Net production/consumption of fluorescent coloured dissolved organic matter by natural bacterial assemblages growing on marine phytoplankton exudates

Running title: Production and consumption of FDOM by marine bacteria

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ABSTRACT

Understanding the distribution of coloured dissolved organic matter (CDOM) in the oceans and its role in the global carbon cycle requires a better knowledge of the coloured materials produced and consumed by marine phytoplankton and bacteria. In this work we examined the net uptake/release of CDOM by a natural bacterial community growing on DOM derived from four phytoplankton species cultured in axenic conditions. Fluorescent humic-like substances exuded by phytoplankton (Ex/Em: 310 nm/392 nm; P. Coble's peak-M) were utilised by bacteria in different proportions depending on the phytoplankton species of origin. Furthermore, bacteria produced humic-like substances that fluoresce at Ex/Em: 340 nm/440 nm (Coble's peak-C). Differences were also observed in the Ex/Em wavelengths of the protein-like materials (Coble`s peak-T) produced by phytoplankton and bacteria. The induced fluorescent emission of CDOM produced by prokaryotes was an order of magnitude higher than that of CDOM produced by eukaryotes. We have also examined the final composition of the bacterial community growing on the exudates, which differed markedly depending on the phytoplankton species of origin. *Alteromonas* and *Roseobacter* were dominant during all the incubation on *Chaetoceros* sp. and *P. minimum* exudates, respectively. *Alteromonas* was the dominant group growing on *S. costatum* exudates during the exponential growth phase, but it was replaced by *Roseobacter* afterwards. On *M. pusilla* exudates, *Roseobacter* was replaced by *Bacteroidetes* after the exponential growth phase. Our work shows that fluorescence excitation-emission matrices of CDOM can be a helpful tool for the identification of microbial sources of DOM in the marine environment but further studies are necessary to explore the association of particular bacterial groups with specific fluorophores.

INTRODUCTION

Coloured dissolved organic matter (CDOM) is receiving increasing attention due to its important role in aquatic ecosystems. It regulates UV and visible light penetration in the water column thus influencing primary productivity (2) and preventing cellular DNA damage (16, 17). CDOM can also form complexes with metals reducing the concentrations of free ions in seawater (28). In addition, changes in the optical properties of CDOM are suitable to trace microbial and photochemical degradation processes (34, 44) and, more specifically, the *in situ* formation of bio-refractory humic materials from bio-available DOM (26). CDOM has also been the focus of remote sensing studies given that the absorption spectrum of CDOM overlaps with that of chlorophyll *a*, affecting the satellite-derived estimates of phytoplankton biomass and activity in the oceans, especially in coastal areas (51).

The fraction of CDOM that emits induced fluorescent light is called fluorescent dissolved organic matter (FDOM). Two main groups of fluorophores have been differentiated (7): protein-like substances, which fluoresce around Ex/Em: 280 nm/350 nm (peak-T); and humic-like substances which fluoresce at two pairs of wavelengths, Ex/Em: 312 nm/380-420 nm (peak-M), and Ex/Em 340 nm/440 nm (peak-C). Both peaks, -M and -C, have been found in samples from freshwater and seawater environments. However, a shift to longer wavelengths of the humic-like peaks in freshwater samples has been reported (6) because terrestrial humic substances are more aromatic than marine humic substances (3). Additionally, and independently of their origin, humic substances also fluoresce in the UV-C region of the spectrum, at Ex/Em: 250 nm/450 nm (6, 7).

Humic materials have been traditionally considered photodegradable but resistant to bacterial degradation. In fact, when humic-like FDOM is not exposed to

natural UV radiation levels, it can accumulate in the ocean at centennial to millennial time-scales within the global conveyor belt (11, 60). Therefore, the formation of bio-resistant CDOM during the degradation of bio-available DOM would allow the sequestration of anthropogenic CO_2 in a dissolved organic form for hundreds to thousands of years, as part of the recently coined "microbial carbon pump" sequestration mechanism (19).

Global carbon flux estimates require quantitative information about the degradation rates of biogenic organic matter. Although the bioavailability of phytoplankton exudates has been recurrently studied (36, 55, 56), few works have dealt with the exudation of CDOM by phytoplankton (45). In fact, marine FDOM has been mainly considered a by-product of the bacterial metabolism (11, 34, 60) until some recent studies have shown that it can be produced by zooplankton (54, 59), krill (37) and also by phytoplankton cells (45). Among these FDOM producers, phytoplankton is the most abundant organisms in terms of biomass. Therefore, the quantitative and qualitative analysis of the FDOM produced by phytoplankton and its comparison with that produced by bacteria is essential to better understand the distributions of CDOM in the oceans as well as the role of CDOM in the global carbon cycle.

In addition, while bacterioplankton structure is modulated by the amount and quality of the available substrates (eg., 41, 46), activity of different group of bacteria can shape organic matter composition. However, to the best of our knowledge, there is a lack of studies examining the changes in FDOM and bacterial community composition in controlled conditions. Since fluorescence excitation-emission matrices (EEMs) are fingerprints of DOM structure, the study of these EEMs in relation to different bacterial groups could be a step further in the characterization of DOM produced by bacteria and in the identification of DOM sources in the environment.

The objective of the present work is to quantify the net production/consumption of CDOM by a natural bacterial community growing on exudates derived from four phytoplankton species (*Chaetoceros* sp., *Skeletonema costatum*, *Prorocentrum minimum* and *Micromonas pusilla*) cultured in axenic conditions. To this end, the time course of the induced fluorescence excitation-emission properties of CDOM were followed as well as its relationship with changes in the structure of the bacterial community. Moreover, we investigated whether the bacterial groups selected by the treatments can be associated to characteristic FDOM signals.

MATERIALS AND METHODS

Phytoplankton cultures. Algal exudates were produced from axenic cultures of four phytoplankton species. The axenic species obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) (https://ccmp.bigelow.org/) were cultured in axenic conditions as described in Romera-Castillo et al. (45). The strains used were the diatoms *Chaetoceros* sp. (CCMP199) and *Skeletonema costatum* (CCMP2092) (Greville) Cleve, the dinoflagellate *Prorocentrum minimum* (CCMP1329) (Pavillard) J. Schiller, and the prasinophyte *Micromonas pusilla* (R.W. Butcher) (CCMP1545) I. Manton and M. Parke. The four species produced significant amounts of FDOM (45).

The cultured volume (1.4 L) of each phytoplankton species was filtered through a 0.2 µm Sterivex cartridge and inoculated with 150 mL of natural seawater filtered twice through 0.6 µm polycarbonate filter to eliminate predators of bacteria, such as small flagellates. The inoculum was taken on 7 May 2008 from the Blanes Bay

Microbial Observatory (http://www.icm.csic.es/bio/projects/icmicrobis/bbmo/). Then, each 1.55 L inoculated sample was distributed in 36 polycarbonate bottles of 60 mL filled with 40 mL of the inoculated exudates.

The bottles were incubated for 34 days and sampled at times 0, 4, 12, 21 and 34 days. At each sampling time, 4 of the initial 36 bottles per treatment were sacrificed: three replicates were used for dissolved organic carbon (DOC) and FDOM analysis and bacterial counts and one for Catalyzed reporter deposition Fluorescence In Situ Hybridization (CARD-FISH).

Bacterial community biomass and structure. Heterotrophic bacteria were counted in the three bottles with a FacsCalibur (Becton & Dickinson) flow cytometer equipped with a 15 mW Argon-ion laser (488 nm emission) as described in Gasol and del Giorgio (15). For each sample, 4 mL of water were collected and fixed immediately with cold glutaraldehyde 10% (final concentration 1%), left in the dark for 10 min at room temperature and then stored at -80°C. Later, 400 µL of sample received a diluted SYTO-13 (Molecular Probes Inc., Eugene, OR, USA) stock (10:1) at 2.5 µmo L^{-1} final concentration, left for about 10 min in the dark to complete the staining and run in the flow cytometer. At least 30000 events were acquired for each subsample (usually 90000 events). Fluorescent beads (1 µm, Fluoresbrite carboxylate microspheres, Polysciences Inc., Warrington, PA) were added at a known density as internal standards. The bead standard concentration was determined by epifluorescence microscopy. Heterotrophic bacteria were detected by their signature in a plot of side scatter (SSC) vs. FL1 (green fluorescence). Data analysis was performed with the Paint-A-Gate software (Becton & Dickinson).

