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Phytoplankton species-specific release of dissolved free amino acids and their selective consumption by bacteria

Hugo Sarmento,[a,b,*] Cristina Romera-Castillo,[a] Markus Lindh,[c] Jarone Pinhassi,[c] M. Montserrat Sala,[a] Josep M. Gasol,[a] Cèlia Marrasé,[a] and Gordon T. Taylor[d]

[a] Institut de Ciències del Mar–Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Catalunya, Spain
[b] Federal University of Rio Grande do Norte, Department of Oceanography and Limnology, 'Pós-graduação em Ecologia', Natal, Brazil
[c] Centre for Ecology and Evolution in Microbial model Systems (EEMiS), Linnaeus University, Kalmar, Sweden
[d] School of Marine & Atmospheric Sciences, Stony Brook University, Stony Brook, New York

Abstract

Despite representing only a small fraction of the ocean's dissolved organic matter pool, dissolved free amino acids (DFAA) have high turnover rates and are major nitrogen and carbon sources for bacterioplankton. Both phytoplankton and bacterioplankton assimilate and release DFAA, but their consumption and production are difficult to quantify in nature due to their short residence times (min) as dissolved monomers. We segregated DFAA production by phytoplankton and bacterial consumption by measuring individual DFAA concentrations in four axenic phytoplankton cultures during the exponential growth phase, and also after 4 d incubations in the presence of a natural bacterioplankton community. The amounts and composition of the DFAA pool varied widely among phytoplankton species. The proportion of dissolved organic carbon attributed to DFAA varied among cultures. The picoeukaryotic prasinophyte, *Micromonas pusilla,* released higher amounts of DFAA than the other species tested (diatoms and dinoflagellate), especially alanine, which has been reported as the dominant individual DFAA in some oligotrophic environments. Community structure of heterotrophic prokaryotes responded to differences in the quality of organic matter released among microalgal species, with *Roseobacter*-related bacteria responding strongly to exudate composition. Our results demonstrate the specificity of DFAA extracellular release among several algal species and their preferential uptake by members of bacterial communities.

Interactions between oceanic microbes and dissolved organic matter (DOM) are expected to influence the global carbon cycle (Kujawinski 2011). Only a small fraction of the largely uncharacterized oceanic DOM pool is composed of dissolved free amino acids (DFAA). However, the importance of these molecules is manifested by their rapid turnover (Fuhrman 1987) and the fact that they can satisfy significant portions of bacterioplankton's nitrogen and carbon demands (Bronk 2002). For example, DFAA can sustain 5% to 24% of heterotrophic prokaryote carbon production (Simon 1991) and up to 51% of the nitrogen demand (Keil and Kirchman 1991). Previous studies in natural communities indicated that phytoplankton release is a primary source of DFAA and that heterotrophic prokaryotes are the major consumers of these low molecular weight molecules (Fuhrman 1987; Kirchman 1994).

Released amino acids vary in composition and proportions among phytoplankton species (Hellebust 1965) and even among growth phases and environmental conditions within a single species (Admiraal et al. 1986). In early studies, the methods used to measure organic compounds at low concentrations were not very precise. Moreover, the algal cultures used in most published studies were not axenic, and consequently observed DFAA compositions were probably the residual pools resulting from continuous modification by co-occurring prokaryotes (Hammer and Brockmann 1983; Martin-Jezequel et al. 1988). In fact, heterotrophic bacteria themselves have been shown to release DFAA under certain conditions (Kaiser and Benner 2008).

Selective consumption of individual DFAA by heterotrophic bacterioplankton has received little attention in the oceanographic literature. Low molecular weight compounds, such as DFAA, are generally highly labile and rapidly consumed by small heterotrophs, and thus only low concentrations of DFAA can be expected in the marine environment. In fact, total DFAA pool size is known to vary from 0.001 μmol L^{-1} to 0.70 μmol L^{-1} in seawater (reviewed by Bronk 2002) and very often shows similar individual DFAA proportions (*see* Discussion).

The aim of this study was to quantify the production of individual DFAA by different phytoplankton species under controlled, axenic conditions, and then measure the consumption of these monomers by a natural community of marine heterotrophic prokaryotes. We studied DFAA production by four axenic phytoplankton cultures representing major functional groups of phytoplankton occurring in coastal oceans and particularly in the northwestern Mediterranean (Margalef 1978). We separated production from consumption processes by removing microalgal cells after the production phase (Phase I), and incubating the filtrate with a predator-free suspension of a heterotrophic prokaryote community from the northwestern Mediterranean coast (Phase II). We also determined the abundance and the phylogenetic identity of the major heterotrophic

* Corresponding author: hsarmento@icm.csic.es

prokaryotic groups at the end of the incubations in order to explore possible relationships between DOM quality, specific DFAA utilization, and net growth of particular prokaryotes.

Methods

Experimental design—Phase I: This experiment was carried out as described in Romera-Castillo et al. (2010). Essentially, axenic cultures of four phytoplankton species were obtained from the Provasoli-Guillard National Center of Marine Algae and Microbiota (https://ncma.bigelow.org) and cultured under axenic conditions as described in Romera-Castillo et al. (2010). 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* (CCMP1545; R.W. Butcher) I. Manton and M. Parke. For each species, an inoculum was added to 2 liters of F/2 culture medium prepared with aged, filtered, and autoclaved coastal Mediterranean seawater. After gentle shaking, 600 mL of each cell suspension was distributed into triplicate polystyrene bottles, which were incubated at 20°C under artificial photosynthetically active radiation of 100 μmol photon m^{-2} s^{-1}, in a 16 : 8 h light : dark cycle, until cell density increased about one order of magnitude (Romera-Castillo et al. 2010). Aliquots for phytoplankton counts, and dissolved organic carbon (DOC) concentration, were taken at the beginning and end of the incubation period. The incubations lasted 3 d for all microalgae except for *P. minimum,* which required 7 d due to its slower growth rate. Samples for DFAA analyses were taken at the end of the incubation period. Control samples of the F/2 medium were also taken before adding the inoculum. The F/2 medium was the same for all four cultures; therefore, the differences between the initial and final compositions reflect differences in materials released by the cultures incubated in the F/2 medium.

The algal cultures were axenic, as guaranteed by the Provasoli-Guillard National Center of Marine Algae and Microbiota, and checked by repeated epifluorescent microscopic observations. To determine phytoplankton cell abundance and to check that the algal cultures remained axenic, aliquots of 1 mL of each culture were fixed with 1% paraformaldehyde + 0.05% glutaraldehyde (final concentration), stained with 4,6 diamidino-2-phenylindole (DAPI) at 10 μg mL^{-1} final concentration, filtered on 0.2 μm pore size polycarbonate filters and examined with an Olympus BX61 epifluorescence microscope under blue and ultraviolet (UV) wavelength excitation at the beginning and at the end of the experiment.

Samples for DOC and DFAA were filtered through 0.2 μm pore size polyethersulfone membrane Sterivex disposable cartridge filters at the beginning and end of the incubations. Two liters of Milli-Q water was filtered through this filtration system and a 10 mL sample was taken before and after this procedure. No significant differences were observed between the DOC values in the Milli-Q water and proving that contamination did not occur during filtration. Still, we discarded the first 100 mL of filtrate of each culture.

Phytoplankton biomass (B), in pg C $cell^{-1}$, was estimated with the following conversion factors: $B = 0.216 \cdot V^{0.939}$ for *P. minimum* and *M. pusilla,* and $B = 0.288 \cdot V^{0.811}$ for diatoms (Menden-Deuer and Lessard 2000), where V is the cell volume in μm^3. These values where multiplied by cell abundance to obtain biomass values.

