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Bacterial uptake of DOM released from P-limited phytoplankton

Alberto Puddu [a,*], Annamaria Zoppini [a], Stefano Fazi [a], Michela Rosati [a], Stefano Amalfitano [a], Erika Magaletti [b]

[a] *Istituto di Ricerca Sulle Acque, CNR, Via Reno 1, 00198 Rome, Italy*
[b] *Central Institute for Marine Research, ICRAM, Via di Casalotti 300, 00166 Rome, Italy*

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Abstract

The growth and the structure of a coastal bacterioplankton community were monitored in short-term bottle experiments in order to investigate the bacterial uptake of extracellular organic carbon released by the diatom *Cylindrotheca closterium* grown under P-balanced and P-depleted conditions. Bacterial specific growth rates and carbon demand were significantly lower in the exudates from P-depleted algae (24% and 30% reduction, respectively). The origin of the extracellular carbon appeared also to affect the taxonomic composition of the bacterioplankton assemblage, mainly reducing the development of γ-Proteobacteria. This pattern of bacterial carbon uptake could contribute to a longer persistence of the exudates released in P-depleted conditions affecting the dynamics of the carbon cycle in marine environments.
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Keywords: Carbon cycle; Algal excretion; *Cylindrotheca closterium*; Bacterial activity; Phylogenetic diversity; Flow cytometry

1. Introduction

In the aquatic environment phytoplankton cells are not only an important source of autochthonous particulate organic matter, but they also significantly contribute to the production of dissolved organic matter (DOM). Up to 50% of photosynthetically fixed carbon circulates in the dissolved compartment as a result of cell lysis (by sloppy feeding and viral infection) and direct exudation [1–3]. Extracellular release of organic carbon is a physiological process occurring in healthy as well as in stressed phytoplankton cells [4,5]; it can comprise a relevant fraction of primary production ranging from 2 to 10% during rapid growth, increasing up to 10–60% in the stationary phase [6]. The rate of per cell release of extracellular organic carbon (EOC) is higher in rapidly growing cells (exponential growth) than in stationary phase cells [7]. The dominant algal species and the physiological state of phytoplankton seem to strongly influence the quantity of photosynthetic extracellular release [8,9]. Nagata [3], summarizing the percent extracellular release relative to total primary production in various marine environments, reports a wide range of values (from 10 to 80%); other authors [10,11] suggest that several methodological artifacts in EOC measurement could be responsible for the large range of reported values.

The fate of freshly produced DOM in the aquatic environment, extremely relevant for the global carbon cycle, is mainly determined by bacterial uptake. The rates of DOM uptake largely depend on the physiological state of bacteria and the chemical composition of the organic substrate [12]. Most DOM in seawater is considered to be resistant to microbial utilization and its labile fraction, usually less than 5% of the total, is confined to the upper levels of the water column [13]. Algal exudates, highly bioavailable in comparison with other DOM sources [14], represent a main component of this fraction.

Information on chemical composition and fate of algal exudates is still not exhaustive [15,16]. Little is known about the bacterial utilization of extracellular organic matter produced by phytoplankton. The characterization of this photosynthetically produced dissolved carbon has shown that either high or low molecular mass substances can dominate its composition with a high within- and

* Corresponding author. Tel.: +39 (06) 8841451; Fax: +39 (06) 8417861.
E-mail address: puddu@irsa.rm.cnr.it (A. Puddu).

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between-system variation [17,18]. From culture studies, carbohydrates are reported as the major components of phytoplankton exudates [19] but their molecular composition is highly variable, depending on species and nutrient status [15]. The extracellular production by healthy cells of *Skeletonema costatum* is characterized by 33% polysaccharides and 15% monosaccharides [20]. Dissolved carbohydrates are mainly glucan, a homopolysaccharide containing only glucose, and heteropolysaccharides, composed of rhamnose, fucose, galactose, mannose and xylose. The relative proportion of these two groups is likely to vary depending on factors such as phytoplankton biomass and nutrient availability and may have important implications for the bioreactivity of EOC [21,22]. Glucan is considered one of the most bioreactive compounds, while heteropolysaccharides are more resistant to microbial degradation and hence can accumulate in seawater [23]. Meon and Kirchman [24], from mesocosm experiments, showed that extended nutrient depletion could explain the presence of glucose-dominated sugar polymers. Other minor components of extracellular DOM are amino acids [25,26], proteins [27], lipids [28] and vitamins [29]. The composition of extracellular amino acids can differ from the intracellular one and change considerably from exponential to stationary algal growth phase [20].

In several areas of the Mediterranean Sea there is evidence that P limitation affects both primary productivity and bacterial uptake of dissolved organic carbon (DOC), the latter due to a low assimilation of dissolved organic substrates when P is not available for bacterial metabolism [30–33]. P limitation has also been hypothesized as one of the causative factors leading to the hyperproduction and massive accumulation of organic matter in the Northern Adriatic Sea during summer [34–41]. The imbalance between growth and photosynthesis, driven by nutrient deficiency, can induce/accelerate the exudation of assimilated carbon from several algal species. Numerous investigations have focused on the 'production term', by looking at phytoplankton physiological responses to nutrient limitation in terms of quantitative carbohydrate exudation [38,42–44].

Benthic diatoms secrete copious amounts of extracellular polymeric substances or mucilage [21,45] that provide a food source for other organisms [46] and stabilize the sediment surface [47]. Due to resuspension phenomena, benthic diatoms can be recovered in the water column of shallow coastal environments. The epipelic benthic diatom *Cylindrotheca closterium* is frequently recovered in coastal waters of the northern Adriatic Sea, mainly in mucilaginous aggregates [48–50]. Some authors hypothesized a direct role of this species in mucilage hyperproduction [43,51,52]. Alcoverro et al. [43] showed that nutrient deficiency reduces the investment in growth and increases carbohydrate release during the exponential growth phase of *C. closterium*. The extracellular release is higher under phosphorus than under nitrogen limitation, but the carbohydrate composition remains to be investigated. Staats et al. [53] showed that *C. closterium* produces considerable amounts of exopolymers with uronic acids and sulfated sugars for the adhesion in diatom biofilms.