Bacterial cell size (V, in μm^3 $cell^{-1}$) was estimated using the relationship between the average bacterial size and the average fluorescence of the SYTO-13–stained sample relative to beads (FL1 bacteria/FL1 beads) reported by Gasol and del Giorgio (15):

$$V = 0.0075 + 0.11 \cdot (\text{FL1 bacteria/FL1 beads})$$

Bacterial biomass (BB, in Pg C $cell^{-1}$) was calculated by using the carbon-to-volume (V, in $\mu m^3.cell^{-1}$) relationship derived by Norland (35) from the data of Simon and Azam (52):

$$BB = 0.12 \cdot V^{0.7}$$

Bacterial community composition (BCC) was obtained by CARD-FISH. Three to five mL samples were fixed with paraformaldehyde (1% final concentration), and bacterial cells were collected onto 0.2 µm polycarbonate filters (Whatman) and kept at -80°C until hybridization. Four horseradish peroxidase probes were used, following the protocol of Pernthaler et al. (39): ALT1413, CF319a, ROS537 and SAR11, targeting respectively the *Alteromonas*, the *Bacteroidetes*, the *Roseobacter* and SAR11 clades (further details in Sarmento & Gasol, submitted). The NON338 probe was used as a negative control. After permeabilization with lysozyme and achromopeptidase, several filter sections were cut and hybridized for 2h at 35°C (39). Probe GAM42a was used with competitor oligonucleotides as described in (27). Filter portions were then counterstained with DAPI (1µg mL^{-1}) before enumeration with an epifluorescence microscope. A minimum of 10 fields per filter-portion were counted, and at least 400 cells per group were counted. The selected groups were expressed as a percentage relative to the total DAPI-stained bacterial cells.

Dissolved organic carbon (DOC) and fluorescent dissolved organic matter (FDOM). Triplicate DOC and FDOM samples were filtered through GF/F filters with an acid-cleaned syringe. Milli-Q water was filtered through the filtration system and no significant enrichment was observed in DOC and FDOM concentrations, discarding contamination during filtration.

Approximately 10 mL of water were collected in pre-combusted (450ºC, 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 4ºC until analysis. DOC was measured with a Shimadzu TOC-V organic carbon analyser. The system was standardized daily with potassium hydrogen phthalate. Each ampoule was injected 3–5 times and the average area of the 3 replicates that yielded a standard deviation < 1% were chosen to calculate the average DOC concentration of each sample after subtraction of the average area of the freshly-produced UV-irradiated milli-Q water used as a blank. The performance of the analyser was tested with the DOC reference materials provided by Prof. D. Hansell (University of Miami). We obtained a concentration of 45.2 ± 0.3 μmol 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 (Certified Reference Materials for DOC analysis, Batch 04, 2004) is 45 μmol C L^{-1}.

Samples for FDOM analysis were measured immediately after collection. Single measurements at specific excitation-emission wavelengths as well as excitation-emission matrices (EEMs) of the aliquots were performed with a LS 55 Perkin Elmer Luminescence spectrometer, equipped with a xenon discharge lamp, equivalent to 20 kW for 8 μs duration. The detector was a red-sensitive R928 photomultiplier, and a photodiode worked as reference detector. Slit widths were 10.0 nm for the excitation

and emission wavelengths and the 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. To compare with other works, the Ex/Em wavelengths used for single measurements were those established by Coble (6): Ex/Em: 280 nm/350 nm (peak-T) as indicator of protein-like substances and Ex/Em: 320 nm/410 nm (peak-M) and Ex/Em: 340 nm/440 nm (peak-C) as indicators of humic-like substances. Following Coble (6), fluorescence measurements were expressed in quinine sulphate units (QSU) by calibrating the LS 55 Perkin Elmer at Ex/Em: 350 nm/450 nm against a quinine sulphate dihydrate (QS) standard made up in 0.05 mol L^{-1} sulphuric acid.

EEMs were performed to track for possible changes in the position of the protein- 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 1st 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 EEM of pure water (Milli-Q) from the EEM of the sample.

Paired Student-*t*-test was used to check for significant differences in the measured variables between the initial, intermediate and final incubation times (53).

RESULTS

Time course of bacterial biomass. Bacterial biomass increased similarly in the four cultures throughout the initial 4 days of exponential growth (Fig. 1) reaching concentrations that ranged from 193 ± 51 to 220 ± 5 µg C L^{-1}. It kept increasing until day 12 only in the treatment with *P. minimum* exudates, reaching 269 ± 3 µg C L^{-1}.

Bacterial biomass slightly decreased after day 4 except on *P. minimum* exudates, where it decreased after day 12. The growth of the natural bacterial community (ΔBB) was not significantly different ($p > 0.05$) among the four treatments amended with exudates of different phytoplankton species.

Time course of dissolved organic carbon. Two phases can be distinguished in the time course of DOC concentrations (Fig. 2a). A sharp decrease of DOC occurred during the initial 4 days of bacterial exponential growth, reaching concentrations that were from 47.1 ± 0.3 % to 59.5 ± 0.9 % of the initial DOC. Diatom exudates presented the most abrupt DOC decrease and *P. minimum* exudates presented the smallest one. In the second phase, from days 4 to 34, DOC concentrations tended to increase until final stagnation or small decrease.

Time course of fluorescent CDOM. To account for the net production/consumption of any CDOM fluorophore after 4 days of exponential growth, the fluorescence excitation-emission matrices (EEMs) at initial time were subtracted from the corresponding EMMs at day 4 for each culture. At day 4, all EEMs showed the production of a humic-like fluorophore that peaked around Ex/Em: 354 nm/424 nm (Figs. 3b, 4b, 5b and 6b); i.e. Coble's peak-C (7). After 34 days of incubation, peak-C was still present in the EEMs of all the cultures but its fluorescence intensity had increased (Figs. 3c, 4c, 5c and 6c). On the contrary, a second humic-like fluorophore that peaked around Ex/Em: 310 nm/392, Coble's peak-M (7), decreased after 4 days of exponential growth in 3 of the 4 cultures (Figs. 3b, 4b and 6b). In the *P. minimum* exudates peak-M took a bit longer to diminish (Figs. 5b and c).

Coble's UV protein-like peak-T (7), which peaks at around Ex/Em: 275 nm/ 361 nm, was also present in the cultures grown on the four phytoplankton species (Figs. 3a, 4a, 5a and 6a). After 4 days of incubation, the intensity of peak-T increased in the cultures grown on *S. costatum*, *M. pusilla* and *P. minimum* exudates (Fig. 4c, 5c and 6c) but decreased slightly in the culture grown on *Chaetoceros* sp. exudates (Fig. 3c). In addition, all the EEMs showed the production of the Coble's UV humic-like peak-A (7) at around 261 nm/463 nm (Fig. 3b, 4b, 5b and 6b).