Phase II: This experiment was carried out as described in Romera-Castillo et al. (2011). Phytoplankton cells were removed from the cultures by filtration through a 0.2 μm Sterivex cartridge, and the filtrate was inoculated with 10% volume of natural seawater filtered twice through 0.6 μm polycarbonate filters to eliminate most bacterivores. Thus, this experiment exclusively examines free-living bacteria. The inoculum was collected on 07 May 2008 from the surface of the Blanes Bay Microbial Observatory (Blanes Bay MO, http://www.icm.csic.es/bio/projects/icmicrobis/bbmo). Then, each mixture was distributed into several polycarbonate bottles (60 mL) filled with 40 mL of the inoculated exudates. For comparison we also incubated Blanes Bay MO seawater filtered through 0.2 μm Sterivex cartridge with 10% volume of seawater filtered twice through 0.6 μm polycarbonate filter as performed with the algal exudates. Three replicates for each treatment were incubated over 4 d in the dark and then were processed for DOC and DFAA analyses, heterotrophic prokaryote counts by flow cytometry, and for catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH), denaturing gradient gel electrophoresis (DGGE), and ectoenzymatic activity.

Dissolved free amino acid analysis—DFAA concentrations were determined in 5 mL filtrates (*see* filtration procedure for DOC) to which 0.5 mL of 100% methanol was added immediately after sampling; these were then stored at −20°C. Quantification by high performance liquid chromatography (HPLC) was achieved by precolumn derivatization with o-phthaldialdehyde (OPA) and fluorescence detection using a ThermoQuest HPLC, including a P2000 solvent module, an A/S 3000 autosampler, a FL2000 fluorescence detector, and an SN4000 controller.

In most respects, our protocol was similar to those presented in Lindroth and Mopper (1979) and Pantoja and Lee (1999). However, samples had to be derivatized manually prior to loading vials into the refrigerated autosampler because the instrument was incapable of performing automated chemistry. Fluorescence derived from OPA prepared with mercaptoethanol decays rapidly through time, so it was replaced with 3-mercaptopropionic acid (Molnár-Perl and Bozor 1998). Optimization experiments demonstrated that fluorescent response factors of standards remained relatively constant (within experimental error) for samples held at 4°C for at least 14 h. The OPA working solution was prepared by combining 60 mg OPA, 30 μL 3-mercaptopropionic acid, 60 μL 30% polyoxyethylene lauryl ether solution (BRIG-35), and 0.5 mL methanol in a combusted glass 6–8 mL vial. Once OPA completely

dissolved, 4.45 mL of 0.8 mol L^{-1} boric acid (adjusted to pH 10.5 with 6 mol L^{-1} KOH) was slowly added. Daily prepared buffer solution was a 1 : 1 mixture of 0.1 mol L^{-1} sodium acetate (adjusted to pH 4.11 with acetic acid) and 0.05 mol L^{-1} sodium acetate with 5% tetrahydrofuran. All glassware was precombusted (450°C) for $>$ 6 h and plasticware was acid-washed (10% HCl). Just prior to crimp-sealing, 1 mL samples or standards and 100 μL of the acetate buffer was added to all vials followed by 50 μL of the OPA solution.

A 2 μmol L^{-1} working stock of amino acid standard mixture was prepared by diluting the 500 μmol L^{-1} standard (Sigma No. A9906) in Milli-Q water and storing frozen as 0.5 mL aliquots. Peak assignments of amino acids were confirmed by adding individual amino acids to the standard mixture at $>$ 10$\times$ the working stock's concentration. Standards and reagent blanks were placed at the beginning, middle, and end of each run to monitor time-dependent changes in fluorescence response and retention times. Reagent blanks (10% methanol in Milli-Q water) subjected to same derivatization produced no significant fluorescence above baseline at retention times indicative of the 19 amino acids presented herein. DFAA were separated on a 4 μm Waters Nova-Pak C18 4 μm 3.9 $\times$ 150 m preceded by a Phenomenex C18 guard column KJO-4282/AJO-4287 and detected fluorescently using excitation λ = 340 nm, emission λ = 452 nm. At a flow rate of 1 mL min^{-1}, the elution gradient was: 0–18 min 0% to 45% B, 18.01–32 min 45% to 55% B, 32.01–38 min 55% to 73% B, 38.01–40 min, 40.01–42 min ramp down to 0% B, and hold for another 2 min (44 min total). Eluent A was 0.04 mol L^{-1} sodium acetate with 2% tetrahydrofuran and 10% methanol adjusted to pH $\sim$ 7 ($\pm$ 0.05) with glacial acetic acid and filtered through a Whatman Qualitative filter. Eluent B was 90% methanol and 10% 0.04 mol L^{-1} sodium acetate (in Milli-Q water).

Sample concentrations of individual DFAA were calculated from peak areas in the chromatograms, using response factors (fluorescence per nmole amino acid) obtained from standards. Only the L-enantiomers were measured. In cases where declining response factors were noted due to long run-times, time-dependent response factors were calculated from regressions of standards placed at beginning, middle, and end of runs. This permitted estimation of response factors for each DFAA at the time the chromatogram was produced. A portion of some individual peak areas in samples may have derived from unidentified, co-eluting, nonprotein amino acids, but their contributions cannot be evaluated with existing data.

Dissolved organic carbon (DOC)—Triplicate samples were filtered through precombusted (450°C, 4 h) glass microfiber GF/F filters. Approximately 10 mL of filtered water were collected in precombusted (450°C, 24 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 calibrated daily with potassium hydrogen phthalate. Each ampoule was sampled 3–5 times, and the average area of the three replicates that yielded a standard deviation $<$ 1% of the mean was chosen to calculate the average DOC concentration of each sample after subtraction of the average area of freshly produced UV-irradiated Milli-Q water used as a blank. The performance of the analyzer was tested with the DOC reference materials provided by D. Hansell (University of Miami). We obtained a concentration of 45.2 $\pm$ 0.3 μmol L^{-1} C for the deep-ocean reference (Sargasso Sea deep water, 2600 m). The nominal DOC value provided by the reference laboratory (Certified Reference Materials for DOC analysis, Batch 04, 2004) is 45 μmol L^{-1} C.

Heterotrophic prokaryote abundance and biomass by flow cytometry—Heterotrophic prokaryotes were enumerated by flow cytometry with a FACSCalibur (BectonDickinson) flow cytometer equipped with a 15 mW Argon-ion laser (488 nm emission) as described in Gasol and del Giorgio (2000). 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) stock (10 : 1) at 2.5 μmol L^{-1} final concentration, were left for about 10 min in the dark to complete the staining, and were run in the flow cytometer. At least 30,000 events were acquired for each subsample (usually 100,000 events). Fluorescent beads (1 μm, Fluoresbrite carboxylate microspheres; Polysciences) were added at a known density as internal standards. The bead standard concentration was determined by epifluorescence microscopy. Heterotrophic prokaryotes were detected by their signature in a plot of side scatter vs. green fluorescence. Data analysis was performed with the Paint-A-Gate software (BectonDickinson).