Compositional differences in the exudates produced by diatoms under different nutrient regimes are little known, as is the effect of such differences on bacterial assimilation rates and community composition. Recent findings on *Cylindrotheca fusiformis* extracellular carbohydrates (E. Magaletti, unpublished results) suggest that P depletion may not only cause an increase in total polysaccharide abundance compared to nutrient-replete and N-depleted conditions, but can also alter the neutral aldose signature, with heteropolysaccharides being more represented than glucose.

The aim of this work was to investigate the effect of P limitation on the bioavailability of *C. closterium* photosynthetic extracellular released products. This diatom species was grown in P-balanced and P-depleted DOC-free media. Excreted organic carbon produced during the exponential growth phase was inoculated by a grazer-free marine bacterial community. The growth and the structure of the bacterioplankton community were monitored in short-term cultures; estimates of bacterial abundance (BAB), production and oxygen consumption were performed every 24 h for 4 days. Fluorescent in situ hybridization (FISH) and cytometric (apparent DNA content) techniques were applied to investigate community structure.

2. Materials and methods

2.1. Phytoplankton cultures

Non-axenic cultures of the diatom *C. closterium* (Ehrenberg) Lewin and Reimann were isolated from natural populations of the Gulf of Trieste, Northern Adriatic Sea (kindly provided by A. Beran, LBM, University of Trieste). Cells were maintained in a nutrient-enriched medium prepared with artificial seawater [54] at a temperature of 18°C and a salinity of 32, under a 16/8 light/dark photocycle and a photon flux of 112 μE m^{-2} s^{-1}, provided by cool-white lamps. The medium was slightly modified from the original recipe by omitting the EDTA in order to avoid additional sources of DOC. Experimental cultures with an initial algal abundance of about 10^3 cells ml^{-1} were set up in 5-l Erlenmeyer flasks containing 3 l of culture medium each, constantly insufflated with 0.2-μm filtered air. Two experimental conditions were tested, which differed only for the initial content of inorganic phosphorus in the medium: a P-balanced condition, with a P-PO_4 concentration equal to 6 μM and an N:P molar ratio of 48; and a P-depleted condition, with 2 μM of inorganic P and an N:P molar ratio of 145. Cell growth was followed daily by monitoring in vivo chlorophyll fluo-

rescence, while periodic microscopy observations were performed to check for cell integrity. Cells grew exponentially in both media ($y = 12.5e^{0.18x}$, $R^2 = 0.96$ and $y = 7.4e^{0.19x}$, $R^2 = 0.97$, respectively for P-balanced and P-depleted conditions) and were harvested after 12 days of growth, corresponding to the late exponential phase, with the aim of avoiding the contribution of DOC resulting from cell lysis. All glassware and sampling apparatus were carefully sterilized to keep BAB in the cultures very low ($< 10^3$ cells ml^{-1}). Consequently, DOM could be assumed to derive only from phytoplankton activity. Algal cells were removed by gentle filtration through a precombusted Whatman GF/F filter and the EOC was collected by subsequent filtration through a pre-rinsed 0.4 μm PC membrane. Initial DOC concentration in the exudates was 55 and 61 μM, for P-balanced and P-depleted cultures respectively. DOC was assayed by high temperature catalytic oxidation using a Shimadzu TOC-5000 analyzer [55]; samples were measured in triplicate and the relative standard deviation never exceeded 2%.

2.2. Experimental set-up

To estimate the bioavailability of exudates, five different treatments were set up: EOC derived from diatoms grown on P-balanced (EOC_b) and P-depleted (EOC_d) conditions, labile organic C (glucose, 80 μM C) added to DOC-free artificial seawater, EOC_b+glucose and EOC_d+glucose. To avoid N or P limitation in bacterial growth, all treatments were enriched with inorganic nutrients (NH_4Cl, KH_2PO_4) at the ratio C:N:P = 80:10:1, with respect to initial C concentrations. The bacterial inoculum was prepared by filtering 1 l of natural seawater through a 0.8-μm polycarbonate filter (Nuclepore) to remove grazers. The filtrate was then passed through several 0.2-μm polycarbonate filters (Nuclepore) to retain bacterial cells. Thereafter filters were washed in 100 ml DOC-free artificial seawater to obtain the concentrated suspension of bacteria ($\sim 10^6$ cells ml^{-1}). This procedure made it possible to avoid an extra addition of DOM from natural seawater. The bacterial inoculum was added to each treatment in the ratio 1:50 (v:v) and two 250-ml Erlenmeyer flasks were prepared. To avoid DOC contamination, all materials were acid-washed and repeatedly rinsed with ultrapure water (Millipore Milli-Q). Flasks were incubated in the dark at 20 ± 1°C for 96 h under still conditions, manually shaken and sampled every 24 h for the analyses.

