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Bacterial Colonization and Ectoenzymatic Activity in Phytoplankton-Derived Model Particles: Cleavage of Peptides and Uptake of Amino Acids

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ABSTRACT

Phytoplankton-derived model particles were created in laboratory from a mixture of autoclaved diatom cultures. These particles were colonized by a marine bacterial community and incubated in rolling tanks in order to examine the relationship between aminopeptidase activity and leucine uptake. Bacteria inhabiting particles and ambient water were characterized for abundance, biovolume, aminopeptidase activity, leucine uptake, and growth rate. Particles were a less favorable habitat than ambient water for bacterial growth since growth rates of particle-attached bacteria were similar or even lower than those of free-living bacteria. During the first ~100 h of the particle decomposition process, there were not statistically significant differences in the aminopeptidase activity:leucine uptake ratio between attached and free-living bacteria. From ~100 h to ~200 h, this ratio was higher for attached bacteria than for free-living bacteria. This indicates an uncoupling of aminopeptidase activity and leucine uptake. During this period, attached and free-living bacteria showed similar hydrolytic activities on a cell-specific basis. In the free-living bacterial community, variations in aminopeptidase activity per cell were associated with variations in leucine uptake per cell and growth rates. However, in the attached bacterial community, when leucine uptake and growth rates decreased, aminopeptidase activity remained constant. Thus, after ~100 h, particle-attached bacteria were not taking advantage of their high aminopeptidase activity; consequently the hydrolysed amino acids were released into the ambient water, supporting the growth of free-living bacteria. These results demonstrate that over the particle decomposition process, the relationship between hydrolysis and uptake of the protein fraction shows different patterns of variation for attached and free-living bacterial communities. However, in our experiments, this uncoupling was not based on a hyperproduction of enzymes by attached bacteria, but on lower uptake rates when compared to the free-living bacteria.

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Introduction

A large fraction of the particulate matter present in marine systems consists of macroscopic aggregates also known as "marine snow" [2]. These flocculent amorphous aggregates are of mainly phytoplanktonic origin and may develop from aggregation of senescent diatom cells and/or coagulation of colloidal organic matter released by phytoplankton [14, 18, 33]. These settling particles are thought to play a vital role in the transfer of carbon and energy from the euphotic layers of the ocean to the deep sea bed [5, 10].

Amorphous aggregates are ubiquitous components in the ocean, but they are not easily sampled due to their fragile structure [2]. The devices designed for routine sampling in oceanography create turbulence and destroy these delicate structures [42]. The preferential method of collecting aggregates without disturbance is "*in situ* " sampling by SCUBA divers. However, this technique is quite time-consuming if large amounts of aggregates are required. Moreover, a certain volume of ambient water is always collected with the aggregates. Because of these constraints, experimental systems to create phytoplankton-derived model particles in the laboratory, under controlled conditions, constitute a useful alternative for in-depth analysis of the complex interactions between microbial communities and particulate matter [7, 24, 35, 36, 40, 45].

Compared to ambient water, marine aggregates are enriched in both microbial biomass and organic carbon [2]. However, several studies have shown that sinking organic particles are generally poor habitats for bacterial growth [4, 20]. Moreover, measurements of heterotrophic bacterial activity in aggregates indicate that it would take months to years to metabolize the carbon content of the aggregates [3]. These results suggest that particle-attached bacteria may not be responsible for the loss of particulate organic carbon (POC) throughout the water column [8]. Thus, marine aggregates would act as refractory carriers of carbon to the deep ocean. However, it has been reported that particle attached bacteria exhibit high hydrolytic activities [21, 39, 40], but do not take advantage of the low molecular weight compounds produced from their ectoenzymatic activity. Consequently, this uncoupling between hydrolysis and uptake could potentially lead to the release of a large fraction of dissolved organic matter into the ambient water, where it could be available to free-living bacteria [8, 27, 39, 40]. Nevertheless, the results obtained in this respect are contradictory, because other authors did not find differences in the ectoenzymatic activity of the attached and free-living bacteria; they, therefore, suggested that there is no uncoupling between ectoenzymatic activity and uptake of substrates [28].