Changes in the intensity of peaks C, M and T, relative to values at initial time, during the course of the incubations are presented in Figure 2 for an easier comparison between the exudates of the different phytoplankton species. The initial values of these parameters are summarised in Table 1. Peak-C increased significantly ($p < 0.001$) until day 21 in all cultures (Fig. 2b) with the highest variation during the first four days; the corresponding net production rates are summarised in Table 2. On the contrary, peak-M increased significantly ($p < 0.05$) only in the *P. minimum* exudates culture (Fig. 2c). For the other 3 species, a significant decrease ($p < 0.05$) occurred during the initial 4 days of incubation. The proportion of bioavailable FDOM excreted by phytoplankton was extremely different depending on the species: 30.3 ± 0.3 % of the marine humic-like substances (peak-M) excreted by *S. costatum* were bioavailable while the proportion decreased to 5 ± 2 % and 10 ± 2 % in *Chaetoceros* sp. and *M. pusilla*, respectively. Since the incubations were performed in the dark photo-bleaching of peak-M can be discarded. During the stationary phase, peak-M experienced a significant ($p < 0.05$) increase only from days 12 to 21. Net bacterial production rates of peaks C and M per unit of carbon biomass and day were calculated for all treatments, during the exponential phase, to compare them with the net production rates of humic-like substances by marine phytoplankton during the exponential growth already obtained by

Romera-Castillo et al. (45). We obtained net productions of peak-C between 15 ± 2 x 10^{-4} and 75 ± 3 x 10^{-4} QSU µg C^{-1} L d^{-1} and the highest net consumption of peak-M, -117 ± 4 x 10^{-4} QSU µg C^{-1} L d^{-1}, corresponded to the bacteria grown on *S. costatum* exudates.

In general, peak-T increased significantly in all cultures during the exponential growth phase (4 days) and, then, decreased dramatically up to the end of the experiment (Fig. 2d). The bacterial culture on *M. pusilla* exudates underwent the largest increase (77%). However, *Chaetoceros* sp. did not show a significant variation of peak-T until the end of the incubation time (day 34) when it sharply decreased by 41%. The net production of peak-T per carbon biomass and day ranged between 6 ± 7 x 10^{-4} and 164 ± 19 x 10^{-4} QSU µg C^{-1} L d^{-1}.

Time courses of bacterial community structure. The most abundant groups of bacteria were identified and counted by CARD-FISH to examine their response to the different substrates generated by the four phytoplankton species tested in this work (Fig. 7). Initial numbers of these bacterial groups were 2.49 x 10^4 cells mL^{-1}, 6.42 x 10^4 cells mL^{-1}, 1.21 x 10^6 cells mL^{-1} and 2.37 x 10^6 cells mL^{-1} for SAR11, *Alteromonas*, *Roseobacter* and *Bacteroidetes*, respectively. In *Chaetoceros* sp. exudates, *Alteromonas* was the dominant bacterial group during the course of the incubation. Among the studied groups on *S. costatum* exudates, *Alteromonas* was the dominant during the first 4 days, but it was replaced by *Roseobacter* thereafter. *Roseobacter* was also the dominant group in *P. minimum* exudates during all the incubation time. In *M. pusilla* exudates, *Roseobacter* was the most abundant group during the exponential phase but *Bacteroidetes* prevailed after day 12. Subtracting the biomass of each group at initial time from that at day 4, we observed that *Alteromonas* was the group that developed

1 better on diatom exudates (1.1 x 10^6 cells mL^{-1} d^{-1} and 7.3 x 10^5 cells mL^{-1} d^{-1}) while it
2 was *Roseobacter* in the case of the *P. minimum* (9.1 x 10^5 cells mL^{-1} d^{-1}) and *M. pusilla*
3 (3.4 x 10^5 cells mL^{-1} d^{-1}) exudates. SAR11, which was present at initial time, decreased
4 throughout the incubations in all exudates.
5
6 **DISCUSSION**
7 **Net production/consumption of DOC and CDOM by marine bacteria.** The
8 proportions of bacterial carbon consumption during the exponential growth phase
9 (between 41 and 53% of the initial DOC) were similar to the 48% of labile DOM found
10 by Puddu et al. (42) in phytoplankton exudates obtained growing the diatom
11 *Cylindrotheca closterium* in a nutrient balanced medium. Previous works have
12 demonstrated that bacteria can take up humic substances retained by XAD resins (4,
13 18). Indeed, during periods of low primary production bacteria seem to use humic
14 substances as a substrate (48, 56). Shimotori et al. (50) also observed a decrease of the
15 FDOM measured at Ex/Em 315 /410 nm (equivalent to peak-M) in their bacterial
16 cultures, but only from incubation times of 20 to 30 days, when bacterial number started
17 to decay. Their incubations consisted of artificial seawater amended with inorganic
18 nutrients and glucose and inoculated with a natural bacterial community. They
19 attributed the FDOM uptake by bacteria in the stationary phase to the depletion of the
20 amended glucose, the only carbon source for bacterial growth in their cultures. In our
21 experiment we used phytoplankton exudates that are known to be rich in carbohydrates
22 (32) and the FDOM decrease that we observed occurred during the exponential growth
23 phase of the experiments. Given the lability of the fresh materials exuded by
24 phytoplankton, full consumption of the exuded carbohydrates and the subsequent
25 utilisation of marine humic-like substances could have occurred in the short time

elapsed between the initial time and day 4. It has been shown that humic substances retained by XAD resins can be adsorbed onto bacterial surfaces, although this adsorption is negligible at pH typical of marine waters for the number of bacteria that our incubation reached (13). The peak-M fluorophore consumed by bacteria in our experiment had been previously produced by phytoplankton (45). Therefore, phytoplankton cells exude fluorescence humic-like substances that are readily bioavailable to microbial degradation.

The different behaviour of peak-M and peak-C during the exponential phase of our incubations suggests a different origin and fate for both fluorophores. Peak-C was produced continuously in all cultures while peak-M was consumed during the exponential and produced during the senescence phase of the bacterial cultures. Since the ratio peak-M/peak-C did not change significantly from day 12 onwards, it is likely that the fluorescence intensity observed at the Ex/Em wavelengths of peak-M during the senescence phase was mostly due to peak-C tailing.

Regarding peak-T, the release of this fluorophore by marine bacteria has been previously reported (5, 31). A similar pattern observed in our experiment, where peak-T increased until bacterial population reached the stationary growth phase and decreased from then on, was showed by Cammack et al. (5) in incubation experiments performed in lake waters of different trophic status. They suggested that peak-T reflects the balance between bacterial consumption and production of a small fraction of the DOM pool and that it could be a by-product of bacterial metabolism. The significant positive linear correlation that we found between the fluorescence of peak-T and bacterial biomass ($R^2 = 0.41$; $p < 0.01$; $N = 16$) supports this statement. Kawasaki and Benner (20) also found an increase of total dissolved amino acids (TDAA) matching a peak in bacterial biomass. Since we have used GF/F filters that could have not retained 100% of

the BB, a partial contribution of the aromatic amino acids containing cells to the peak-T signal cannot be discarded.

Wavelength shifts in EEMs as indicators of changes in the chemical structure of CDOM. EEMs provide complementary information about the chemical structure, specially the aromaticity, of the FDOM produced or consumed by marine phytoplankton and bacteria. Peak-T produced by bacteria in our experiments was shifted to slightly longer excitation and shorter emission wavelengths than the peak-T reported by Coble (7). In this sense, tryptophan residues that are exposed to water have maximum fluorescence at emission wavelengths of about 340-350 nm, whereas totally buried residues (e.g., being part of peptides) fluoresce at about 330 nm (25). We found maximum intensities in the peak-T region at Ex/Em: 280 nm/335 nm, which is an indication of the release of aromatic amino-acids by bacteria in a more buried combined rather than in a free form. That maximum matches with the fluorescence maximum of the enzyme nuclease, Ex/Em: 280 nm/334 nm (25). This exoenzyme could have been excreted by bacteria to hydrolyze DNA and consume their products (38). It differs from the protein-like substances excreted by phytoplankton since the maximum peak produced by three species of phytoplankton in that region was at Ex/Em: 275 nm/358 nm (45).