Heterotrophic prokaryote cell volume (V, in μm^3 $cell^{-1}$) was estimated using the ratio between the average heterotrophic prokaryote size and the average fluorescence of the SYTO-13-stained sample relative to beads ($FL1_{\text{heterotrophic prokaryote}} : FL1_{\text{beads}}$) reported by Gasol and del Giorgio (2000):

$$V = 0.0075 + 0.11 \times \left(FL1_{\text{heterotrophic prokaryote}} : FL1_{\text{beads}}\right) \quad (1)$$

Heterotrophic prokaryote biomass (BB, in pg C $cell^{-1}$) was calculated by using the carbon-to-volume (V, in μm^3 $cell^{-1}$) relationship derived by Norland (1993):

$$BB = 0.12\ V^{0.7} \quad (2)$$

An estimation of bacterial growth efficiency (BGE = bacterial production / [bacterial respiration + bacterial production]) during the 4 d incubations was calculated assuming that all consumed DOC not incorporated in bacterial biomass was respired (in other words, bacterial respiration = DOC consumed $-$ bacterial biomass production).

Ectoenzymatic activity—We measured the activity of the enzymes aminopeptidase and β-glucosidase, as indicators

of proteolytic and glycolytic activities. The procedures followed those already described elsewhere (Sala et al. 2010). In brief, ectoenzymatic activity was assayed by observing the release of fluorescent molecules from the fluorogenic substrates 4-methylumbelliferyl-β-D-glucoside (for β-glucosidase) and L-leucine-7-amido-4-methyl-coumarin (for aminopeptidase) added at a single final concentration (100 μmol L^{-1}). Fluorescence was measured before and after 1–3 h incubation with a Shimadzu RF-540 spectrofluorometer at λ = 446 nm excitation and λ = 365 nm emission wavelengths. Increases of fluorescence units during the incubation were converted to molar rates with a standard curve. Specific ectoenzymatic activities were calculated by dividing the activities by the number of bacterial cells.

Heterotrophic prokaryote community composition—Heterotrophic prokaryote community composition was examined by CARD-FISH and DGGE. For CARD-FISH, 3 mL samples were fixed overnight with 1% paraformaldehyde + 0.05% glutaraldehyde (final concentration) at 4°C and gently filtered on 0.2 μm polycarbonate filters (Millipore; GTTP, 25 mm diameter) and kept at −80°C until hybridization. After permeabilization with lysozyme and achromopeptidase, several filter sections were cut and hybridized for 2 h at 35°C with several horseradish peroxidase probes to characterize the composition of the heterotrophic prokaryote community, using the CARD-FISH protocol described in Pernthaler et al. (2004). All probes were purchased from www.biomers.net. Specific hybridization conditions were established by addition of formamide to the hybridization buffers (20% formamide for the NON338 probe, 45% formamide for the ALF968 and SAR11-441R probes, 50% for NOR5-730, 60% for Alt1413, and 55% for the other probes). Counterstaining of CARD-FISH preparations was done with DAPI (1 μg mL^{-1}). The slides were then inspected with an Olympus BX61 epifluorescence microscope. Between 500 and 1000 DAPI-positive cells were counted manually in a minimum of 10 fields. CARD-FISH positive cells (hybridized with the specific probe) appear in bright green under blue light excitation.

For DGGE, microbial biomass was collected by filtering ~ 150 mL of seawater through a 0.2 μm filter (Durapore; Millipore). The filters were stored in lysis buffer (50 mmol L^{-1} Tris-HCl pH 8.3, 40 mmol L^{-1} ethylenediaminetetraacetic acid [EDTA] pH 8.0, 0.75 mol L^{-1}sucrose) at −80°C. Filters were treated with lysozyme, proteinase K, and sodium dodecyl sulfate, and the nucleic acids were extracted with phenol and concentrated in a Centricon-100 (Millipore), as described in Schauer et al. (2000). The bacterial 16S ribosomal deoxyribonucleic acid (rDNA) was amplified by polymerase chain reaction (PCR) using primer set GC341F and 907RM (Muyzer et al. 1993; Muyzer 1998). Initial denaturation was at 95°C for 2 min, followed by a thermal cycling program as follows: denaturation for 30 s at 94°C; annealing for 30 s at an initial 63°C, decreasing 1°C every two cycles to a final of 53°C; extension for 90 s at 72°C. Ten cycles were run at 53°C for 30 cycles, followed by final 7 min of incubation at 72°C. The quality and size of the PCR products were verified by agarose gel electrophoresis and quantity was determined in QuantityOne (version 4.6.2; BioRad) by using GeneRuler| DNA ladder (Fermentas) as standard. Subsequently 200 ng of PCR product were analyzed by DGGE on a 6% polyacrylamide gel with a DNA-denaturant gradient ranging from 29% to 52% in the D Gene System (Bio-Rad) at 60°C for 6 h at 150 V in 1× TAE (40 mmol L^{-1} tris, acetic acid, EDTA, pH 7.4). The DGGE bands were excised using a sterile razor blade and eluted in 20 mL of Milli-Q water overnight at 4°C, followed by a freeze–thaw cycle. Five μL of the eluate was used for reamplification with the original primer set. A part of the PCR product was analyzed by DGGE together with the original sample to verify the correct position of the band, and in cases where more than one band was present the target band was processed again as described above. PCR products were purified with the QIAquick PCR-Purification Kit (Qiagen) and quantified fluorometrically (PicoGreen; Molecular Probes) and subsequently sequenced by Macrogen (Korea). Sequence quality was examined using DNAtools Xplorer (2.4.2) and identity determined by comparison with GenBank (National Center for Biotechnology Information) using BLAST (nucleotide - BLASTn). Sequencing of six DGGE bands was successful, from a total of 13 excised bands. Sequences obtained in this study were deposited in GenBank under the accession numbers JQ742966 to JQ742970.

Results

Phytoplankton production of DFAA (Phase I)—We observed production of extracellular DFAA in all the phytoplankton cultures (Fig. 1), but amounts and compositions varied among species. Although total DOC production was similar among the four microalgae, *Micromonas* released about twice as much DFAA as the others (Table 1).

Compositions of DFAA pools produced by the microalgal cultures were remarkably different from those found in Blanes Bay MO seawater (Fig. 2). Furthermore, each assayed algal species produced a different DFAA pool: histidine (His) was the dominant extracellular DFAA in *Skeletonema* and *Prorocentrum* filtrates (Fig. 2). In contrast, *Micromonas* released large amounts of the nonpolar (hydrophobic) amino acid alanine (Ala; Fig. 2). Polar (hydrophilic) threonine (Thr) and glutamine (Gln) were amongst the most abundant DFAA in all culture filtrates, except for *Skeletonema*. The other diatom (*Chaetoceros*) released mainly a mixture of nonpolar glycine (Gly) and polar Thr (Figs. 1, 2).

Bacterial consumption of the excreted DFAA (Phase II)—After 4 d of incubation with bacteria (T_2), total extracellular DFAA concentrations were significantly lower than those observed at the end of the production phase (T_1), illustrating net heterotrophic prokaryotic consumption of DFAA (Table 2). This was not the case with the unamended Blanes Bay MO seawater control, where the DFAA decrease during the incubation was not statistically significant (Table 2).