2.3. Bacterial abundance, apparent DNA content and growth rates

BAB was determined by epifluorescence microscopy (EFM) and flow cytometry (FC). Samples (5 ml) for EFM, fixed with 2% formaldehyde (final concentration), were stained for 5 min with DAPI [56] at a final concentration of 1 μg ml^{-1} and filtered through 0.2-μm black polycarbonate filters (25 mm diameter, Nuclepore). Filters were mounted on microscope slides with non-fluorescent oil (R.P. Cargille Lab) and stored frozen until counted. BAB was determined with a Leica DM LB 30 epifluorescence microscope at 1000× magnification, counting a minimum of 300 cells per sample. Subsamples (1.5 ml) for FC were immediately fixed with freshly prepared 1% paraformaldehyde plus 0.05% glutaraldehyde (final concentrations), incubated for 20 min at room temperature in the dark, and then stored at −80°C [57]. Before the analyses, samples were thawed, 400 μl was stained with SYTO-13 (Molecular Probes) 5 μM final concentration, left in the dark for 15 min and run through a Becton Dickinson FACScalibur flow cytometer with an argon laser emitting at 488 nm. Ten microliter of a standard 0.97-μm fluorescent bead suspension (Polyscience) was added to the sample just before counting as an internal reference. The absolute concentration of beads in the suspension was determined by EFM and the ratio of cells to the beads was used to compute bacterial concentration in the sample. To avoid coincidence, samples were run at various flow rates to keep the number of events below 500 s^{-1}. Data were acquired in log mode until 10 000 events had been reached. Bacteria were detected by their signatures in a plot of 90° side light scatter versus green fluorescence (FL1).

Recently, Button and Robertson [58] described the evidence of a direct relationship between DNA cellular content and DAPI fluorescence detected by FC. The sensitivity of the flow cytometer makes it possible to easily discriminate two or more bacterial groups characterized by an increasing apparent DNA content [58–61]. Furthermore, the intensity of fluorescence emitted by bacterial cells after staining with the SYTO-13 nucleic acid probe can be assumed to be proportional to the cell size [57] and activity [63]. In this experiment, different clusters of low and high DNA bacteria were separated in the SSC versus FL1 plot; cytometric noise was discarded both by setting a threshold on FL1 and by manually separating noise from cells in the FL1 versus FL3 (red) plot [64]. Data acquisition was performed with CELL QUEST software and data analysis with PAINT-A-GATE software (Becton Dickinson).

The specific growth rates (μ) were calculated by regression analysis of the slope of the linear portion of the curves of ln abundance versus time (days), whereas the turnover times (t_t) were equal to $\ln(2)/\mu$ and expressed in hour units.

2.4. Bacterial carbon production, respiration and growth efficiency

Bacterial carbon production (BCP) was measured by the incorporation of [^{3}H]leucine. Triplicate 1.7-ml samples and one killed control (trichloroacetic acid (TCA) 5%) were amended with 20 nM radiotracer and incubated for 1 h at 20°C. The extraction, with TCA 5% and subsequent washing with TCA 5% and ethanol 80%, was carried out

by the micro-centrifugation method, according to Smith and Azam [65]. To estimate BCP the rates of leucine incorporation were transformed into units of C by using the conversion factor of 3.1 kg C produced per mol of leucine incorporated [66].

At the beginning of the experiment oxygen concentration was determined in the initial solutions, in triplicate, by the Winkler method with a Mettler DL 67 autotitrator with potentiometric end-point detection [67]; the relative standard deviation never exceeded 1%. Three acid-washed and rinsed BOD bottles (110 ml) for each treatment were also set up, filled and incubated under the same conditions as the experimental flasks. One bottle for each treatment was sacrificed at times 48, 72 and 96 h to determine oxygen concentration in duplicate. BAB was analyzed in the BOD bottles, at 48 and 96 h, for a direct comparison with the experimental flasks. Bacterial respiration rate (BR) was calculated along the incubation as dissolved oxygen depletion vs. time. BR was then transformed into C units assuming a respiratory quotient of 1.

Bacterial growth efficiency (BGE) in the different samples was calculated during the exponential growth period, to represent the estimation of maximum efficiency [68], from bacterial C production and bacterial respiration as:

$$\mathrm{BGE} = \mathrm{BCP}/(\mathrm{BCP} + \mathrm{BR})$$

BCP plus BR represent the total bacterial carbon demand (BCD).

2.5. Community structure

Community structure was analyzed by FISH at the beginning and at the end (96 h) of the experiment. Bacteria from fixed samples (formaldehyde 4%; overnight) were collected on 0.2-μm polycarbonate membranes (47 mm diameter, Nuclepore) by filtering 5–10 ml (depending on BAB) by gentle vacuum (< 0.2 bar) and washed with 10–20 ml of sterile Milli-Q water. Until further processing, the filters were stored in Petri dishes at −20°C. FISH of harvested cells counterstained with DAPI was performed according to the protocol of Pernthaler et al. [69]. Each filter was cut into about 16 sections and two filter sections were hybridized with the same probes. The following oligonucleotide probes were used: EUB338, EUB338 II, EUB338 III specific for domain Eubacteria [70], ALF1b specific for the α-Proteobacteria subclass; BET42a for β-Proteobacteria; GAM42a for γ-Proteobacteria; CF319a for *Cytophaga-Flavobacterium* [71]. All probes were commercially synthesized (MWG AG Biotech, Germany) and labeled with either fluorescein or Cy3 dye. After hybridization the filter sections were stained with DAPI and mounted on microscope slides in Vectashield medium. The relative abundance of hybridized cells was estimated as a ratio of hybridized cell counts to counts of DAPI-stained cells using EFM. In order to synthesize the information on Eubacteria relative distribution in the four phylogenetic subgroups, the Shannon index ($\mathrm{Hs} = -\Sigma \mathrm{P_i}\ \ln \mathrm{P_i}$; $\mathrm{P_i}$ = phylogenetic subgroup relative abundance), intended as a condensing index, was calculated at the end of the experiment.