Polypeptides are part of the dissolved combined amino acids (DCAAs) pool and some authors have reported that protein and DCAAs are relatively more important as bacterial substrates than DFAAs (8, 22, 24, 47). Using radiolabelled proteins to examine the relative significance of protein versus DFAAs as bacterial substrates, Kiel and Kirchman (21) found that bacteria preferred proteins in the oligotrophic Sargasso Sea (22), but DFAAs in the eutrophic Delaware Bay (21). Since our treatments with

phytoplankton exudates were performed in nutrient-rich conditions, bacteria could have used DFAA and released peptides.

Production of humic-like substances: marine phytoplankton *versus* marine bacteria. Peak-M has traditionally been associated to the humic materials produced in situ in marine ecosystems since the EEMs of natural seawater samples usually present a fluorescence maximum in that region. In contrast, the EEMs of natural samples with a predominant terrestrial origin present a fluorescence maximum at peak-C, shifted to significantly longer excitation and emission wavelengths than peak-M (7). Therefore, in our culture experiments with marine bacteria growing on marine phytoplankton exudates, production of peak-M fluorescence should be expected. However, our study demonstrates that the main humic-like fluorophore produced by marine bacteria grown on marine phytoplankton exudates was peak-C.

Furthermore, our results show that marine phytoplankton is able to produce substances fluorescing at peak-M, which are consumed or transformed by marine bacteria that, in turn, produce other humic-like substances that fluoresce at peak-C. Any shift of a humic-like fluorescent emission maximum to longer wavelengths is an unequivocal indication of increased aromaticity and poly-condensation of humic materials (10). Therefore, attending to the position of both humic-like peak maxima, humic materials exuded by phytoplankton were more aliphatic (blue shifted) than the more aromatic humic materials produced by bacteria (red shifted). Preferential consumption of peak-M by marine bacteria concurs with other studies that reported a higher bioavailability with increasing aliphatic carbon moieties in a compound (30, 54).

Considering our results, we could hypothesise that the more aliphatic humic-like substances that fluoresce at peak-M are mostly produced as a by-product of the

eukaryotes metabolism. Conversely, the more aromatic substances fluorescing at peak-C could be associated to prokaryotes by-products. This hypothesis is also supported by the evidence that copepods exude humic-like substances that fluoresce at peak-M (59) and that a strong signal in the peak-C region was found in cultures of *Synechoccocus* and *Procloroccocus* (data not shown). Furthermore, it has been reported that FDOM intensity at peak-M is higher in the euphotic zone (6), where phytoplankton is abundant, and decreases with depth. On the contrary, the intensity of peak-C increases with depth (6) and it is known that organic matter transformations in the dark ocean are dominated by bacterial activity, which could generate higher fluorescence at this peak.

In general, the production of peaks T and C normalized to the bacterial biomass, during the exponential growth phase was about one order of magnitude higher for marine bacteria than for marine phytoplankton. This fact could explain the lack of correlation between chlorophyll and CDOM that some authors have reported (33, 43), which they attributed to the in situ production of CDOM by bacteria concluding that phytoplankton was not a direct source of CDOM. Indeed, the consumption of the peak-M fluorophore by bacteria also contributes to reduce the expected correlation between phytoplankton biomass and CDOM.

Selection of bacterial groups growing on different phytoplankton exudates. Different bacterial groups were selected depending on the phytoplankton exudates on which they grew. This agrees with previous studies that concluded that bacterioplankton structure is determined by the amount and quality of the substrates available in the ecosystem (e.g., 41, 46). It is well known that both SAR11 and *Roseobacter* are specialised in processing low molecular weight organic substrates (1, 12, 29), but the former prevail in oligotrophic and the latter in meso- and eutrophic environments (14).

On the other hand, the group *Bacteriodetes* is specialised in processing high molecular weight organic substrates (23, 58).

With these general considerations in mind, it can be straightforwardly explained why SAR11, which was present in the natural bacterial community of the (oligotrophic) Blanes Bay Observatory water that we used as inoculum, was not selected in any of our (eutrophic) cultures. The fact that after 4 days of incubation the highest cells numbers corresponded to the group *Alteromonas* grown on *Chaetoceros* sp. exudates could be the result of the production, by this diatom species, of a specific substrate, in which *Alteromonas* is specialised. Even if Schäfer et al. (49) did not find *Gammaproteobacteria* in 6 mono-algal diatom non-axenic cultures, our work indicates that this class can also be associated with diatoms.

Suzuki et al. (57) found that the growth of members of the *Flavobacteriaceae* (subgroup of *Bacteroidetes*) was not affected when inorganic nitrogen was excluded from the medium. On this basis, they suggested that these bacteria could utilize amino acids as sole nitrogen source. Others studies reported that this group of bacteria notably grow when abundant amounts of dissolved proteins are available (9, 40). In our experiment, the negative correlation found between Δpeak-T and Δ*Bacteroidetes* during the exponential phase ($R^2 = 0.98$; $p < 0.01$; $N = 4$) supports this observation. Moreover, in the *M. pusilla* exudates, the largest amount of protein-like substances was produced during the exponential bacterial growth phase (Table 2, Fig. 2d). These protein-like substances were likely in the form of proteins rather than as free amino acids according to the shift of peak-T observed in the EEM (Fig. 5b). This could also explain the dominance of *Bacteroidetes* after day 12 in the sample grown on *M. Pusilla* exudates.

Although our results show that phytoplankton exudates influence bacterial community composition in each treatment, the association of each bacterial group with

a particular fluorescent humic-like peak is not clear. More studies are needed to asses it but from our results it could be suggested that humic-like fluorescent material is a by-product of bacteria metabolism in general and not due to the activity of particular bacterial group(s).

CONCLUSIONS

Marine phytoplankton exudates contain fluorescent CDOM that is bioavailable to bacterial degradation; blue-shifted fluorescent humic-like substances (P. Coble's peak-M) are readily taken up by marine bacteria that, in turn, exude red-shifted humic-like substances (P. Coble's peak-C) during both the exponential and stationary growth phases. Based on this and previous results by other authors, we hypothesize that peak-M could be a by-product preferentially associated to catabolism of marine eukaryotic cells, whereas peak-C could be associated to catabolism by marine prokaryotes.

Marine bacteria produced humic- and protein-like substances an order of magnitude faster than phytoplankton when normalized to their respective biomasses. This fact, together with the photo degradability of aromatic compounds, is likely the reason behind the lack of correlation observed between phytoplankton biomass and CDOM in both, in situ measurements and satellite-derived estimates.

Although bacterial growth was independent of the phytoplankton exudates in which they were grown, the exudates influenced bacterial community structure by preferentially selecting bacterial groups with contrasting substrate preferences.

Bacteria growth on exudates of	BB (μg C L^{-1})	DOC (μmol C L^{-1})	peak-M (QSU)	peak-C (QSU)	peak-T (QSU)
Chaetoceros sp.	0.41 ± 0.02	287.8 ± 2.2	3.12 ± 0.06	2.07 ± 0.01	4.32 ± 0.04
S. costatum	0.23 ± 0.03	304.5 ± 0.4	4.73 ± 0.01	2.22 ± 0.00	4.05 ± 0.02
P. minimum	0.31 ± 0.05	304.9 ± 1.0	2.96 ± 0.02	1.97 ± 0.03	3.77 ± 0.06
M. pusilla	0.35 ± 0.02	242.1 ± 0.6	3.00 ± 0.01	2.03 ± 0.02	2.91 ± 0.02

Table 1. Initial values of average bacterial biomass (BB), dissolved organic carbon (DOC), and fluorescence of CDOM at peak-M, peak-C and peak-T. Average ± SD values are reported.