The aim of this study was to analyze the relationship between the hydrolysis and uptake of the protein fraction during the decomposition of organic particles, created in the laboratory from autoclaved phytoplankton cultures placed in rolling cylinders.

Materials and Methods

Four experiments were carried out to characterize the microbial communities and the concentration of free amino acids (FAA) in the particles and the ambient water, throughout the decomposition process of the phytoplankton-derived model particles in laboratory microcosms (13 liters). We simultaneously analyzed microcosms without particles. The comparison between the metabolic activity of bacteria inhabiting the ambient water in the presence and absence of particles allowed us to investigate the effect that particles have on the growth of free-living bacteria.

Formation of Phytoplankton-Derived Model Particles

A mixture of four species of diatoms (*Chaetoceros muelleri* C.C.A.P.1010/3, *Nitzschia epithemoides* C.C.A.P.1052/18, *Navicula hanseni* C.C.A.P.1050/8, and *Skeletonema costatum* C.C.A.P.1077/1B) commonly found in marine aggregates [32] and in the Bay of Biscay [29] was used to obtain model particles. Batch cultures were grown on Guillard's medium for diatoms (f/2 + Si) [12] at 15°C with shaking, aeration and light (125 μE m^{-2} s^{-1}, 16 h light-8 h dark). Growth was characterized as algal abundance and dissolved organic carbon (DOC) to determine the optimum conditions for the cultures to be harvested. Algal cells and extracellular products were collected in stationary phase. In order to separate the particulate material (algal cells) from the dissolved material (extracellular products), cultures were centrifuged (2,100 × g, 20 min). The pellet was harvested and the supernatant was filtered onto 0.2-μm pore size polycarbonate filters (Millipore) to separate particulate material.

The cells and extracellular products of the four diatom species were mixed so that the contribution of each species to the final algal abundance and DOC concentration in the mixture was the same. Model particles were formed in rolling tanks according to the experimental design proposed by Shanks and Edmonson [35]. Five-liter bottles were filled with artificial seawater (Sigma) and a mixture of algal cells and extracellular products (10^9 cells 1^{-1} and 420 μM DOC 1^{-1} final concentrations), and then autoclaved. After 2–3 d rolling at 2.5 rpm, the particles formed were carefully withdrawn from the bottles and transferred to tanks (13 liter capacity) containing 12.5 liters of autoclaved artificial seawater in order to maintain an air headspace. The final concentration of DOC at the

beginning of the experiments was 1.5–2 mg l^{-1}, within the range observed in the Bay of Biscay, Spain (see below). The microcosms without particles were prepared with artificial seawater and extracellular products, in order to reach the same concentrations of DOC, but without transferring particles.

The bacterial assemblage used as inoculum was obtained by tangential flow filtration (100,000 Da, Nominal Molecular Weight Limit, Millipore) from coastal waters in the Bay of Biscay, 43° 24.5′ N 3° 2.7′ W (North Spain). At the beginning of the experiments, bacterial abundance in the tanks was adjusted to that found in natural seawater (10^5–10^6 bacteria ml^{-1}).

Microcosms were incubated in the dark at room temperature, while rolling at 2.5 rpm. During the decomposition period (0–300 h), subsamples of particles and ambient water were withdrawn to characterize the bacterial and flagellate abundance, bacterial biovolume, aminopeptidase activity, leucine uptake, bacterial production, and FAA concentration. Subsamples of ambient water were removed by micropipette. Particles were carefully collected by using a glass pipe with a plastic pipe at the end, and transferred, without disruption, to Petri dishes containing sterile artificial seawater. Precise volumes of particles without ambient water were collected with micropipettes by placing the tip directly on the particles.