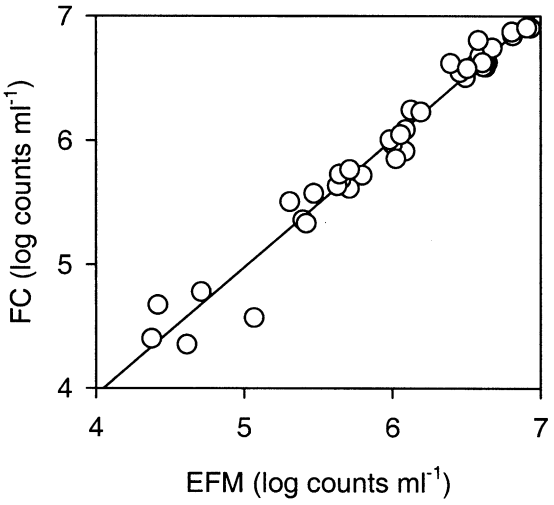

Fig. 1. Relation between BAB estimated by EFM and FC.

3. Results

3.1. Bacterial abundance and growth rates

BAB was estimated using both EFM and FC. As shown in Fig. 1, counts by both methods are highly correlated ($P < 0.0001$) with a relation close to 1:1 (log SYTO = 1.03 ± 0.03 DAPI-0.17; $n = 40$; $r = 0.98$; S.E.M. = 0.13) confirming the validity of using the faster flow cytometric enumeration [72]. All bacterial concentrations presented hereafter were derived from cytometric determinations.

Time courses of BAB are presented in Fig. 2. Bacteria were readily able to take up EOC from *C. closterium* grown on P-balanced medium ($\mathrm{EOC_b}$), reaching their maximum abundance after 3 days (1.5×10^6 cells $\mathrm{ml^{-1}}$). In exudates from P-depleted cultures ($\mathrm{EOC_d}$), bacterial growth showed an apparent 24-h lag phase and maximum yields were about three times lower than those obtained in $\mathrm{EOC_b}$. When glucose was added to the media, alone or in combination with the algal exudates, bacterial growth was differentially stimulated. The highest BABs were observed after 3 days in both replicates with glucose in combination with $\mathrm{EOC_b}$ (8.0×10^6 cells $\mathrm{ml^{-1}}$) with no initial lag phase. In glucose alone or combined with $\mathrm{EOC_d}$ bacterial growth showed the same initial lag phase described above and the peak of BAB, at 72 h, was lower than that observed in glucose plus $\mathrm{EOC_b}$ (5.1 and 4.1×10^6 cells $\mathrm{ml^{-1}}$, respectively).

Cell yield at the end of the experiment (96 h) was compared by analysis of variance (two-way ANOVA) for two levels of phosphorus (balanced–depleted) and two levels of

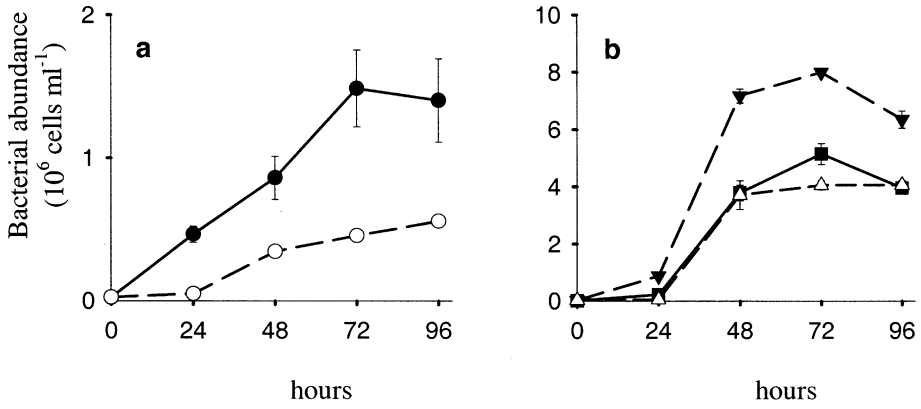

Fig. 2. Time courses of BAB in: (a) exudates from P-balanced (●) and P-depleted (○) *C. closterium* cultures; (b) exudates from P-balanced cultures+glucose (▼); P-depleted cultures+glucose (△); glucose alone (■). Means ± range of two replicate experiments are shown.

glucose. The results showed that there is a statistically significant interaction between phosphorus and glucose (ANCOVA test, $F = 88.2$, $P < 0.001$): the effect of different levels of phosphorus depends on the presence of glucose. We also performed a pairwise multiple comparison procedure (Student–Newman–Keuls method) and all the comparisons were significantly different.

Specific growth rates were 24% lower in EOC_d than in EOC_b treatments and attained maximum values in the glucose enrichments (Table 1). Bacterial cells duplicated fast when glucose was combined with EOC_b while when combined with EOC_d bacterial growth was lower, even with respect to the glucose condition.

The trends in BAB, derived from two replicate flasks in each treatment, were further confirmed by results from BOD bottles (variation among bottles and flasks ranged from 6 to 20%).

3.2. Bacterial carbon production, respiration and bacterial carbon demand

BCP (Fig. 3), strongly increased during the incubation in EOC_b, glucose and EOC_b+glucose conditions. Maximum production rates in EOC_b (2.1 μg C l^{-1} h^{-1}) were reached in 24 h and remained about constant thereafter. With glucose alone or combined with EOC_b, the production rates showed a peak at 24 h and decreased sharply during the following days. BCP of bacteria growing on EOC_d alone or in combination with glucose increased only after 24 h. BCP rates in EOC_d increased slightly until the end of the experiment, reaching a value not statistically different (*t*-test; $P = 0.67$) from EOC_b. Nevertheless, differences in cell abundance determined a higher per cell BCP (BCPs) in EOC_d (4.35×10^{-9} μg C cell^{-1} h^{-1} at 96 h) than in EOC_b (1.87×10^{-9} μg C cell^{-1} h^{-1} at 96 h). The integration of BCP values measured at 24-h intervals allowed us to estimate the total amount of C incorporated by bacteria at the end of the experiment (Table 1).