	BB from days 0 to 4	ΔDOC/ (BB·t) (μg C μg C^{-1} d^{-1})	Δpeak-T/ (BB·t) (QSU μg C^{-1} L d^{-1})	Δpeak-M/ (BB·t) (QSU μg C^{-1} L d^{-1})	Δpeak-C/ (BB·t) (QSU μg C^{-1} L d^{-1})
Bacteria grown on					
Chaetoceros sp.	31.3 ± 0.8	-11.3 ± 0.4	6 ± 7 x 10^{-4}	-13 ± 4 x 10^{-4}	75 ± 3 x 10^{-4}
S. costatum	30.7 ± 1.0	-15.77 ± 0.08	34 ± 7 x 10^{-4}	-117 ± 4 x 10^{-4}	15 ± 2 x 10^{-4}
P. minimum	31.4 ± 1.6	-11.8 ± 0.2	84 ± 8 x 10^{-4}	9 ± 3 x 10^{-4}	34 ± 3 x 10^{-4}
M. pusilla	34.1 ± 0.8	-9.9 ± 0.1	164 ± 19 x 10^{-4}	-21 ± 3 x 10^{-4}	63 ± 2 x 10^{-4}
Production by					
Chaetoceros sp.		116 ± 11 x 10^{-3}	9.1 ± 0.8 x 10^{-4}	4.8 ± 0.1 x 10^{-4}	2.4 ± 0.05 x 10^{-4}
S. costatum		93 ± 13 x 10^{-3}	5.8 ± 0.1 x 10^{-4}	4.5 ± 0.1 x 10^{-4}	1.3 ± x 0.02 10^{-4}
P. minimum		97 ± 9 x 10^{-3}	3.2 ± 0.2 x 10^{-4}	1.3 ± 0.03 x 10^{-4}	0.8 ± 0.04 x 10^{-4}
M. pusilla		86 ± 5 x 10^{-3}	2.6 ± 0.02 x 10^{-4}	3.0 ± 0.1 x 10^{-4}	1.7 ± x 0.02 10^{-4}

Table 2. Bacterial versus phytoplankton net production of dissolved organic carbon (DOC) and fluorescent CDOM in the four cultures. BB is average bacterial biomass.

ACKNOWLEDGEMENTS

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Figure captions:

Figure 1. Time course of the bacterial biomass during the incubation time.

Figure 2. Time course of a) DOC, b) peak-C, c) peak-M, and d) peak-T during the incubation time. Values are reported as % of their respective initial concentrations.

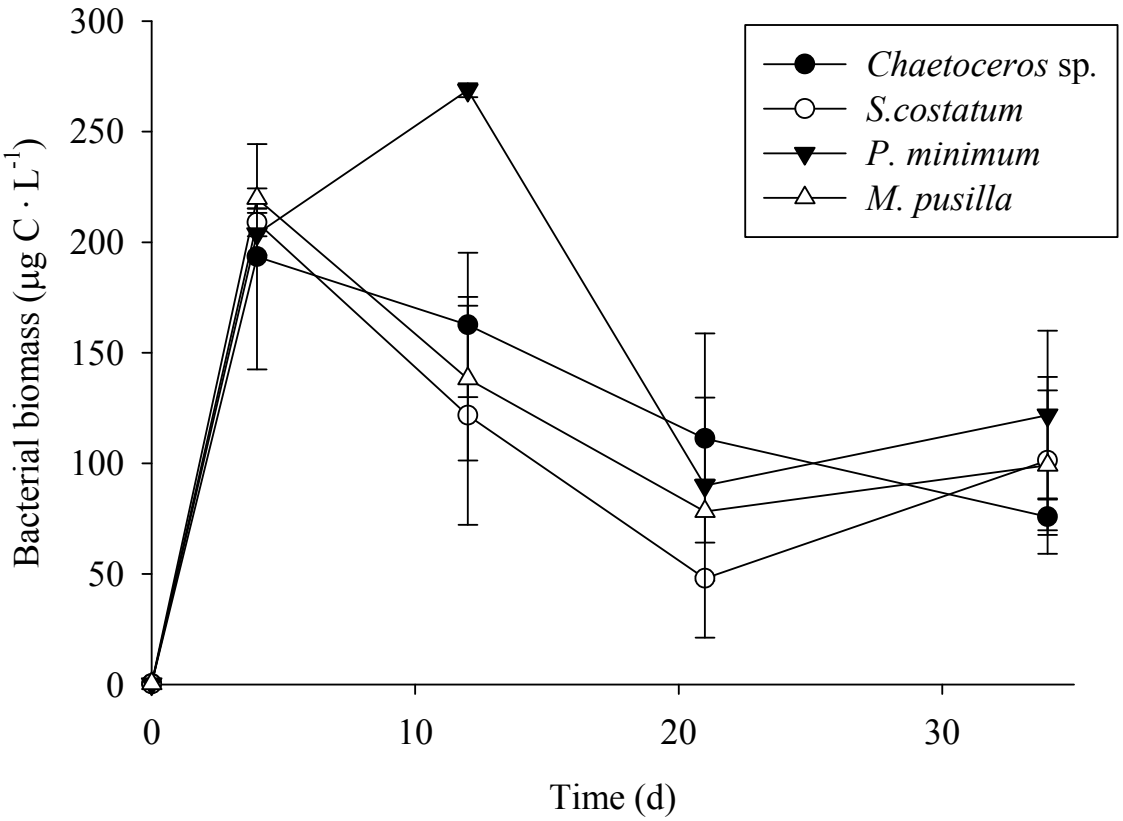
Figure 3. FDOM excitation-emission matrices of the incubation on *Chaetoceros* sp. exudates at a) Day 0, b) Day 4 minus Day 0, c) Day 34 minus Day 0. Different peaks are indicated.

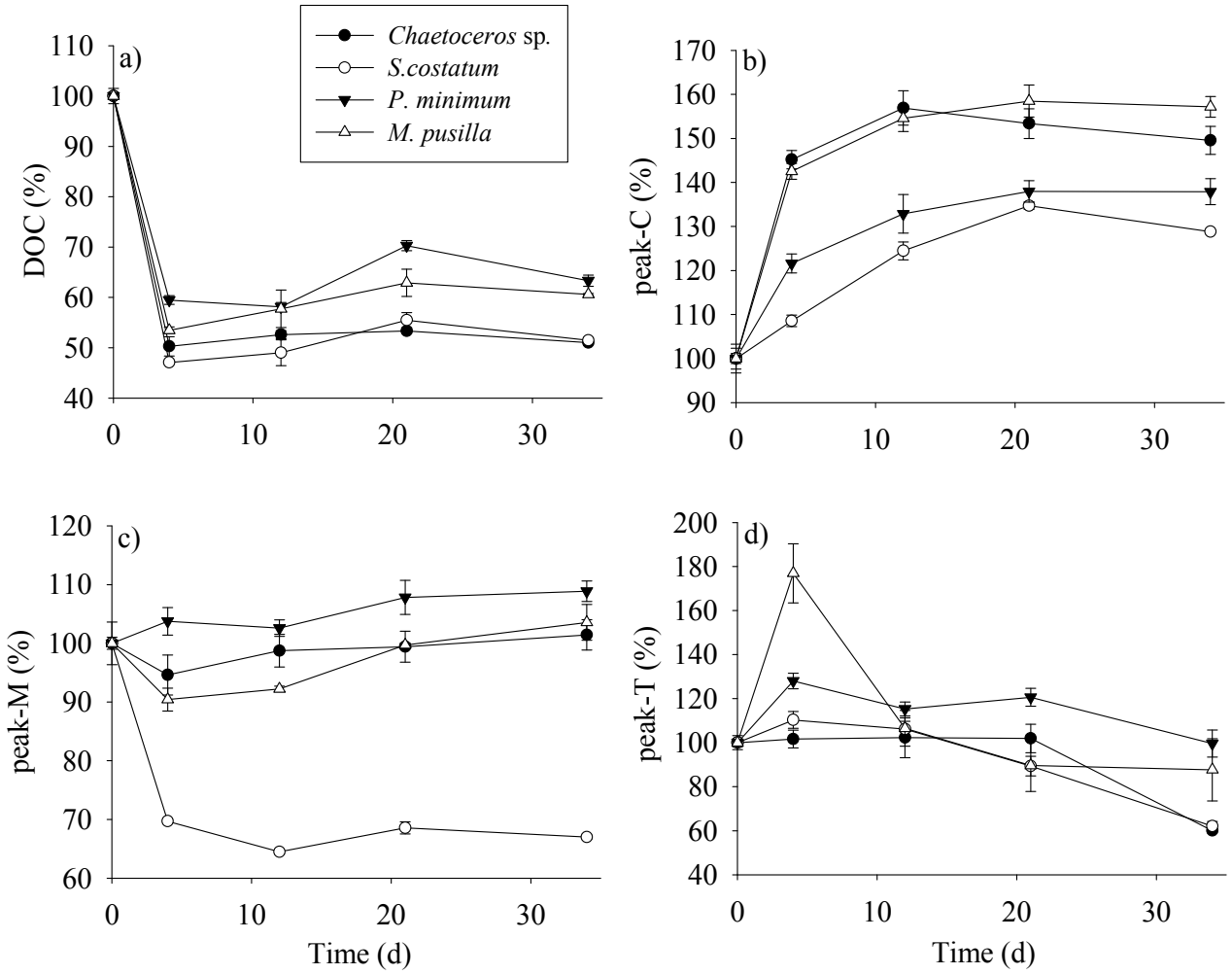
Figure 4. FDOM excitation-emission matrices of the incubation on *S. costatum* exudates at a) Day 0, b) Day 4 minus Day 0, c) Day 34 minus Day 0. Different peaks are indicated.