Bacterial Counts

Three subsamples of particles (7 µl in 1 ml of sterile artificial seawater) and one sample of ambient water (10 ml) were taken and fixed with formalin (2% v/v final concentration). Bacterial abundance was measured by acridine orange epifluorescence direct counting (AODC) [15]. Immediately before staining, samples were sonicated (100 W, 6 pulses of 5 s) to disperse the particles [44]. Subsamples were stained with acridine orange (0.01% w/v final concentration) for 2 min, and filtered using 0.2-µm pore size black Millipore polycarbonate filters. The wet filters were placed on microscope slides and mounted in low-fluorescence immersion oil. The filters were examined under a Nikon epifluorescence microscope, at a magnification of 1,250×. Bacteria present in at least 30 randomly selected fields, with 20–30 bacteria per field, were counted.

Bacterial abundance in particles was not estimated during the first 12 h of the experiments, because there were dead bacteria attached to the particles (originating from the phytoplankton cultures), and it was not possible to distinguish dead from living bacteria (originating from the inoculum). After 12 h, the number of bacteria in particles increased significantly as a result of colonization and growth; therefore, the abundance of dead bacteria became negligible.

Flagellate Counts

Three replicates of particles (7 µl in 1 ml of sterile artificial seawater) and ambient water (10 ml) were taken. Diamidino-phenylindol (DAPI)-stained preparations for epifluorescence microscopy [31] were used for protistan enumeration. Alkaline Lugol (10 g of I_2, 20 g of IK and 10 g of sodium acetate in 140 ml of distilled water; 0.5% v/v final concentration)–formalin (3% v/v final concentration) [37] preserved subsamples were stained with DAPI (0.2-µg ml^{-1} final concentration) for 7 min and filtered on 0.8-µm pore-size prestained (Irgalan black, 0.2% w/v in 2% v/v acetic acid for 24 h) polycarbonate filters. The wet filters were placed on microscope slides and mounted in low-fluorescence immersion oil. The filters were examined through a Nikon epifluorescence microscope. Heterotrophic flagellate counts were made under UV light at a magnification of 1,250×.

Bacterial Biovolume

Bacterial biovolume was determined from acridine orange–stained samples. Cell size was calibrated with a stage micrometer, at a magnification of 1,250×, on a Nikon epifluorescence microscope, equipped with a video camera (Hamamatsu 2400) and a semiautomatic analysis system (VIDS IV). To calculate bacterial biovolume, bacteria were classified as spheres (cocci) or cylinders with hemispherical caps (rods). The diameter (cocci) and the maximum length and width (rods) of 200 attached bacteria and 200 free-living bacteria were measured.

Aminopeptidase Activity

Ectoenzymatic aminopeptidase activity was measured using a fluorogenic substrate [16]. L-leucyl 4-methylcoumarinyl-7amide was added to triplicate subsamples of particles (10.5 µl in 3 ml of sterile, artificial seawater) and ambient water (3 ml) at saturating concentrations (350 µM) determined in previous studies. Subsamples were incubated in the dark at room temperature in a rotatory shaker (120 rpm). The increase of fluorescence caused by the enzymatic cleavage of 7-amino-4-methylcoumarine (MCA) over 30–60 minutes was measured with a Perkin Elmer LS 50B spectrofluorometer, at 360 nm excitation and 445 nm emission. Relative fluorescence units were calibrated with 100 µM MCA standards. Subsamples without substrate were used as blanks to determine the background fluorescence of the samples. Previous experiments showed that abiotic hydrolysis of the substrate was not significant. Aminopeptidase activity per cell was estimated by dividing aminopeptidase activity by the bacterial abundance.

