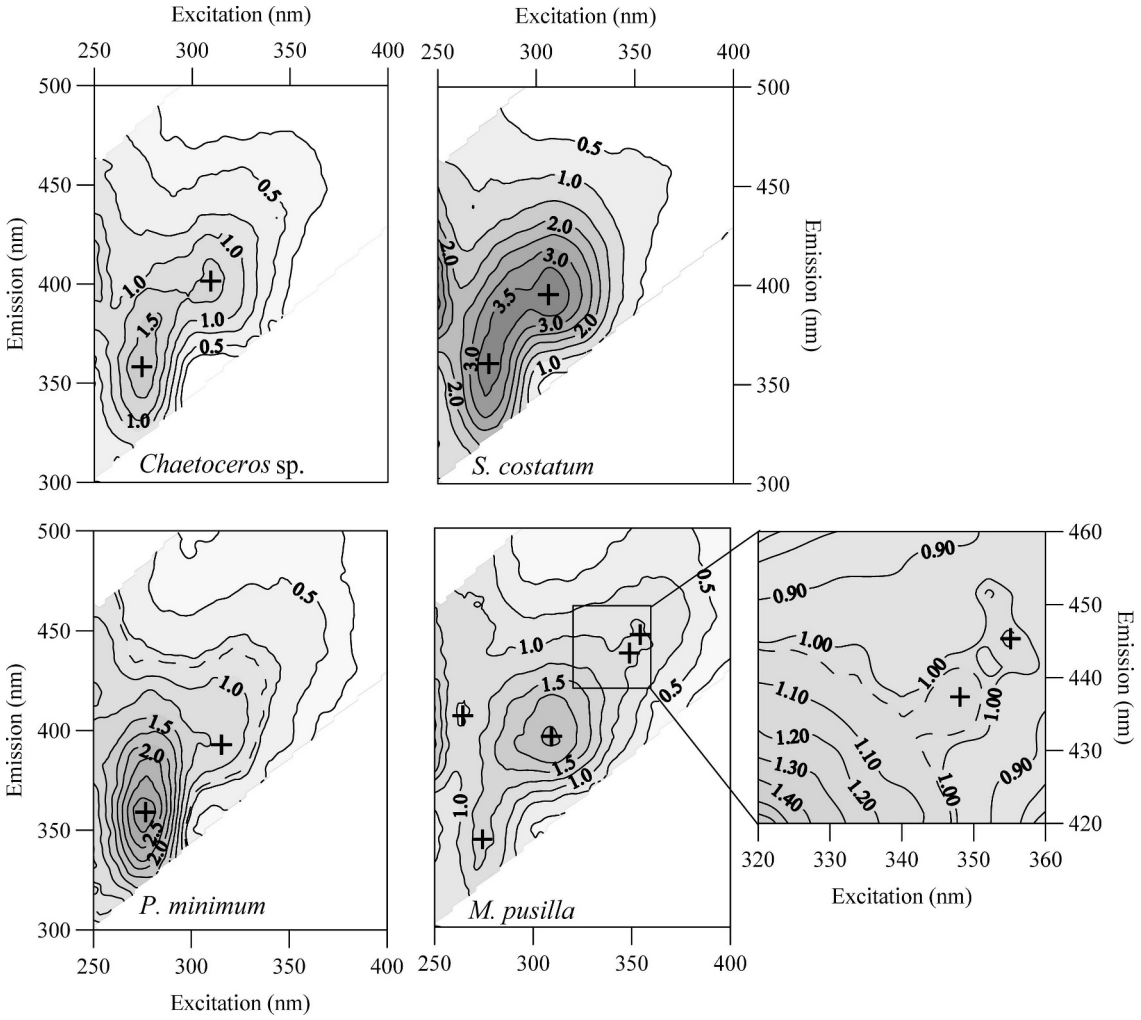


Fig. 2. Excitation emission matrices (EEMs) of the FDOM produced in the cultures, in quinine sulfate units (QSU). Note that these values are normalized to neither incubation time nor to biomass. All the species were incubated for 3 d, except *P. minimum,* which was incubated for 7 d.