Dissolved oxygen respired in the BOD bottles at the end of the experiment (96 h) was higher in the exudates from P-balanced algal cultures (12.3 ± 0.4 μM O_2, mean and range of two replicate measurements) than in EOC_d (8.9 ± 0.2 μM O_2). Total respiration by bacteria fed with glucose rose to 19.0 ± 1.0 μM O_2. The non-significant differences between BAB in the experimental flasks and in BOD bottles allowed us to extrapolate respiration data from BOD bottles in order to estimate BCD and BGE in the flasks. BCD and DOC uptake values are reported in Table 2. BCD in EOC_d was 30% lower than in EOC_b. At the end of the incubation, 48% of EOC_b and only 30%

Table 1
Bacterial specific growth rates (μ), turnover times (t_t) and total incorporated carbon (C_{inc})

Treatment	μ (day^{-1})[a]	t_t (h)[a]	C_{inc} (μM)[b]
EOC_b	1.91	8.6	14.1
EOC_d	1.46	11.5	9.7
Glucose	3.03	5.5	32.8
EOC_b+glucose	3.10	5.3	42.5
EOC_d+glucose	2.80	6.0	35.2

Values are means of two replicate experiments.
[a]μ and t_t were estimated over a period of 48 h (exponential growth phase).
[b]Derived from BCP integrated up to the end of the experiment.

Table 2
BCD and the percentage of DOC taken up (% DOC_{upt}) at the end of the experiment

Treatment	BCD derived from total incorporated (C_{inc}) and total respired C (C_{resp})			% DOC uptake derived from BCD and initial DOC concentration	
	C_{inc} [a]	C_{resp} [b]	BCD	Initial DOC[c]	% DOC_{upt}
EOC_b	14.1	12.3	26.4	55	48
EOC_d	9.7	8.9	18.6	61	30
Glucose	32.8	19.0	51.8	80	65

All values are expressed in μM C.
[a]As in Table 1.
[b]Values, derived from oxygen respiration assuming a respiratory quotient of 1, are means of two replicate experiments.
[c]Mean of three replicates.

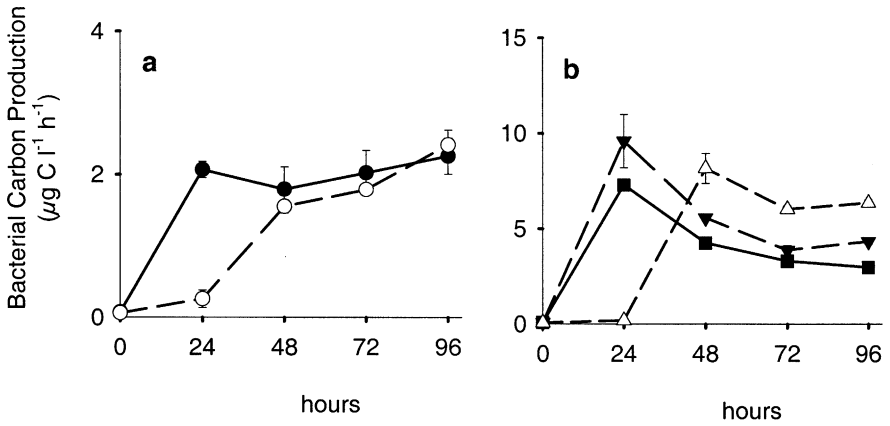

Fig. 3. Time course of BCP, as determined by [^{3}H]leucine incorporation. Means ± range of two replicate experiments are shown. Symbols as in Fig. 2.

of EOC_d were taken up. We assume that these percentages represent the fraction of bioreactive DOC, readily available for bacterial metabolism in these short-term experiments.

BGEs are reported in Table 3. The organic substrate was utilized more efficiently when derived from algae grown under P-balanced conditions: in EOC_b the quantity of C transformed into biomass was higher than that respired (BGE = 0.56), while in EOC_d only 0.44 of assimilated C was utilized for the production of new biomass. Glucose stimulated higher growth efficiency than EOC_b.

3.3. *Bacterial community structure (apparent DNA concentration and FISH)*

FC allowed the discrimination of different bacterial subgroups, based on their apparent DNA content. Results showed relevant changes in subgroup composition depending on time and treatments. Since the fluorescence units depend on the instrument used, quantitative results are expressed as the ratio of cell fluorescence vs. standard bead fluorescence. Taking into account the relative fluorescence range of the bacterial cells in the inoculum (0.1–0.9 standard bead fluorescence), we assumed 0.6 to be the