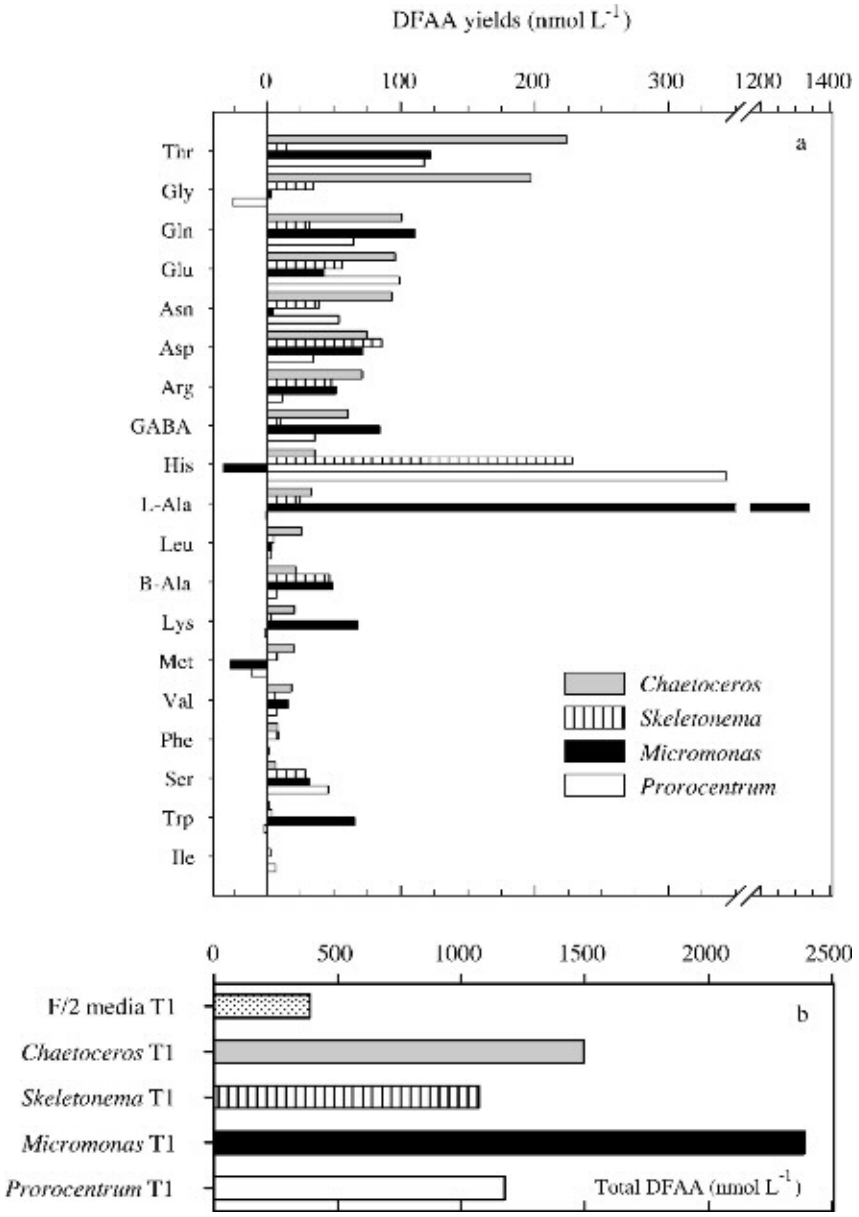

Fig. 1. (a) Yields of individual dissolved free amino acids (DFAA) produced by four microalgal species in F/2 medium ($T_1 - T_0$). Individual DFAA concentrations in sterile F/2 medium were subtracted from culture filtrates after 4 d incubations (except for *Prorocentrum minimum,* which was incubated for 7 d, *see* Methods). (b) Total DFAA pool sizes at the beginning of the experiment (i.e., F/2 media), and at the end of the phytoplankton growth phase (T_1), Phase I. *See* Fig. 2 for abbreviations.

The consumption rates of individual DFAA by heterotrophic prokaryotes were very different among algal exudates. The upper bar plots in Fig. 2 illustrate that the consumption rate of each individual DFAA over the 4 d incubations (dDFAA/dt) was roughly proportional to the initial DFAA pool sizes (shown by the circles in the same figures). The most abundant DFAA were generally consumed most rapidly (Fig. 2) in all algal exudate treatments, but this was not the case for the control Blanes Bay MO seawater (Fig. 2). Pearson product-moment correlations between pool size and net fluxes derived from concentration differences between T_2 and T_1 were significant in all treatments ($p < 0.0001$), except in the unamended Blanes Bay MO seawater control, where the DFAA consumption rates did not correlate with initial DFAA concentrations. These results indicate that the most abundant DFAA produced by microalgae during Phase I were also preferentially consumed by heterotrophic prokaryotes during Phase II. Apparently, rates of individual DFAA consumption were a response to resource availability.

Table 1. Summary of dissolved pool sizes and cell standing stocks throughout the experiments (average and standard deviation from triplicates where available, na stands for not available). DOC is dissolved organic carbon, BBMO is the Blanes Bay Microbial Observatory, T_0 corresponds to the beginning of the phytoplankton growth phase (Phase I), T_1 is the end of the phytoplankton growth phase prior to inoculation of a heterotrophic prokaryote natural community into the filtrates, T_2 correspond to the fourth day of Phase II (prokaryote consumption phase), and DFAA is dissolved free amino acids.

	BBMO control	*Chaetoceros*	*Skeletonema*	*Micromonas*	*Prorocentrum*
DOC (μmol C L^{-1})					
T_0	—	233.4	222.7	191.9	201.6
T_1	114.1	287.8	304.5	242.1	304.9
T_2	68.0(±0.9)	144.7(±4.4)	152.3(±15.6)	132.1(±4.7)	181.3(±2.0)
Algal abundance (10^3 cell mL^{-1})					
T_0	—	na	na	na	na
T_1	—	162(±52)	303(±21)	3342(±29)	12(±0.5)
T_2	—	0	0	0	0
Algal biomass (μg C L^{-1})					
T_0	—	na	na	na	na
T_1	—	990(±32)	2272(±158)	1975(±13)	1254(±136)
T_2	—	0	0	0	0
Bacterial abundance (10^5 cell mL^{-1})					
T_0	—	0	0	0	0
T_1*	0.16*	0.18*	0.10*	0.15*	0.13*
T_2	28.6(±1.4)	92.7(±3.9)	87.9(±4.7)	89.0(±5.5)	92.1(±6.6)
Bacterial biomass (μg C L^{-1})					
T_0	—	0	0	0	0
T_1*	0.33	0.41*	0.23*	0.35*	0.31*
T_2	66.4(±3.0)	218.3(±7.8)	207.1(±10.4)	210.7(±12.3)	213.6(±15.5)
Total DFAA (nmol L^{-1})					
T_0†	—	388.5†	388.5†	388.5†	388.5†
T_1	569.4	1496.8	1072.7	2388.5	1175.8
T_2	390.2(±33.6)	612.5(±32.4)	631.8(±124.5)	589.1(±22.3)	723.5(±11.9)
% of DOC attributed to DFAA					
T_0	—	0.7	0.7	0.8	0.8
T_1	2.2	2.2	1.6	3.7	1.9
T_2	2.6	1.9	1.9	1.9	1.7

* Values at T_1 immediately after adding the natural community inoculum.
† Values of the F/2 medium, before inoculating the algae.

During the consumption phase (Phase II), bacteria in the *Micromonas* treatments had the lowest specific rates of β-glucosidase (0.33 ± 0.01 fmol bac^{-1} h^{-1}) and the highest of aminopeptidase activities (51 ± 1.6 fmol bac^{-1} h^{-1}). These results yielded markedly higher ratios of aminopeptidase : β-glucosidase (AMA : β-GLU) for bacteria growing in *Micromonas* exudates (154 ± 1.8) than in those growing on other algal exudates (range = 50–96; Table 2), suggesting that *Micromonas* exudates were richer in peptides and relatively poor in polysaccharides.