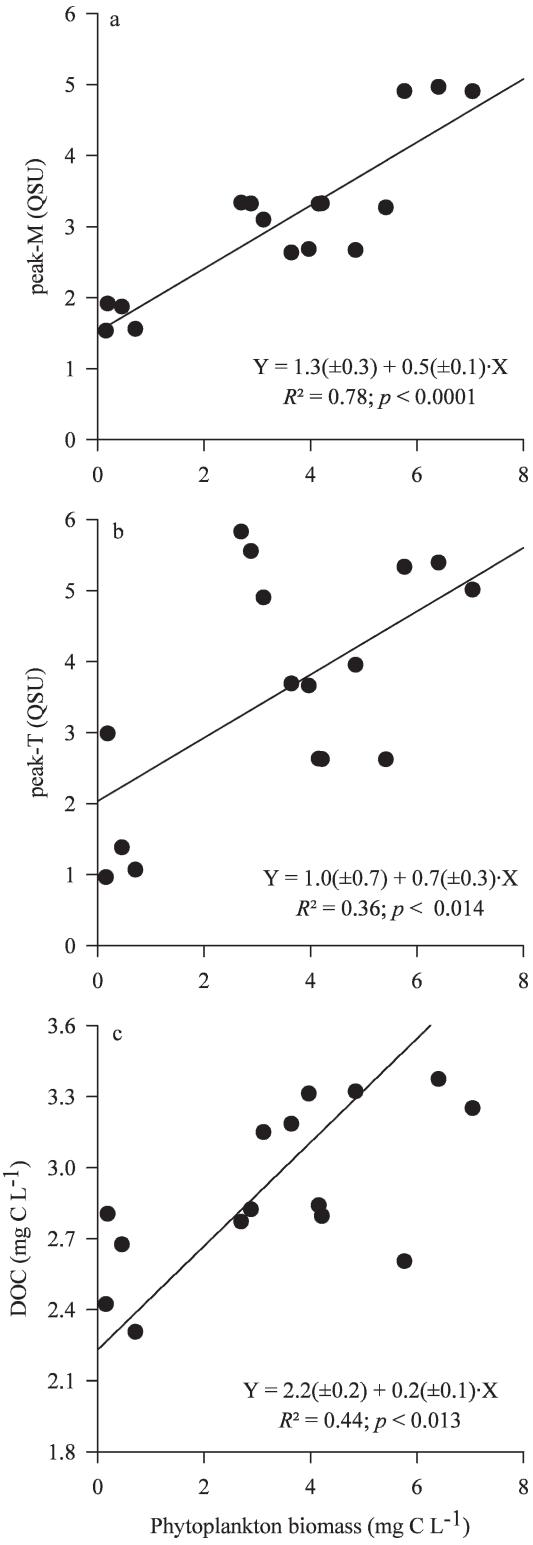


Fig. 3. (a) Plot of peak-M fluorescence (QSU) vs. phytoplankton biomass (mg C $L^{-1}$); (b) Plot of peak-T fluorescence (QSU) vs. biomass (mg C $L^{-1}$); and (c) Plot of DOC (mg C $L^{-1}$) vs. biomass (mg C $L^{-1}$).

In all cultures, humic-like and protein-like substances increased during the course of the incubations. Because bacteria were absent, it implies that the increases in DOC and FDOM were exclusively due to phytoplankton metabolism. The amount and quality of FDOM produced were different depending on the species. Factors such as nutrients, light, and growth phase can affect PER (Obernosterer and Herndl 1995), so it is likely that FDOM, as a fraction of the released DOM, is affected by the same factors. Here, the four species were incubated under identical experimental conditions, and, therefore, it can be assumed that the differences found on the excreted organic substances between cultures were attributable to specific differences in metabolic activity. The Δpeak-T : Δpeak-M ratio was significantly different among species, indicating that the selected dinoflagellates exuded relatively more protein-like substances than humic-like substances as compared with the diatoms, and the latter produced relatively more than the prasinophyte. A low value of this ratio would indicate a relative dominance of the exudation of the products of early degradation (respiration) over the synthesis products by healthy marine phytoplankton, since it has been reported that humic-like substances are a by-product of microbial respiration (Nieto-Cid et al. 2006).

The variations in the position of the peak-T and peak-M fluorescence maxima in the EEMs, indicate that different substances are being produced by each culture (i.e., that the quality of the organic matter produced by phytoplankton was different depending on the species). It is difficult to know the cause of those variations in the fluorescence maxima without concurrent molecular analysis. For example, tryptophan is highly sensitive to the polarity of its surrounding environment. Frequently, spectral shifts are observed as a result of binding ligands, protein–protein associations, and denaturation, among other causes (Lakowicz 1983).