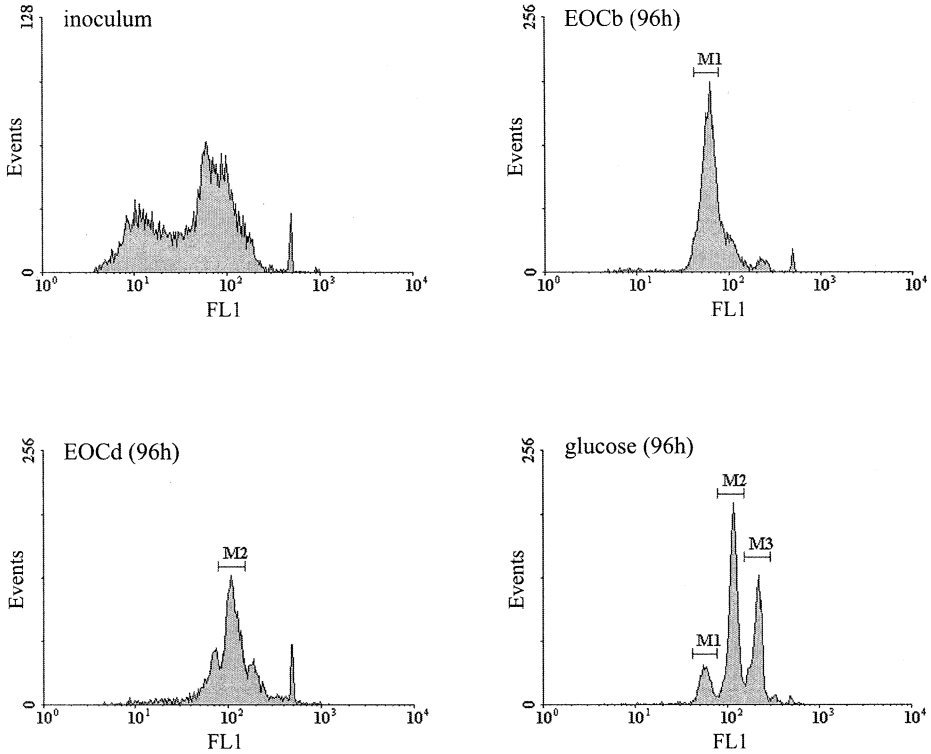

Fig. 4. Frequency distribution of apparent DNA fluorescence (FL1 = relative units of SYTO-13 green fluorescence cell^{-1}) in the initial inoculum and in different treatments at the end of the experiment (96 h). Markers identify HDNA1 (M1), HDNA2 (M2) and HDNA3 (M3) clusters. The highest fluorescence corresponds to the microbead signal.

Table 3
BGEs estimated from BCP and BR integrated over a period of 48 h (exponential growth phase)

Treatment	BCP[a]	BR[a]	BGE
EOC_b	6.00	4.69	0.56
EOC_d	2.13	2.69	0.44
Glucose	18.95	11.13	0.63

[a]Values, expressed in μM C, are means of two replicate experiments.

threshold value between low DNA (LDNA) and high DNA (HDNA) bacteria.

At the beginning of the experiment 41% of bacteria belonged to the LDNA cluster and 59% to the HDNA cluster. The relative abundance of LDNA decreased rapidly during the first 24 h of incubation to 8% in EOC_b, 22% in EOC_d and 16% in the glucose treatment. Fast-growing HDNA bacteria became dominant, reaching 90% of the population in all treatments after 2 days. Frequency distributions of green fluorescence, due to SYTO-13 DNA staining, at the beginning and at the end of the experiments are shown in Fig. 4. Within the HDNA cluster three different subpopulations were easily distinguished, hereafter named HDNA1, HDNA2 and HDNA3 bacteria, with a mean DNA fluorescence over the standard bead fluorescence equal to 0.66, 0.77 and 0.87, respectively. At the end of the experiment, bacteria growing in the EOC_b medium were strongly dominated by the HDNA1 cluster (75%), while in the EOC_d treatment HDNA2 bacteria became dominant; when bacteria were fed on glucose, all three subpopulations were represented. Temporal changes in the relative distribution of the HDNA subclasses are shown for all treatments in Fig. 5. The HDNA1 subpopulation, which rapidly became dominant in EOC_b, was outmoded by HDNA2 in EOC_d at 48 h. In the glucose treatments, HDNA2 similarly dominated but 30–40% of bacteria were grouped in the HDNA3 cluster until the end of the incubation. Bacteria growing on EOC enriched with glucose showed intermediate trends with respect to the substrate alone added.

FISH showed that in the inoculum 66.7% (range ±2.6) of the bacteria enumerated with DAPI hybridized with the Eubacteria-specific probes; this percentage increased up to 70–80% in the algal exudates and 80–95% in glucose at the end of the incubation (96 h). Analysis of the bacterial community with a comprehensive suite of specific rRNA target probes revealed the phylogenetic composition to be governed by substrate quality. The communities, exposed to different experimental conditions, showed substantial changes in their phylogenetic composition at 96 h (Fig. 6). In the EOC_b condition cells affiliated to γ-Proteobacteria were numerically the most important subgroup (64% of the total DAPI cell counts). The abundance of the remaining subgroups ranged between 3 and 14%. In the exudates produced under P-depleted conditions (EOC_d) there was not such an evident predominance of any of the subgroups. In the glucose treatment a marked dominance of γ-Proteobacteria was also found (average 85%). Members of the γ-cluster constantly accounted for the