Growth responses of bacterioplankton populations varied among treatments. Results from CARD-FISH analyses (Fig. 3) showed that *Rhodobacteraceae* bacteria were particularly responsive to *Prorocentrum* and *Skeletonema* exudates, while *Gammaproteobacteria* of the *Alteromonas* clade and Bacteroidetes increased in all exudates, but somewhat less in those produced by *Micromonas*. In the *Micromonas* filtrates, *Alpha-proteobacteria* dominated the prokaryotic community (Fig. 3). In the unamended Blanes Bay MO seawater control, the most relevant changes were the drop in the contribution of Bacteroidetes (from 36.2% to $< 1\%$), and a small increase in the proportion of *Roseobacter* (from 3.9% to 18.6%).

In agreement with the CARD-FISH results, DGGE analysis showed that the bacterial assemblage changed substantially in response to exudate source. For example, several bacterial populations present in the initial seawater sample disappeared (Fig. 4). Moreover, partial sequencing of the 16S ribosomal ribonucleic acid (rRNA) gene from excised DGGE bands identified two of the most dominant phylotypes arising in all exudates as belonging to the *Roseobacter* (*Nereida* sp. phylotype E2) and *Alteromonas* (*Alteromonas* sp. phylotype E8) groups (Fig. 4; Table 3). *Nereida* sp. phylotype E2 reached the highest relative abundance in the *Prorocentrum* and *Skeletonema* exudates. The less abundant *Roseobacter* group phylotypes E5, E10, and E11 differed in abundance among treatments, where phylotype E5 flourished most in *Micromonas* exudates. The less abundant *Alteromonas* phylotype E9 achieved a higher relative abundance in the *Skeletonema* filtrate. The

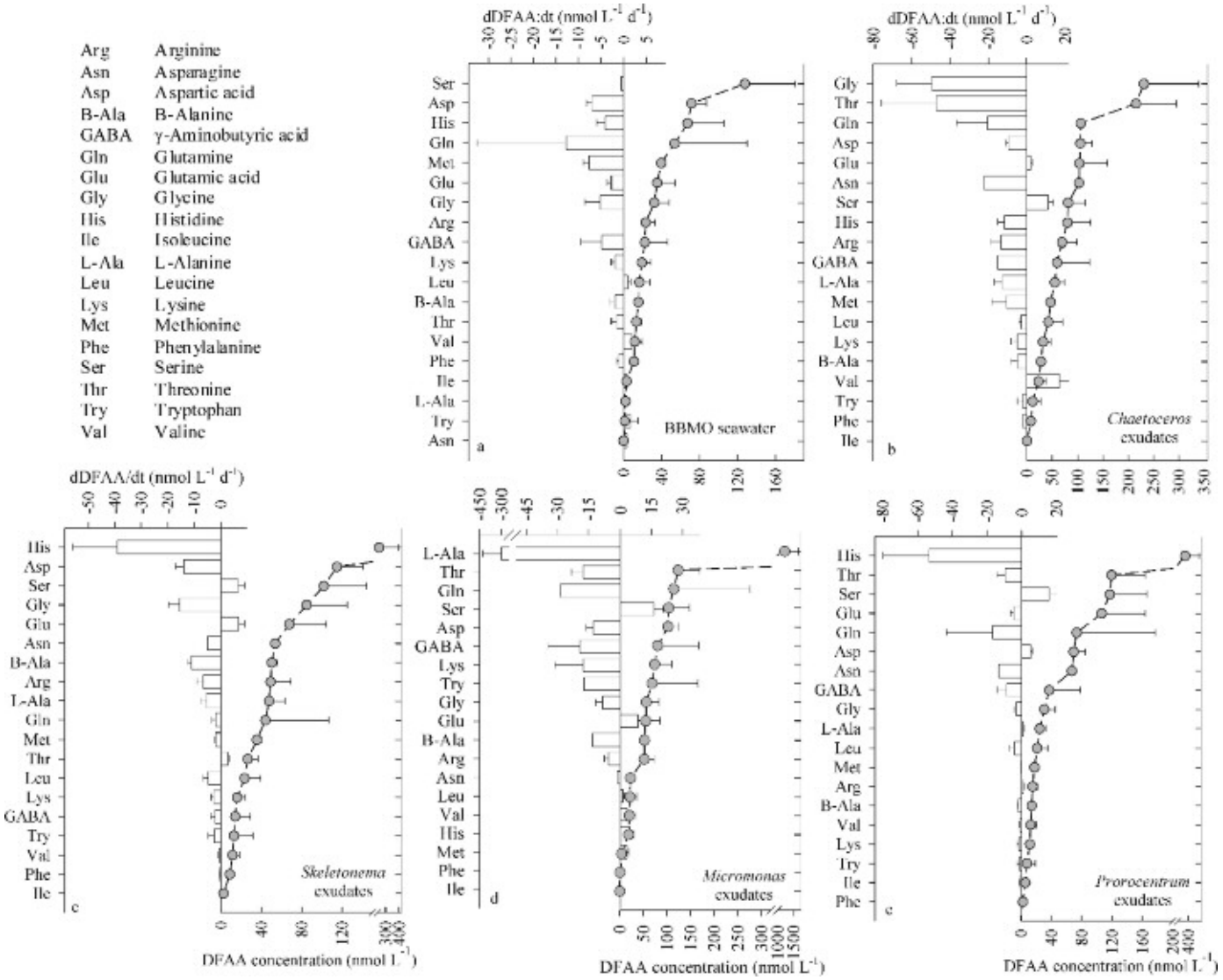

Fig. 2. Initial pool sizes of individual DFAA present in Blanes Bay microbial observatory (BBMO) seawater (right) compared with individual DFAA net fluxes (consumption) over 4 d incubations (left). (a) BBMO seawater, (b) *Chaetoceros* sp. exudates (c) *Skeletonema costatum* exudates, (d) *Micromonas pusilla* exudates, and (e) *Prorocentrum minimum* exudates.

presence of bands in the upper part of the DGGE gel suggested that Bacteroidetes phylotypes were also present, but verification of these bands by sequencing was not successful (Fig. 4).

Discussion

It is well-established that both phytoplankton and bacterioplankton assimilate (Keil and Kirchman 1999) and release (Myklestad et al. 1989; Kaiser and Benner 2008) DFAA during their metabolic activities. DFAA are a highly labile source of organic nitrogen and carbon for heterotrophic metabolism (Bronk 2002). Under some circumstances, DFAA can be synthesized in excess of biosynthetic demands and released to the medium, mainly during the photosynthetic process (Mague et al. 1980). By separating the autotrophic and heterotrophic microbial communities under controlled conditions, we could distinguish the net DFAA production by microalgae from the net DFAA bacterial consumption. We cannot account for possible DFAA production by prokaryotes in Phase II of the experiment; however, total DFAA consumption surpassed production. Similarly, we cannot account for phytoplankton uptake of DFAA during Phase I; if it took place, it certainly did so at a lower rate than DFAA production.

In a literature review, Myklestad (2000) ranked the most prominent DFAA released by different phytoplankton species, in culture and in mesocosm studies, as follows: serine (Ser), Gly, lysine (Lys), Ala, glutamic acid (Glu), aspartic acid (Asp), and His. In our study, the four axenic algal species released an altogether different combination of dominant DFAA: His, Ala, Gly, and Thr (Fig. 2). In the studies cited by Myklestad (2000), most cultures were not

Table 2. Summary of net production of dissolved organic carbon (DOC) and total dissolved free amino acids (DFAA; $T_2 - T_1$) among treatments; Specific aminopeptidase and β-glucosidase activities, and AMA : β-GLU ratios at the end of the consumption phase (T_2; mean and standard deviation of two replicate measurements, na stands for not available); Mass balances for the 4 d incubations ($T_2 - T_1$), bacterial growth efficiency = bacterial production : DOC consumed. An asterisk indicates that the concentration is significantly different ($p < 0.05$) from initial values. BBMO is the Blanes Bay Microbial Observatory.