Both diatoms showed a peak at Ex/Em 280 nm/381 nm for *Chaetoceros* sp. and slightly displaced at longer wavelengths, 289 nm/391 nm, for *S. costatum*. A similar peak (Ex/Em 280 nm/370 nm) was found by Coble et al. (1998) in samples collected in the upper 100 m of a coastal upwelling system, where chlorophyll was high. That peak (called peak-N) had not been previously reported, and the authors concluded that it could not be a humic-like peak but appeared to be associated with production of new CDOM in that marine environment. Our results support their conclusion and also show that peak-N is produced by marine phytoplankton. The absence of this peak in the cultures of the other two species (*P. minimum and M. pusilla*) could indicate that the compounds associated with this peak are only produced by diatoms, or that the peak was masked by other fluorescence peaks in the other cultures. In future experiments it would be worthwhile to explore the possible specificity of FDOM compounds produced by different groups of phytoplankton.

The EEM of *M. pusilla* was compared with the EEM of a natural seawater sample, taken at the Blanes Bay Microbial Observatory (http://www.icm.csic.es/bio/projects/icmicrobis/bbmo/) in the northwest Mediterranean Sea

during a *Micromonas* bloom (R. Massana pers. comm.) in February 2007 (details not shown). A peak at Ex/Em 283 nm/358 nm, which could correspond to the peak-T by Coble (1996), was found in the natural sample. This peak is slightly displaced to longer wavelengths than the peak-T of the *M. pusilla* culture (Ex/Em 275 nm/345 nm). The EEM of the natural sample also showed a maximum at Ex/Em 348 nm/434 nm that coincided with the peak at Ex/Em 348 nm/436 nm detected in the axenic culture. These maxima coincide with the peak-C defined by Coble et al. (1998). Certainly, it should not be expected that the EEM of a natural sample, even if it was taken during an almost-monospecific *Micromonas* bloom, would match completely the peaks found in a *Micromonas* axenic culture. The EEM of the *Micromonas* culture shown in Fig. 2 reflects the production of fluorescent materials only by this species. On the other hand, the fluorescence fingerprint of the natural sample results from the production and/or accumulation of FDOM due to the activity of plankton assemblages, the transformations by photochemical and biological reactions, and/or the inputs of terrestrial FDOM. With these considerations in mind, the correspondence between the EEM of a natural *Micromonas* bloom and that from a *Micromonas* axenic culture is indeed remarkable.

Several studies have confirmed that carbohydrates constitute the major fraction of compounds released by phytoplankton. The next largest fraction is composed of N-compounds, with the most prominent members being amino acids, peptides, and proteins (Myklestad 2000). Studies of dissolved free amino acids excreted by phytoplankton (Martin-Jézéquel et al. 1988) have found that the most prominent excreted free amino acids are Ser, Gly, Lys, Ala, Glu, Asp, Orn, and His (Myklestad 2000). Yamashita and Tanoue (2003) observed a high correlation between protein-like fluorescence intensities and total amino acids in the ocean, meaning that fluorescence intensities (i.e., peak-T) may be a useful indicator of the dynamics of dissolved amino acids. We found a significant increase of protein-like fluorescence per unit of biomass during the experiments for all the species examined, indicating that phytoplankton excretes fluorescent protein-like material during growth. Stedmon and Markager (2005) applied parallel factor analysis to EEMs from a phytoplankton bloom sample and found two components in the protein-like region. One of them, with a maximum at Ex/Em 280 nm/338 nm, was more susceptible to microbial degradation than the one that peaks at Ex/Em 275 nm/306 nm. Note that our peak-T is close to the former, more labile, component.