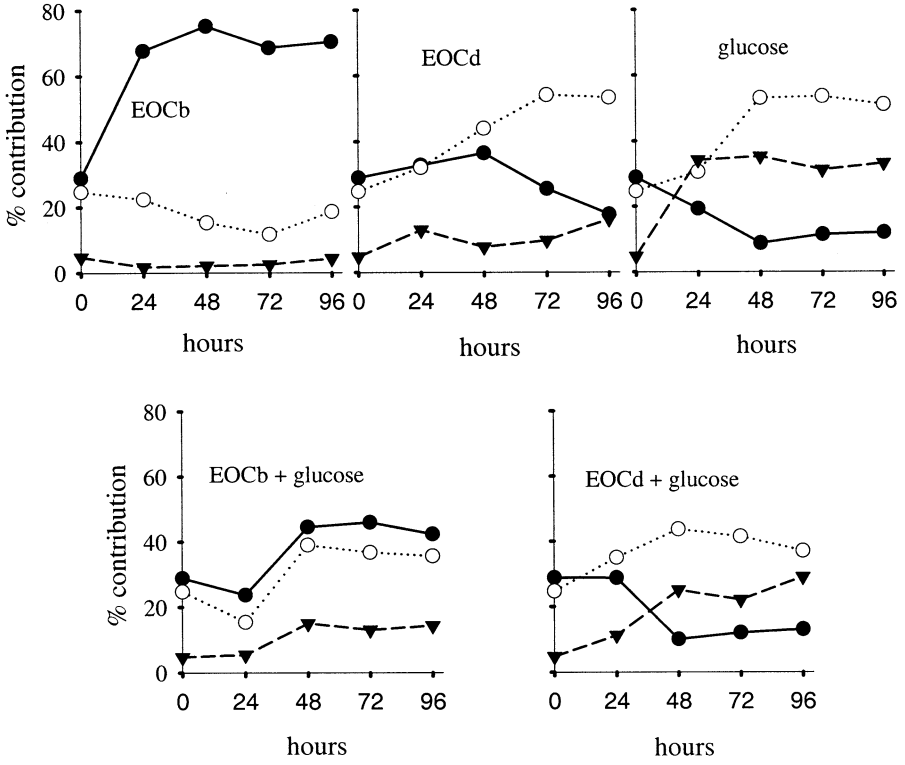

Fig. 5. Time courses of the relative distribution of three subpopulations of HDNA bacteria in the experimental treatments. (●) HDNA1, (○) HDNA2, (▼) HDNA3 clusters.

majority of cells also when glucose was combined with exudates.

By comparing all the experimental conditions at the end of the incubation period (96 h), we found a negative correlation between BAB (cells ml^{-1}) and Shannon index (Hs) applied to the relative abundance of phylogenetic subgroups. Hs was also positively correlated with BCPs. An inverse relation between abundances and BCPs was also found (Table 4).

Table 4
Regression analyses among Hs[a], BAB[b] and BCPs[c] at the end of the experiment ($n = 10$)

	R^2	P
$Hs = -1 \times 10^{-7}$ BAB+1.16	0.77	< 0.05
Hs = 220 BCPs+0.37	0.89	< 0.05
$BAB = 7.7 \times 10^{6}\ e^{-619\ BCPs}$	0.82	< 0.05

[a]Calculated to synthesize the Eubacteria relative distribution in the four phylogenetic subgroups.
[b]Values are expressed as cells ml^{-1}.
[c]Values are expressed as μg C $(10^6$ cells$)^{-1}$ h^{-1}.

4. Discussion

The phytoplankton extracellular production of organic carbon is enhanced under low nutrient concentration because carbon fixation may exceed incorporation into cell material [73]. This is widely supported by field [8,74] and culture studies [26,75]. In addition, Obernosterer and Herndl [35] demonstrated that extracellular products released from P-limited phytoplankton could not be efficiently utilized by heterotrophic bacteria, their metabolism being affected by the same P limitation. Our study provides some evidence that P-limited algal growth affects the characteristics of released DOC, in terms of lability and persistence, reducing bacterial uptake even when this occurs under no P-limited conditions.

Growth, carbon production and respiration of bacteria, not constrained by nutrient limitation, showed a reduction when cultures were fed on algal exudates produced under P-limited conditions. The percentage of DOC uptake in the present work (48–65%) was higher than the average percentage of labile DOC (19%) reported for various environments by Søndergaard and Middelboe [76], but it is comparable with the results from recent mesocosm experiments [24] where only 32% of the DOC accumulated during experimental phytoplankton blooms was found to be recalcitrant. Such differences in DOC uptake may be the result of changes in DOC composition [17,77].

BGE, defined as the quantity of biomass synthesized per unit of substrate assimilated, can help to elucidate the quality of EOC in terms of lability. When there are no differences among all the other variables affecting bacterial metabolism, the nature of the organic substrate will be reflected in the ratio between anabolic and catabolic reac-

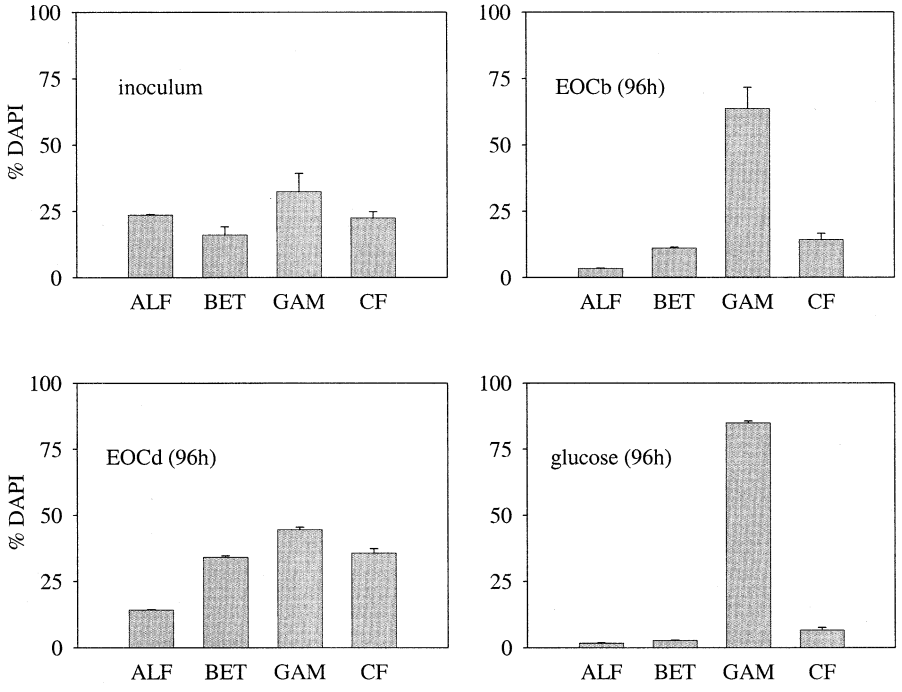

Fig. 6. Percentage of cells hybridized with a suite of specific rRNA target probes in the initial inoculum and in different treatments at the end of the experiment (96 h). Data are reported as percentage of hybridized cells over DAPI-stained cells. ALF = α-Proteobacteria, BET = β-Proteobacteria, GAM = γ-Proteobacteria, CF = *Cytophaga-Flavobacterium*. Means ± range of two replicate experiments are shown.