^{14}C-Leucine Uptake

^{14}C-leucine uptake was determined in triplicate subsamples of particles (17.5 µl in 5 ml of sterile artificial seawater) and ambient water (5 ml). [U-^{14}C] leucine (313 mCi $mmol^{-1}$, Radiochemical Centre, Amersham, England) was added at saturating concentrations determined in a previous study: 0.24-µM for the ambient water and 1.8 µM for the particles. Subsamples were incubated in the dark for 2 hours at room temperature in a rotatory shaker (120 rpm). After incubation, H_2SO_4 (final concentration, 0.02N) was

added through the cap in order to stop the incubation and to trap the CO_2 respired in the wicks containing phenethylamine. After 12 hours, the wicks were placed in scintillation vials and radioassayed by liquid scintillation counting (liquid scintillation spectrofluorometer Tri-Carb 2000CA, Packard Instrument Co., Inc.). The subsamples were filtered through 0.2-μm pore size filters (applying a vacuum pressure of 150 mm Hg). The filters were rinsed three times with 5 ml of distilled water, placed in scintillation vials, and radioassayed by liquid scintillation counting. These measurements represented the assimilated ^{14}C-leucine. Formaldehyde-killed controls (2% final concentration) were processed in a similar manner. Leucine uptake per cell was estimated by dividing leucine uptake (assimilation plus respiration) by the bacterial abundance.

Bacterial Production

Bacterial production was estimated from the uptake of [methyl-^{3}H]thymidine [11]. Triplicate subsamples of particles (17.5 μl in 3 ml of sterile artificial seawater) and ambient water (3 ml) were incubated with 20 nM of [methyl-^{3}H]thymidine (80–90 Ci mmol^{-1}, Radiochemical Centre, Amersham, England). Subsamples were incubated in a rotatory shaker (120 rpm) for 1 h at room temperature, in the dark. The saturating thymidine concentration and the appropriate incubation time were previously determined. After incubation, subsamples were chilled on ice and 3 ml of ice-cold 10% (w/v) trichloroacetic acid (TCA) was added to each subsample. The mixtures were kept on ice for 5 min and then filtered through 0.2-μm membrane filters (applying a vacuum pressure of 150 mm Hg). The filters were rinsed five times with 3 ml of ice-cold 5% (w/v) TCA, dried, and placed in scintillation vials with 1 ml of ethyl acetate. After 20 minutes, 10 ml of scintillation liquid (BCS, Amersham) was added to the vials and radioassayed by liquid scintillation (liquid scintillation spectrofluorometer Tri-Carb 2000CA, Packard Instrument Co., Inc.). Formaldehyde-killed controls (2% final concentration) were processed in a similar manner.

Bacterial cell production was calculated from thymidine incorporation by using the conversion factor: 1.1×10^{18} cell mol^{-1} [34]. Bacterial cell production was transformed into bacterial biomass production by multiplying the biovolume with the conversion factor 0.38 g C cm^{-3} [25]. Bacterial turnover rates were estimated by dividing bacterial cell production by the bacterial abundance.

Chemical Analyses

Subsamples of particles (70 μl in 100 ml of deionized water) and ambient water (100 ml) were taken in order to determine the FAA concentration, according to Parsons et al. [30]. This technique actually measures the concentration of primary amines and, therefore, the FAA concentrations are overestimated because they include ammonium. The concentration of organic carbon was estimated according to the technique proposed by Sugimura and Suzuki [41], using an organic carbon analyzer (Shimadzu TOC-5000/Shimadzu ASI-5000).

Results

Attached Bacteria versus Free-Living Bacteria

Particles were round-shaped, and their size and number varied over time. The diameter of the particles at the start of the experiments was 2 ± 0.4 mm (mean ± SE), and the initial concentration was about 200 particles L^{-1}. During the first 50 h, particles collided, and consequently, increased their diameter up to 8 ± 2.1 mm (mean ± SE), while the number of particles decreased to approximately 4 particles L^{-1}. From 50 to 100 h, particle size decreased to the initial values, and remained constant until the end of the experiment.

The development of bacterial and flagellate abundance, aminopeptidase activity, ^{14}C-leucine uptake, bacterial production, and FAA concentration during the incubations followed a similar trend in four decomposition experiments. Therefore, only one of the experiments was used to illustrate changes in variables over time. Mean values ± standard errors, ranges, and statistical analysis, however, are given for all the experiments. Phytoplankton-derived particles were rapidly and heavily colonized by bacteria. During the first 20 h of the experiments, bacterial abundance in particles reached maximum values of 10^9–10^{10} bacteria ml^{-1} of particle. In ambient water, bacterial abundance was three orders of magnitude lower than in particles, with maximum values within the initial 50 h of the experiments (Fig. 1A). Flagellates showed maximum abundances of 10^4 flagellates ml^{-1} in ambient water, and 10^5–10^6 flagellates ml^{-1} in particles (Fig. 1B).