	BBMO control	*Chaetoceros*	*Skeletonema*	*Micromonas*	*Prorocentrum*
DOC consumed (T_2-T_1) (μmol L^{-1})	−46.1±0.9	−143.1±4.4	−152.2±15.6	−110.0±4.7	−123.6 ±2.1
Total DFAA consumed (T_2-T_1) (nmol L^{-1})	−179.2±33.6	−884.3±22.9*	−440.9±124.5*	−1799.4±22.3*	−452.2±11.9*
Specific aminopeptidase activity (T_2) (fmol bac^{-1} h^{-1})	na	47.6±6.6	27.1±2.6	50.7±1.6	40.8±9.2
Specific β-glucosidase activity (T_2) (fmol bac^{-1} h^{-1})	na	0.50±0.08	0.54±0.02	0.33±0.01	0.57±0.10
Aminopeptidase:β-glucosidase (T_2)	na	96.1±1.5	50.2±6.8	154.3±1.8	71.2±3.3
Bacterial production (μmol L^{-1})	5.5±0.3	20.3±0.5	17.4±0.9	18.3±7	17.0±1.4
Bacterial respiration (μmol L^{-1})	40.6±1.3	122.8±3.9	134.8±15.8	91.7±5.0	106.6±2.4
Bacterial growth efficiency (%)	11.9±1.0	14.2±0.1	11.5±1.5	16.7±1.1	13.7±1.1

axenic, and the DFAA composition was probably the net result of competing algal production and prokaryotic consumption processes. The DFAA pool compositions reported in that review are actually more similar to what we found in filtered seawater than in our axenic phytoplankton cultures (Table 4).

The only similar experiments addressing DFAA composition in axenic culture filtrates were performed with two *Chaetoceros* species: *C. affinis* (Myklestad et al. 1989) and *C. debile* (Poulet and Martin-Jezequel 1983). Our strain was catalogued as *Chaetoceros* sp. (CCMP199), and it produced a DFAA pool distinctive from other species of *Chaetoceros: C. affinis* mainly released Gln, Glu, and Ser (Myklestad et al. 1989); while *C. debile* released phenylalanine (Phe) and NH_4^+ (could not be distinguished with the method used), Thr, and Ser (Poulet and Martin-Jezequel 1983). In both experiments, the individual DFAA concentrations were determined at different stages of the growth curve and, although the relative proportions changed, the ranking of the main DFAA released was constant. Our *Chaetoceros* culture mainly released Gly and Thr. The dominant DFAA concentrations were relatively low in all three independent experiments (200–400 nmol L^{-1}). These results suggest that different species of *Chaetoceros* produce different combinations of DFAA but at similar and relatively low amounts.

The only picoeukaryote used in our study (*Micromonas*) released a significantly larger amount of extracellular DFAA (Table 1) and also a significantly different DFAA pool compared with the other cultures of larger cells (*Chaetoceros*, *Prorocentrum,* and *Skeletonema*). *Micromonas* produced large amounts of Ala. This amino acid was

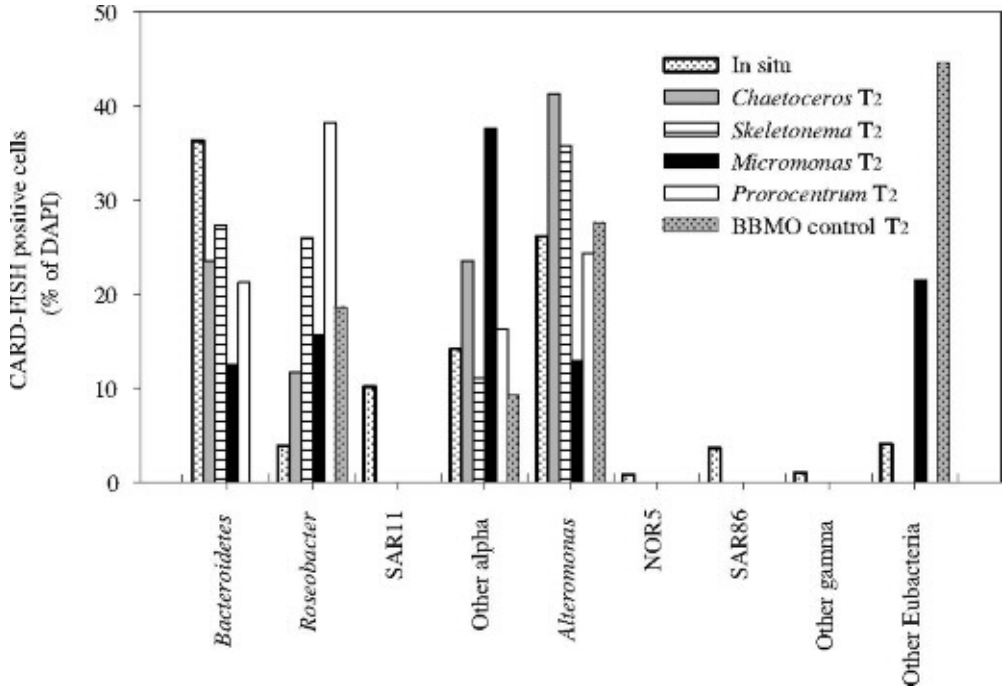

Fig. 3. Relative abundance of heterotrophic prokaryote groups, as enumerated by CARD-FISH, in the inoculum (initial heterotrophic prokaryote natural community), in the unamended BBMO seawater control, and 4 d later after growth on the different exudates (T_2).