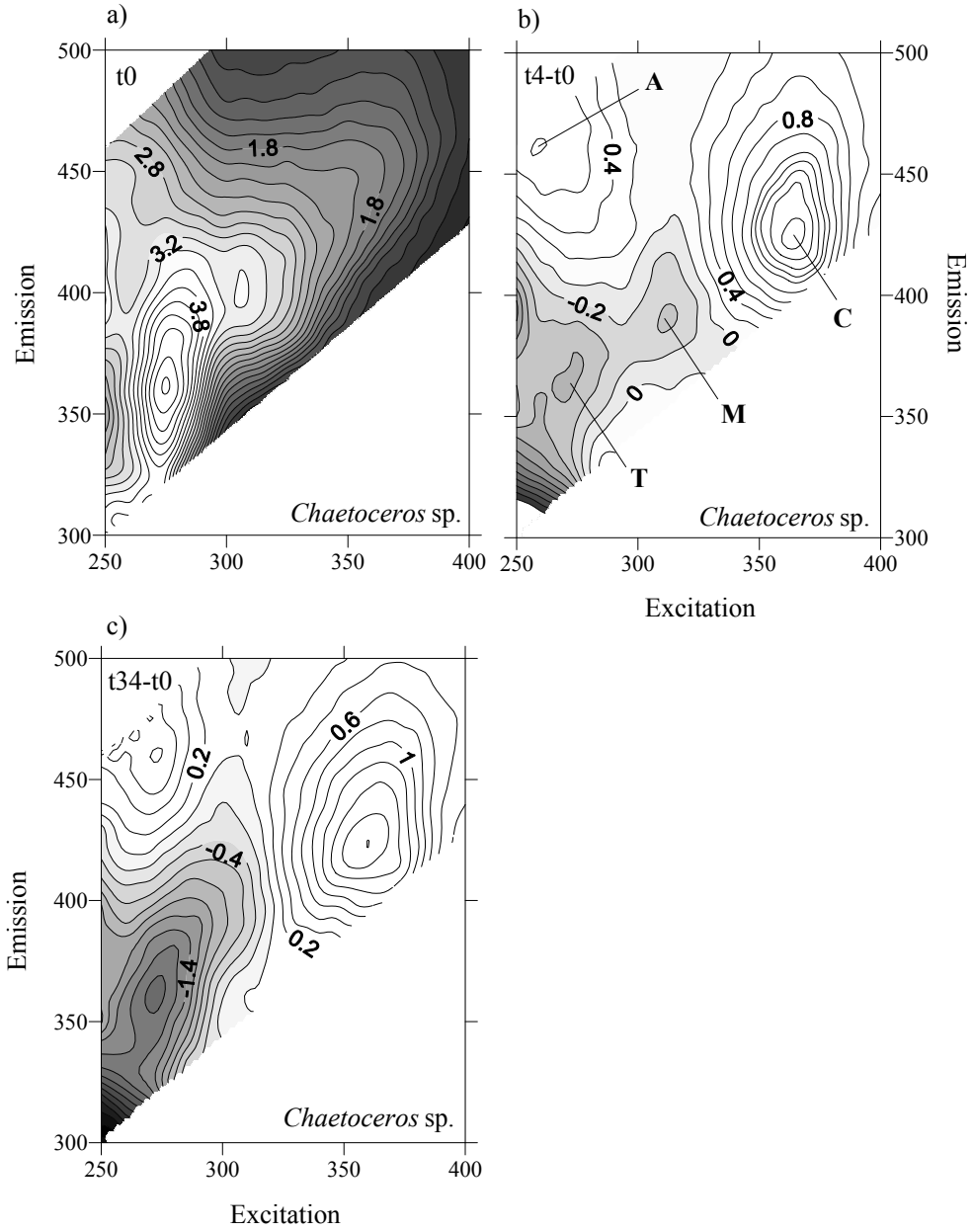
Figure 5. FDOM excitation-emission matrices of the incubation on *P. minimum* exudates at a) Day 0, b) Day 4 minus Day 0, c) Day 34 minus Day 0. Different peaks are indicated.

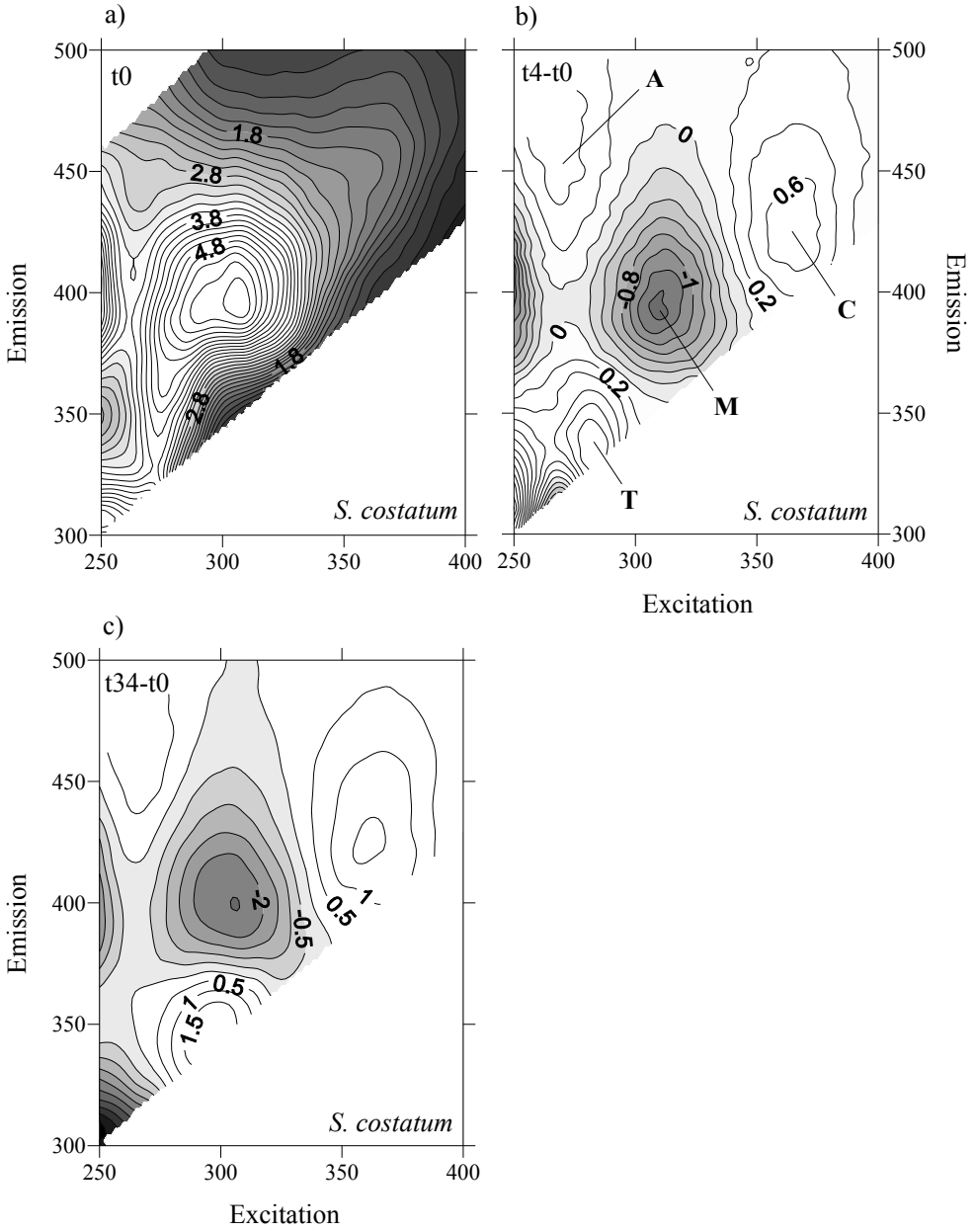
Figure 6. Figure 4. FDOM excitation-emission matrices of the incubation on *M. pusilla* exudates at a) Day 0, b) Day 4 minus Day 0, c) Day 34 minus Day 0. Different peaks are indicated.