Microbial processes are an important source of humic-like compounds (Yentsch and Reichert 1961). However, previous experimental and field studies have concluded that phytoplankton cannot be a direct source of fluorescent humic-like substances, mainly because of a lack of correlation between phytoplankton biomass and CDOM (Nelson et al. 1998; Rochelle-Newall and Fisher 2002). However, our study shows a clear increase of humic-like substances during the incubation of four different phytoplankton axenic cultures. This contrast may be attributable to a variety of causes. In field studies it is difficult to isolate the variables influencing CDOM dynamics and bacterial activity and UV radiation could mask the actual humic-like CDOM production role of phytoplankton, as it has been shown that bacteria do produce such compounds (Tranvik 1993; Nieto-Cid et al. 2006) and that natural UV radiation photodegrades them (Moran et al. 2000; Nieto-Cid et al. 2006). To our knowledge, ours is the first study focusing on FDOM production in which phytoplankton cultures were maintained in an axenic state until the end of the experiment. Therefore, the lack of a connection between humic-like CDOM and phytoplankton in previous experimental approaches could be caused by bacterial activity. Rochelle-Newall and Fisher (2002) analyzed fluorescence at longer wavelengths (Ex/Em 335 nm/450 nm) than those we used in a region where we observed smaller increases in the intensity of fluorescence (data not shown). In our experiment, in the absence of bacteria and UV radiation, the good correlation obtained between algal biomass and marine humic-like CDOM ($n = 16$, $R^2 = 0.78$, $p < 0.001$) indicates that phytoplankton is indeed a net source of humic-like CDOM.

Previous studies with natural samples had indicated that phytoplankton could release protein-like compounds, measured as peak-T (Nieto-Cid et al. 2006). The correlation between peak-T and biomass found here was also significant ($R^2 = 0.36$, $p < 0.01$), but lower than that between peak-M and biomass, indicating that the dynamics of protein-like substances are species specific. Determann et al. (1998) observed the same pattern in nonaxenic algae cultures that included cells and exudates. They found that fluorescence intensities (Ex/Em 230 nm/330 nm) normalized to phytoplankton cell numbers were a function of the species and were also dependent on the physiological status. Moreover, it has been demonstrated that phytoplankton excretes amino acids but can take them up too (Paerl 1991). So, we cannot exclude the possibility of some uptake of protein-like substances by phytoplankton, and this could also contribute to a decrease in the correlation between peak-T and phytoplankton biomass.

How and why healthy phytoplankton cells release amino acids and humic substances to the medium is unclear. The DOC excretion by phytoplankton has been widely discussed. Bjørnsen (1988) proposed passive diffusion as the most likely mechanism. It is known that phytoplankton exudes low-molecular-weight photosynthetic metabolites and by-products of the degradation of cellular polymeric material (Myklestad 2000). In this sense, phytogenic marine humic substances can be considered a by-product of aerobic respiration.

To assess the relevance of our results in nature, we have roughly estimated the contribution of phytoplankton to the production of colored humic-like substances in the highly productive coastal upwelling system of the Ría de Vigo (northwest Iberian Peninsula). Seasonal cycles of peak-M (Nieto-Cid et al. 2006) and autotrophic carbon biomass (Teixeira and Figueiras 2009) were obtained in the surface layer of this ecosystem during year 2002. A significant positive linear relationship ($n = 8$, $R^2 = 0.78$, $p < 0.001$) was obtained between peak-M and biomass, with a slope of $2.9 \pm 0.6$ $\mu$g eq QS mg $C^{-1}$. Considering that the slope of the linear regression of the curve in Fig. 3a is $0.5 \pm 0.1$ $\mu$g

eq QS mg $C^{-1}$, the contribution of phytoplankton to the buildup of peak-M in the surface layer of the coastal upwelling system of the Ría de Vigo should be 18% ± 9% if the relationships obtained in cultures were transferable to nature. Although this is certainly a rough estimation, it indicates that the contribution of phytoplankton to the production of colored humic-like substances is potentially important; thus, further studies are necessary to assess the contribution of phytoplankton to the autochthonous CDOM pool in the marine ecosystem.

The optical characterization of exudates produced by axenic cultures of four common phytoplankton species showed that these organisms produced CDOM, detected by the buildup of fluorescent protein-like and humic-like materials. Whereas the exudation of protein-like substances by healthy phytoplankton cells has been recognized as a common but intriguing process, the in situ production of marine humic–like substances has been traditionally associated with bacterial respiration. Although further studies are needed to examine the composition of the FDOM and its role in the physiology of phytoplankton, this work demonstrates unequivocally for the first time that phytoplankton cells in exponential growth also exude fluorescent humic-like substances and that the quality of this material is different for each species. As for the case of bacteria, it is hypothesized that the exudation of humic materials by phytoplankton could ultimately be derived from phytoplankton respiration. These results have clear implications for understanding the cycle of CDOM in the photic layer of coastal and open ocean waters by providing evidence for a previously unrecognized process contributing to the in situ production of CDOM.

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