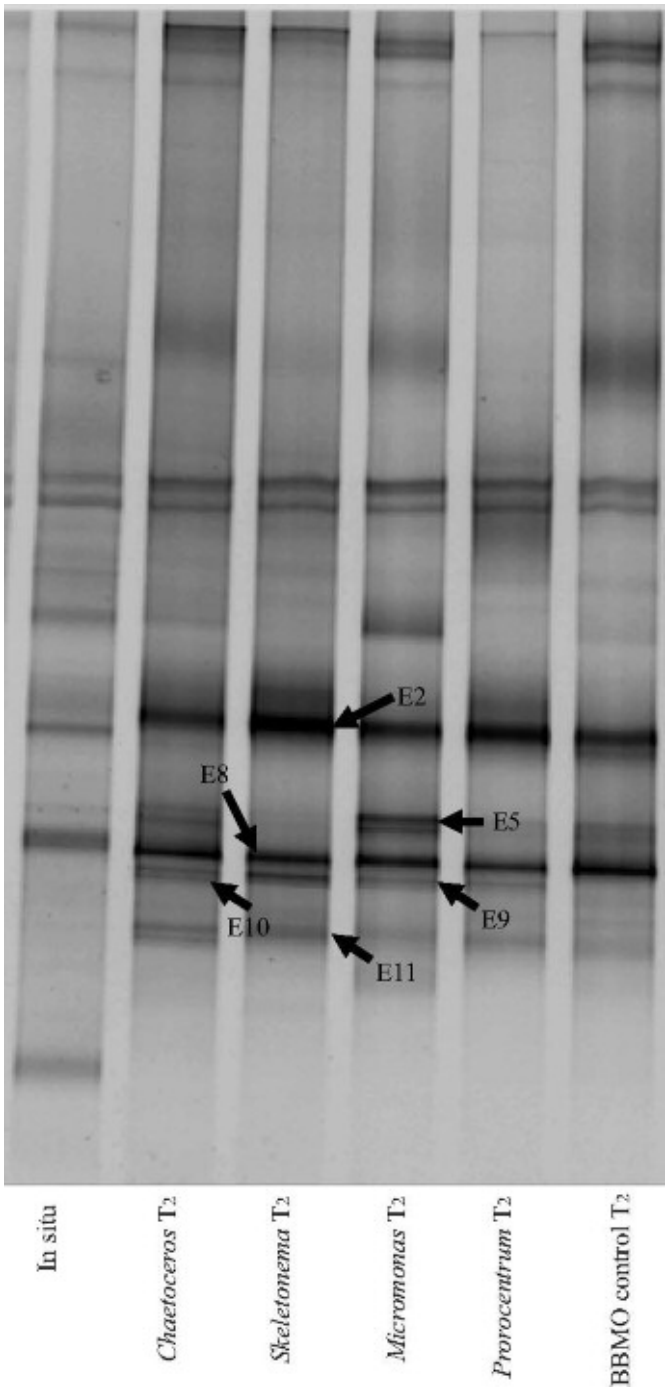

Fig. 4. DGGE profiles of the heterotrophic prokaryote community at T_0 and T_2 (at the end of the heterotrophic prokaryote consumption phase), with indication of excised and sequenced bands (*see* Table 3).

also the most common one observed in the oligotrophic northern Sargasso Sea (Keil and Kirchman 1999). Phytoplankton communities in the oligotrophic open-ocean are usually dominated by picoeukaryotes and cyanobacteria. We did not use any cyanobacteria in this experiment, but previous studies showed that cyanobacteria usually release much higher amounts of DFAA than other phytoplankton (Myklestad 2000), due to fundamental differences in metabolic pathways (Behrenfeld et al. 2008). Altogether, we have some indications that in oligotrophic regions, extracellular DFAA release per phytoplankton biomass unit might be higher than that of more productive coastal regions. If confirmed, this could partially explain the stronger coupling between phytoplankton and bacterioplankton in the open ocean compared with coastal regions (Morán et al. 2002). On the other hand, the lower extracellular release of small labile molecules (i.e., DFAA) by large phytoplankton (*Chaetoceros*, *Prorocentrum,* and *Skeletonema*) that we show here could partially explain the weaker coupling between phytoplankton and bacterioplankton observed in places where larger phytoplankton dominate, such as the Southern Ocean (Duarte et al. 2005).

Bacterial growth efficiencies (BGE) in marine systems typically range between 10% and 30% depending on nutrient regimes, substrate quality, and temperature (reviewed by del Giorgio and Cole 1998). Previously observed values at Blanes Bay MO range from 3% to 42% (Alonso-Sáez et al. 2008), encompassing values derived from the present study (Table 2). BGE appeared to co-vary with DFAA consumption (Table 2): heterotrophic prokaryotes in the *Micromonas* filtrates had the highest consumption of DFAA and the highest BGE, in contrast to those growing on *Skeletonema* filtrates that exhibited the lowest consumption of DFAA and the lowest BGE. In our experiment, the availability and consumption of DFAA were more useful for the interpretation of the BGE values than was bulk DOC availability. DFAA are likely to be a better indicator of available labile material and hence bear a more predictable relationship with BGE than do bulk DOC measurements. In marine systems, DOC is a complex, dynamic, and variable mixture of refractory, semi-labile, and labile organic substrates (Kirchman et al. 1993).

Patterns of ectoenzymatic activity can provide insights into the types of organic substrates being used in the system (Sala and Güde 1999). Ratios between different ectoenzyme activities are often used to compare processes (Sala et al. 2001). The AMA : β-GLU ratio has been used often as an indicator of the prevalence of proteolysis vs. saccharification in contrasting oceanic regions (Christian and Karl 1995; Misic et al. 2006). In the Blanes Bay MO, the annual range of AMA : β-GLU ratios is 4–39 (Alonso-Sáez et al. 2008), lower than the values observed in our experiment (between 50 for *Skeletonema* and 154 for *Micromonas*), suggesting that exudates were more enriched in nitrogenous polymers than the natural environment. Interestingly, *Micromonas* exudates stimulated activity of aminopeptidases more than glucosidases. This is also observed in situ in Blanes Bay MO during the winter months when *Micromonas* cells are a large fraction of the primary producers (Alonso-Sáez et al. 2008). It is plausible that algal cultures growing in nutrient-replete conditions could excrete more N-rich organic compounds, compared with natural nutrient-limited conditions, even during a bloom. It is known that DOC and dissolved organic nitrogen (DON) turnover rates are not necessarily coupled, and N-rich organic molecules (except DFAA that are a minor compo-

Table 3. Phylogenetic affiliation of the 16S rRNA gene sequences from excised denaturing gradient gel electrophoresis (DGGE) bands (identified in Fig 4).

DGGE band	Closest relative in GenBank	Accession number	% identity	Family (subgroup)	Class
E10	*Thalassobius aestuarii* strain D7019	FJ161326.1	95	*Rhodobacteraceae* (*Roseobacter* clade)	*Alphaproteobacteria*
E5*	*Uncultured marine* bacterium clone B-SW95	HM437646.1	79	*Rhodobacteraceae* (*Roseobacter* clade)	*Alphaproteobacteria*
E2	*Nereida ignava* strain MED479	FJ482233.1	99	*Rhodobacteraceae* (*Roseobacter* clade)	*Alphaproteobacteria*
E11	*Antarctobacter* sp. JLT354-W	EU734592.1	94	*Rhodobacteraceae* (*Roseobacter* clade)	*Alphaproteobacteria*
E9	Uncultured *Alteromonas* sp. isolate DGGE gel band M1-A3	HQ836378.1	96	*Alteromonadaceae*	*Gammaproteobacteria*
E8	Uncultured *Alteromonas* sp. isolate DGGE gel band M2-A7	HQ836401.1	99	*Alteromonadaceae*	*Gammaproteobacteria*

* Low-quality sequence.

nent of the total nitrogen pool) are not as extensively assimilated as DOC (Kirchman et al. 1991). A parallel experiment focused on fluorescent DOM showed that bacteria were using DFAA rather than amino acids in combined forms (Romera-Castillo et al. 2011), an observation that is consistent with our discussion of DOC and DFAA consumption. Another possible explanation is the fact that in our experiments, all the DOM was added at once, which is rather different from natural conditions where N-rich organic compounds are produced and removed continuously. Consequently, it is probable that field measurements could underestimate what is actually released.

The extremely high AMA : β-GLU ratios found in the *Micromonas* treatment indicate that bacteria growing on these exudates are less oriented toward the utilization of compounds containing only C (i.e., glucose), than toward N-containing carbon sources. In a previous experimental study of bacteria growing in exudates from several axenic phytoplankton cultures, stimulation of aminopeptidase was higher than β-glucosidase activity (Grossart 1999). However, that study included only diatom species and three bacterial strains growing in the same medium as the algae, and is thus not directly comparable to our study. Laboratory experiments have obvious limitations when it comes to comparisons with field data, especially using bulk approaches that treat the DON as a single pool. Our findings support the view that it is important to discriminate among the different DON forms, primarily DFAA, in experiments of microbial degradation.