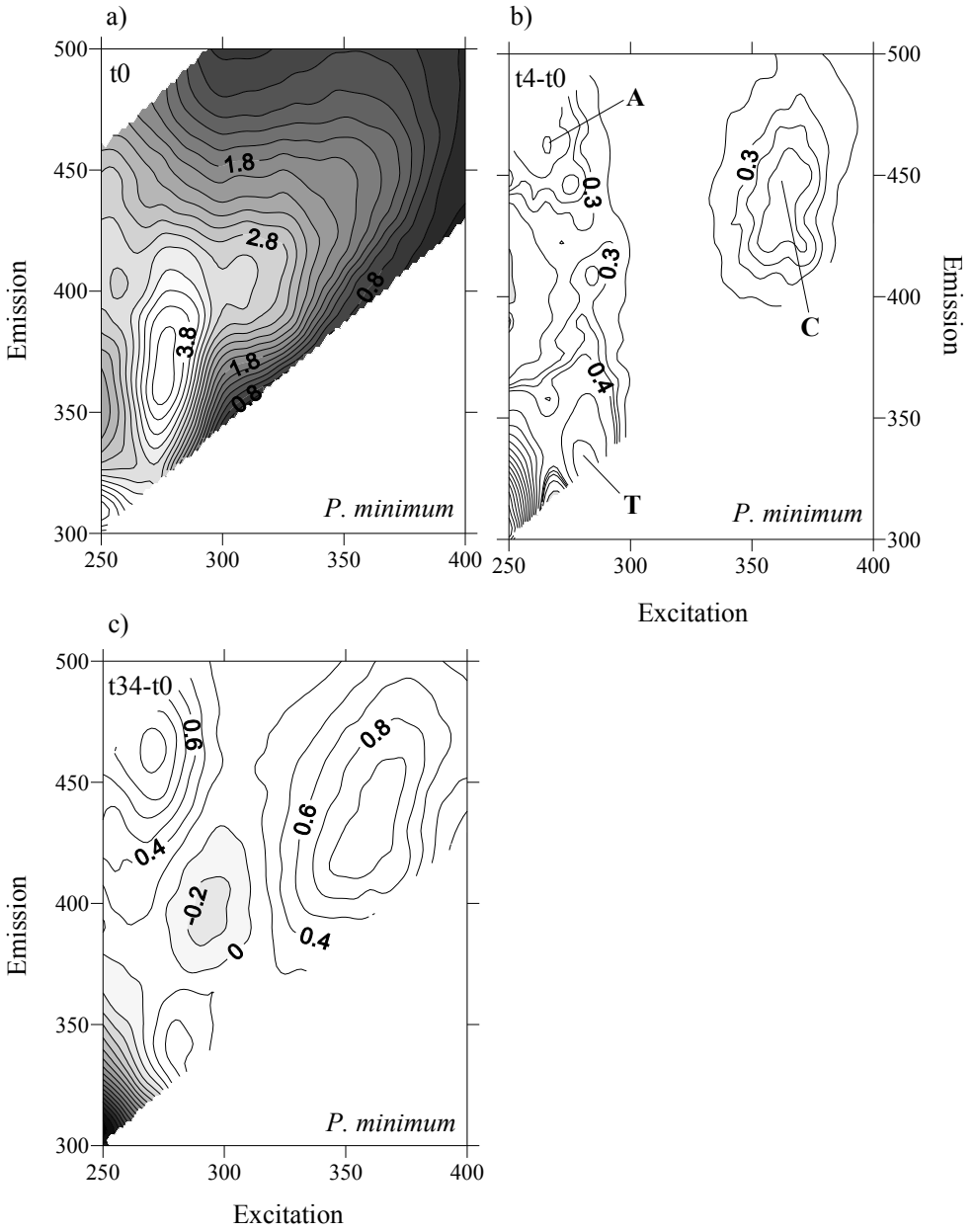
Figure 7. Time course of the abundances of the main bacterial groups grown on a) *Chaetoceros* sp., b) *S. costatum*, c) *P. minimum* and d) *M. pusilla* exudates during the incubation time.