In the four experiments, bacterial biovolume ranged from 0.104 μm^3 to 0.886 μm^3 for ambient water, and from 0.168 μm^3 to 0.775 μm^3 for particles. Bacteria colonizing particles and ambient water did not show significant differences in biovolume (Wilcoxon signed-rank test, $P > 0.05$). In some experiments, bacterial biovolume was similar for attached and free-living bacteria, while in other experiments, attached bacteria were bigger than free-living bacteria. Differences were not statistically significant.

Bacterial turnover rates among the four experiments ranged from 0.002 h^{-1} to 0.216 h^{-1} in ambient water, and from 0.0005 h^{-1} to 0.117 h^{-1} in particles. Over the incubation time particle-attached bacteria showed similar, and even lower turnover rates than free-living bacteria. From 0 h to ~70 h, bacteria colonizing particles and ambient water did not exhibit statistically significant differences in aminopeptidase activity per cell or leucine uptake rate per cell (Wilcoxon signed-rank test, $P > 0.05$) (Fig. 2). Two ratios

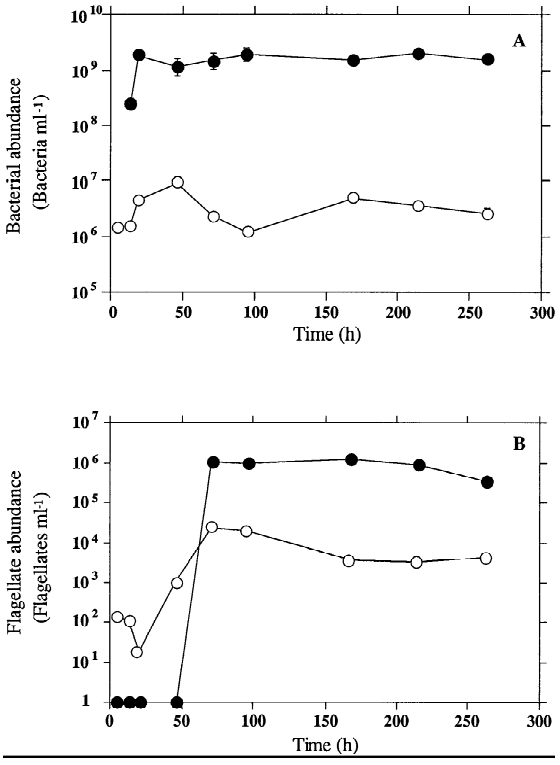

Fig. 1. Bacterial (A) and flagellate abundance (B) in particles (●) and ambient water (○) during the decomposition process. Bars represent standard errors of three replicate subsamples.

were calculated, aminopeptidase activity:bacterial production and aminopeptidase activity:leucine uptake. The comparison of these two ratios between attached and free-living bacteria allowed the determination of whether hydrolysis of macromolecules and uptake of the hydrolysate were uncoupled processes. From the beginning of the experiments to ~70 h, particle-attached bacteria and free-living bacteria did not show statistically significant differences in these ratios (Wilcoxon signed-rank test, $P > 0.05$; Fig. 3).

From ~70 h to ~100 h, attached bacteria showed an increase in the aminopeptidase activity and leucine uptake per cell, as well as in turnover rates. After ~100 h, attached bacteria showed a decrease in their leucine uptake per cell and turnover rates, although their aminopeptidase activity per cell remained constant until ~200 h (Fig. 2). After ~100 h, free-living bacteria showed an increase in their aminopeptidase activity and leucine uptake on a cell-specific basis, as well as their turnover rates. From ~100 h to ~200 h, the aminopeptidase activity:bacterial production ratio was significantly higher for bacteria inhabiting particles than for free-living bacteria (Wilcoxon signed-rank test, $P < 0.05$; Fig. 3A). The aminopeptidase activity:leucine uptake ratio was also higher for attached bacteria during the same period (Wilcoxon signed-rank test, $P < 0.05$; Fig. 3B).