The bacterioplankton phylogenetic groups targeted by CARD-FISH probes grew in different proportions among the treatments, indicating that these groups differentially responded to varying components of the DOM pool (Fig. 3). Considering the experimental procedure used, we cannot discern whether increases in different bacterial populations were due to different initial concentrations of particular DFAA or to general differences in the DOC from the algal species investigated. However, it seems

Table 4. Total dissolved free amino acids (DFAA) concentration and identity of the dominant individual DFAA in filtered estuarine or seawater from the literature compared with residuals after 4 d incubations in this study (except for Blanes Bay MO, which are values of in situ filtered seawater).

Location	Total DFAA concentration (nmol L^{-1})	Dominant individual DFAA	Reference
Delaware estuary	50–1400	Gly, Asp, Ser, Glu, Ala	Coffin 1989
Northern Sargasso Sea	3–9	Ala, Gly-Thr, Ser	Keil and Kirchman 1999
North Atlantic Ocean	4–132	Gly, Ala, Glu, Ser, Asp	Kuznetsova et al. 2004
Kysing Fjord estuary (North Sea)	200–2500	Ser, Glu, Gly, Ala	Jørgensen 1982
Northern North Sea	100–300	Ser, Gly, Glu, Ala	Hammer and Kattner 1986
Arctic waters	500(±140)	Gly, Asp, Ala, Ser, Glu	Hubberten et al. 1995
Antarctic waters	440(±160)	Gly, Ala, Ser, Glu, Asp	Hubberten et al. 1995
Florida Coastal Everglades estuary	na	Gly, Ala, Asp, Ser, Thr	Maie et al. 2006
Blanes Bay MO (northwestern Mediterranean Sea)	351–412	Ser, Asp, His, Gln	this study
Chaetoceros filtrates at T_2	612(±32)	Ser, Glu, Val, Asp	this study
Micromonas filtrates at T_2	589(±22)	Ser, Glu, Thr, Asp	this study
Prorocentrum filtrates at T_2	723(±12)	Ser, His, Asp, Glu, Thr	this study
Skeletonema filtrates at T_2	631(±124)	Ser, His, Glu, Asp	this study

reasonable to assert that the successful bacteria (e.g., *Roseobacter* and *Alteromonas*) participated in the quantified consumption of the most abundant DFAA in this experiment.

The CARD-FISH results indicated high abundance of *Gammaproteobacteria* (especially *Alteromonas*), *Alphaproteobacteria* (mainly *Rhodobacterales*), and Bacteroidetes growing on phytoplankton exudates. However, compared with the inoculum, *Roseobacter* was the group that increased the most, as it had in previous experimentally induced phytoplankton blooms in Blanes Bay MO (Allers et al. 2007). *Alteronomanadales*, *Rhodobacterales,* and Bacteroidetes are known to appear associated to high chlorophyll levels and algal blooms (Fuhrman and Hagström 2008). However, not all these groups respond similarly to the different sources of organic matter: *Rhodobacterales* tend to prefer low-molecular-weight substrates, while Bacteroidetes tend to avoid them (Cottrell and Kirchman 2000). In nature, the Bacteroidetes seem to be the group most responsive to high chlorophyll *a* levels (Wietz et al. 2010), but the response of the different groups seems to depend on the type of substrate dominating in each bloom. As an example, in nutrient-amended marine mesocosms that elicited modest phytoplankton growth, an initial *Alteromonadaceae* response was observed, and was superseded by a *Rhodobacterales* response coincident with maximum chlorophyll *a* values (Allers et al. 2007). In this case, the *Alteromonas* response was assigned to a response to mesocosm filling and water manipulation rather than as a response to the algae.

The DGGE analysis provided further detail of the identity of the bacteria growing in the exudates. In a general way, the identified DGGE bands agreed with the CARD-FISH results, although sequencing of bands belonging to the Bacteroidetes was unsuccessful. The finding that individual phylotypes differed in relative abundance among treatments indicated that differences in initial chemical conditions induced changes in bacterial community structure.

Interestingly, some bacterial groups, such as SAR11, SAR86 and NOR5, were practically absent at the end of the experiment (Fig. 3). The collapse of SAR11 and SAR86 populations was not surprising. It is well-known that these phylotypes are well-adapted to oligotrophic conditions and are hardly encountered in enriched conditions (Eilers et al. 2000; Rappé et al. 2002). However, the NOR5 clade, usually associated with high phytoplankton biomass (Yan et al. 2009), could have increased in abundance during our incubations. One possible explanation for the fact that NOR5 did not grow is that this clade develops only in association with certain phytoplankton species. Although not established, there are some independent reports of high NOR5 abundances in association with dinoflagellate blooms (Yan et al. 2009). There are also reports of members of NOR5 growing attached to phytoplankton cells (Fuchs et al. 2007). In Phase II of our experiments no phytoplankton cells were present, and this could have limited the growth of NOR5 members if they follow that life strategy. Using the same phytoplankton strains, Sarmento and Gasol (2012) detected the use of *Prorocentrum minutum*–derived DOC by NOR5 in short incubations (5 h), but no significant growth of this bacterial group was observed in longer incubations.

The *Alphaproteobacteria* retrieved from the excised DGGE bands belonged to the *Roseobacter* clade, which is well-targeted by the ROS537 probe in the CARD-FISH counts. Among the four responsive *Roseobacter* clade phylotypes, the most dominant phylotype E2 was closely related to the characterized species *Nereida ignava* that preferentially grows on complex media rather than monomeric substances (Pujalte et al. 2005). The *Alteromonas* phylotype E8 that dominated all filtrates was nearly identical to a phylotype from the Mediterranean Sea, which recently was shown to be a generalist that rapidly forms abundant populations in response to many different carbon compounds (Gómez-Consarnau et al. 2012). A recent study used microautoradiography to quantify the preferences of the major heterotrophic prokaryote phylogenetic groups on DOC derived from the same algal strains used in this experiment, and also found a large proportion of *Gammaproteobacteria* (especially *Alteromonas*) using diatom exudates, and mainly *Alphaproteobacteria* taking up *Micromonas*-derived DOC (Sarmento and Gasol 2012).

The most common extracellular DFAA in the sea seems to be Ser (appearing at or near the top of DFAA lists in all studies), Gly, and Ala (both appearing in seven out of eight studies presented in Table 4). The residual DFAA composition after 4 d of Phase II incubations was similar to that found in the natural environment (Table 4), and very different from the DFAA pools observed after the production phase (Phase I). This points toward varying degrees of lability among individual DFAA, and ultimately some selectivity in the uptake or preferential extracellular release by heterotrophic prokaryotes. Interestingly, Ser concentration increased systematically during Phase II in all treatments, except for Blanes Bay MO control. This increase during the incubations could be related to bacterial activity and could explain the relatively high concentrations observed in situ, compared with the axenic phytoplankton cultures.

In summary, our findings indicate that the extracellular release of DFAA by phytoplankton is species-dependent: the amount and composition of the DFAA pool was highly variable among four phytoplankton species grown under identical conditions. The picoeukaryote *Micromonas* released significantly higher amounts of DFAA to the medium, especially Ala, which has been reported as the dominant individual DFAA in some oligotrophic environments (Keil and Kirchman 1999) where picoeukaryotes are a large component of the phytoplankton community (Worden et al. 2004). In our experiments, the heterotrophic prokaryote community structure shifted in response to the varying compositions of the released organic matter. DFAA availability and consumption explained more of the variance in observed BGE values among treatments than the bulk DOC pool itself. This fact emphasizes the need for devoting more effort to determining the sources and the quality of organic matter in order to better

understand and predict carbon pathways in marine systems.

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