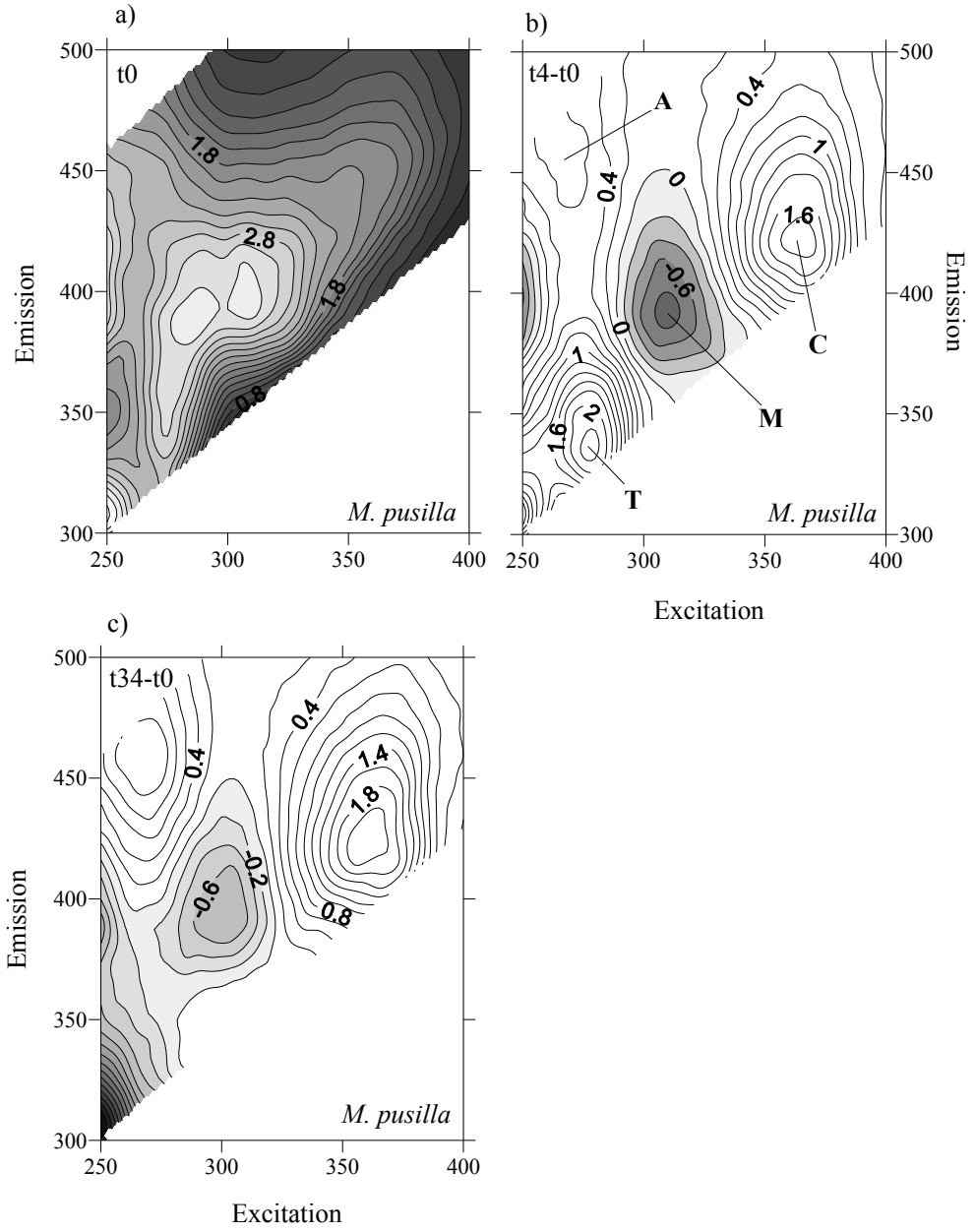

300
250
200
150
100
50
0
Bacterial biomass (µg C · L-1)
0
10
20
30
Time (d)
Chaetoceros sp.
S.costatum
P. minimum
M. pusilla

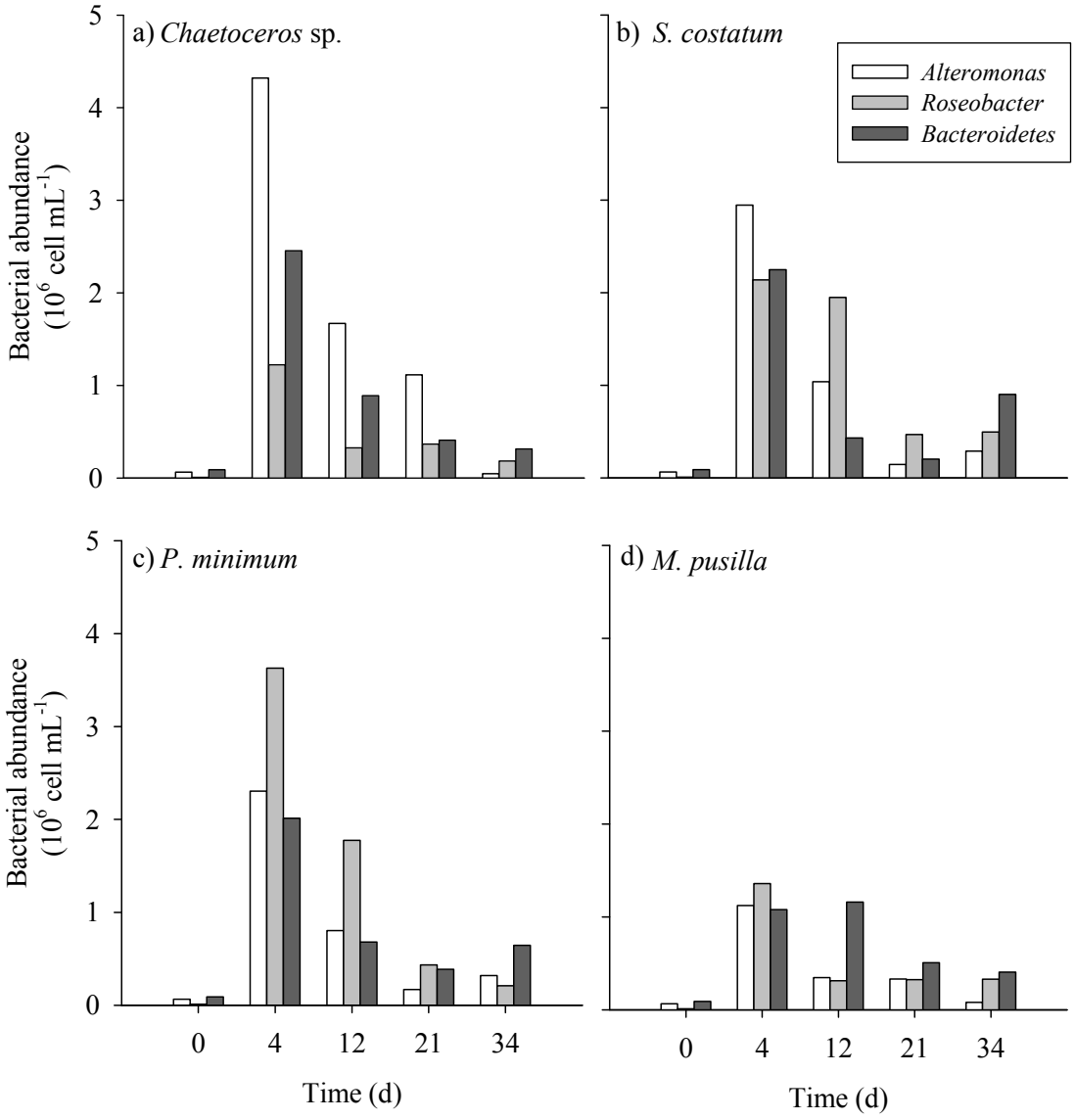

a)
b)
c)
d)
Chaetoceros sp.
S.costatum
P. minimum
M. pusilla
DOC (%)
peak-C (%)
peak-M (%)
peak-T (%)
Time (d)
Time (d)


a)
t0
1.8
2.8
1.8
3.2
3.8
Chaetoceros sp.
b)
t4-t0
A
0.8
0.4
0.4
-0.2
0
C
0
M
T
Chaetoceros sp.
c)
t34-t0
0.2
0.6
1
-0.4
0.2
-1.4
Chaetoceros sp.
Emission
Excitation
500
450
400
350
300
250
300
350
400


a)
t0
1.8
2.8
3.8
4.8
1.8
2.8
S. costatum
b)
t4-t0
A
0
0.6
-0.8
-1
0.2
C
0
0.2
M
T
S. costatum
c)
t34-t0
-2
-0.5
0.5
1
0.5
1
1.5
S. costatum
Emission
Excitation
500
450
400
350
300
250
300
350
400


a)
t0
1.8
2.8
3.8
1.8
0.8
P. minimum
b)
t4-t0
A
0.3
0.3
0.3
0.4
C
T
P. minimum
c)
t34-t0
0.6
0.8
0.4
0.6
-0.2
0
0.4
P. minimum
Emission
Excitation
500
450
400
350
300
250
300
350
400


a)
t0
1.8
2.8
1.8
0.8
M. pusilla
b)
t4-t0
A
0.4
0.4
0
1
1.6
-0.6
0
1
2
1.6
C
M
T
M. pusilla
c)
t34-t0
0.4
0.4
1.4
1.8
-0.6
-0.2
0.8
M. pusilla
Emission
Excitation
500
450
400
350
300
250
300
350
400


a) Chaetoceros sp.
b) S. costatum
c) P. minimum
d) M. pusilla
Alteromonas
Roseobacter
Bacteroidetes
Bacterial abundance
(10^6 cell mL^-1)
0
1
2
3
4
5
0
4
12
21
34
Time (d)