tions. BGE is generally low (< 30%) for many categories of DOM except for the organic matter excreted by phytoplankton in which most values are above 50% [78]. The BGE observed in EOC_b (56%) confirms the high availability of this source of DOM. The lower BGE found in EOC_d (44%) is a further demonstration of the reduced availability of this substrate. When bacteria fed on a single labile substrate, such as glucose, they showed a higher BGE value (63%), similar to that observed on the EOC_b substrate. This pattern confirms the characteristics of lability of freshly produced DOM from P-balanced *C. closterium* cultures. BGE values ranging from < 5% to 69% have been reported for marine ecosystems [78]; our values lie within the upper portion of this range.

Reduction of bacterial activity and consequent DOC uptake might lead to a longer persistence in the water environment of EOC_d. This could probably imply that in order to assimilate EOC_d a higher degree of metabolic specialization is needed that none of the members of our bacterial consortium is ready to express.

To further understand the differences in bioreactivity of the organic substrates, we focused our attention on the dynamics of the community structure, investigated by means of apparent DNA content analysis and FISH.

As only recently shown by Fuchs et al. [79] and Massana et al. [80] from short-term bottle experiments, the composition of the bacterial assemblage can change over a period of days of incubation. Our results confirm these observations and provide additional evidence that short-term experiments, even though they do not exactly reproduce the in situ growth, can indeed be used for a comparative analysis to assess differences in organic substrate uptake.

Flow cytometric observations showed that HDNA bacteria soon became predominant in all the experimental conditions. Gasol et al. [64] suggested that HDNA cells are the active and dynamic members of the bacterioplankton community. Lebaron et al. [81] confirmed that these cells are responsible for most of the community activity, even though they found a low correlation between HDNA counts and bacterial production, possibly due to the heterogeneity of specific activities within this group. Differences among treatments in the ratio LDNA/HDNA cells at 24 h (0.1, 0.3 and 0.2, in EOC_b, EOC_d and glucose, respectively) might be a further confirmation of the different composition of the organic matter: a rapid activation of bacteria occurred only in EOC_b and, to a lesser extent, in glucose. In addition, as the incubation proceeded, bacterial assemblages distributed differently among the HDNA subclass. Lebaron et al. [62], measuring the distribution of the specific activity within the fraction of HDNA bacteria in coastal waters, found that both nucleic acid content of cells and their biovolume were positively correlated with specific activity and this may be partly due to the structure of bacterial communities.

Detection yields of Eubacteria, relative to total cell counts, increased from 67% in the inoculum up to 95% after 96 h of growth. Taking into account that only cells having a sufficient cellular rRNA content and a good cell wall permeabilization hybridize with specific probes, it is not surprising that the percent of Eubacteria fluorescing cells was higher in actively growing assemblages.

In our experiment, the different treatments strongly affected the final composition of the Eubacteria assemblage. It was consistent in all the treatments with highly degradable substrata (P-balanced and glucose conditions) that γ-Proteobacteria became the most abundant group, reaching a relative abundance of 50–85%. Fuchs et al. [79] speculated that the fast-growing γ-Proteobacteria fill the niche of typical r-strategists, which rapidly exploit extra nutrients when they become available. Members of this group are adapted to high nutrient concentrations and therefore grow well under culture conditions [82]. In our natural samples the *Cytophaga-Flavobacterium* cluster accounted for about 20% of the cells; its relative abundance decreased at the end of the incubation with the exception of the P-depleted condition. This could be interpreted as a sign of k-strategy [79] and might also be due to the lack of particulate organic C in the substrata. As many studies have demonstrated, *Cytophaga-Flavobacterium* are enriched on particulate organic detritus in marine habitats [83], and they could be specialists for particulate organic matter degradation, which was removed by filtration in this experiment. The relative abundance of α-Proteobacteria also decreased with time. Jurgens et al. [84] reported that this group shows higher resistance to grazing pressure. The lack of grazing in our cultures could have resulted in a disadvantage for these bacteria in competing with dominant γ-Proteobacteria. It is interesting to note that although β-Proteobacteria are reported to be the less abundant group in marine waters (< 4% [82]), in our natural samples they represented 16% of the total and comprised up to 34% of the assemblage at the end of the incubation in the P-depleted condition.

In the treatments with easily degradable substrates a higher BAB was associated with a numerical explosion of γ-Proteobacteria and a lower BCPs. This result might suggest that in EOC_b one phylogenetic group had a selective advantage in substrate utilization. After an initial phase of fast growth and short turnover time, a strong *intra-cluster* competition, due to the reduction of the bioavailable organic resources, could have led to a decrease of the specific production. In the EOC_d treatment, bacterial assemblages showed lower growth rates and longer turnover times with no dominance of any particular phylogenetic group. The per cell carbon production remained high possibly due to the persistence of some labile compounds.

In conclusion, our results seem to indicate that DOM excreted by P-stressed *C. closterium* is less available to bacterial uptake, and affects the taxonomic composition of the bacterioplankton community in short-term bottle experiments. Carefully extrapolating these results to the

natural environment, we might expect that under P-limited conditions some phytoplankton species release DOM whose properties slow down bacterial uptake. The presence in the water column of long-lived organic compounds released from P-starved algal cells could represent an additional factor affecting the DOM cycle in the Northern Adriatic Sea where high N:P ratios are observed all through the year.

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