In our experiments, aminopeptidase activity was correlated with bacterial abundance both in ambient water ($r^2 = 0.909$, $P < 0.05$) and in particles ($r^2 = 0.952$, $P < 0.05$). However, we did not find significant correlation between aminopeptidase activity and flagellate abundance.

The FAA concentration was 3 orders of magnitude higher in particles than in ambient water (Fig. 4). In particles, the FAA concentration decreased from the beginning to the end of the experiments. In ambient water, the concentration of FAA decreased from the beginning to ~100 h, and then increased until ~200 h.

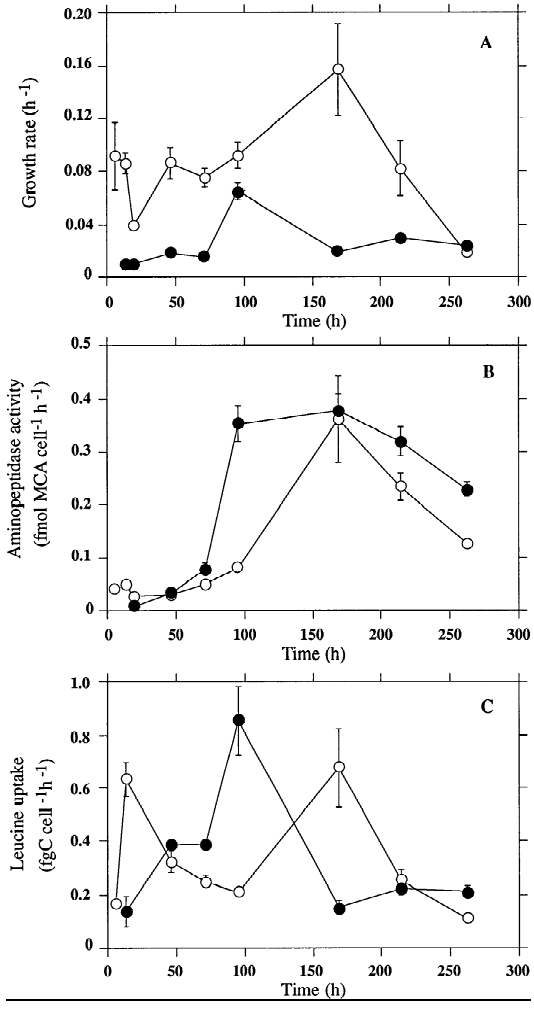

Fig. 2. Bacterial growth rate (A), aminopeptidase activity per cell (B), and leucine uptake per cell (C) for attached (●) and free-living bacteria (○) during the decomposition process. Bars represent standard errors of three replicate subsamples.

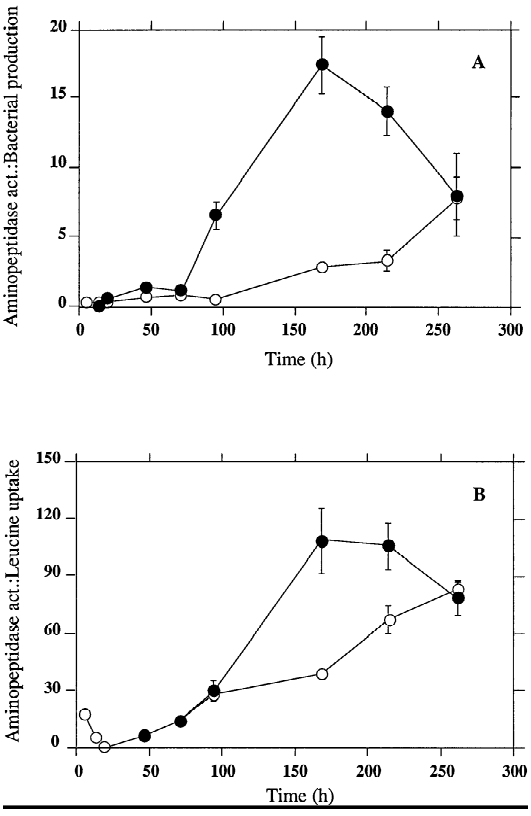

Fig. 3. Aminopeptidase activity:bacterial production (A) and aminopeptidase activity:leucine uptake (B) ratios for attached (●) and free-living bacteria (○) during the decomposition process. Bars represent standard errors of three replicate subsamples.

Free-Living Bacteria in the Presence and Absence of Particles

Bacteria growing in the absence of particles reached the same abundance as in the presence of particles. Bacterial biovolume was higher in the presence of particles (Wilcoxon signed-rank test, $P < 0.05$). From ~100 h to ~200 h, free-living bacteria increased their biovolume in the presence of particles. Without particles, no increase was detectable (Fig. 5A). Flagellate abundance was similar in the presence and absence of particles (10^4 flagellates ml^{-1}).

When comparing turnover rates of free-living bacteria in the presence and absence of particles, two time periods should be considered: The first period, from 0 h to ~100 h, without significant differences; and the second period, from 100 h to 200 h, with higher turnover rates in the presence of particles (Wilcoxon signed-rank test, $P < 0.05$; Fig. 5B).

Aminopeptidase activity per cell, in the presence and absence of particles was not significantly different over the incubation period (Wilcoxon signed-rank test, $P > 0.05$; Fig. 5C). Leucine uptake rates per cell were similar during the first 100 h, but from ~100 h to ~200 h they were higher in the microcosm with particles (Wilcoxon signed-rank test, $P < 0.05$; Fig. 5D). Both ratios, aminopeptidase activity:bacterial production and aminopeptidase activity:leucine uptake, were similar in the presence and absence of particles during the first ~100 h, but from ~100 h to ~200 h, they were higher in the microcosms without particles (Wilcoxon signed-rank test, $P < 0.05$; Fig. 6).

The concentration of FAA in the ambient water declined, both in the presence and absence of particles, until ~70 h; from ~70 h to ~200 h, however, the concentration of FAA increased in the presence of particles (Fig. 7).

Discussion

The experimental system used to create phytoplankton-derived model particles in rolling cylinders is a useful approach for examining the metabolic activities of marine bacterial assemblages during particle decomposition, under well-defined conditions. There are, however, some limitations. Phytoplankton-derived model particles were made from dead and autoclaved diatom cultures. Thus, the material might be altered physically and chemically. In addition, physical, chemical, and biological conditions of the sea are certainly different from those in laboratory microcosms. Despite these limitations, the use of artificial microcosms offers some advantages: first, it allows one to define, control, and modify the experimental conditions; second, it allows high number of particles to be sampled for extensive analysis of bacterial processes, which is not always possible with natural aggregates; third, it is well known that sampling natural aggregates can be difficult, due to the fragile nature of aggregates.

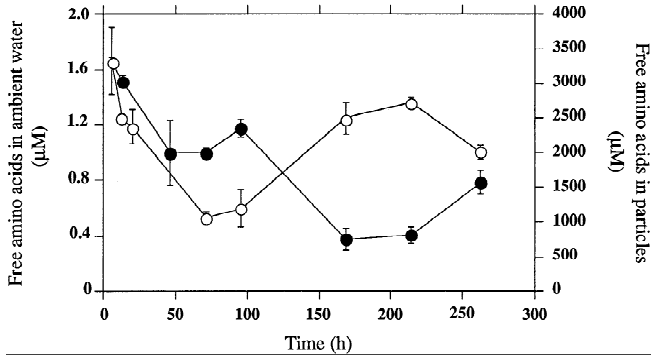

Fig. 4. Concentration of free amino acids in particles (●) and ambient water (○) during the decomposition process. Bars represent standard errors of three replicate subsamples.

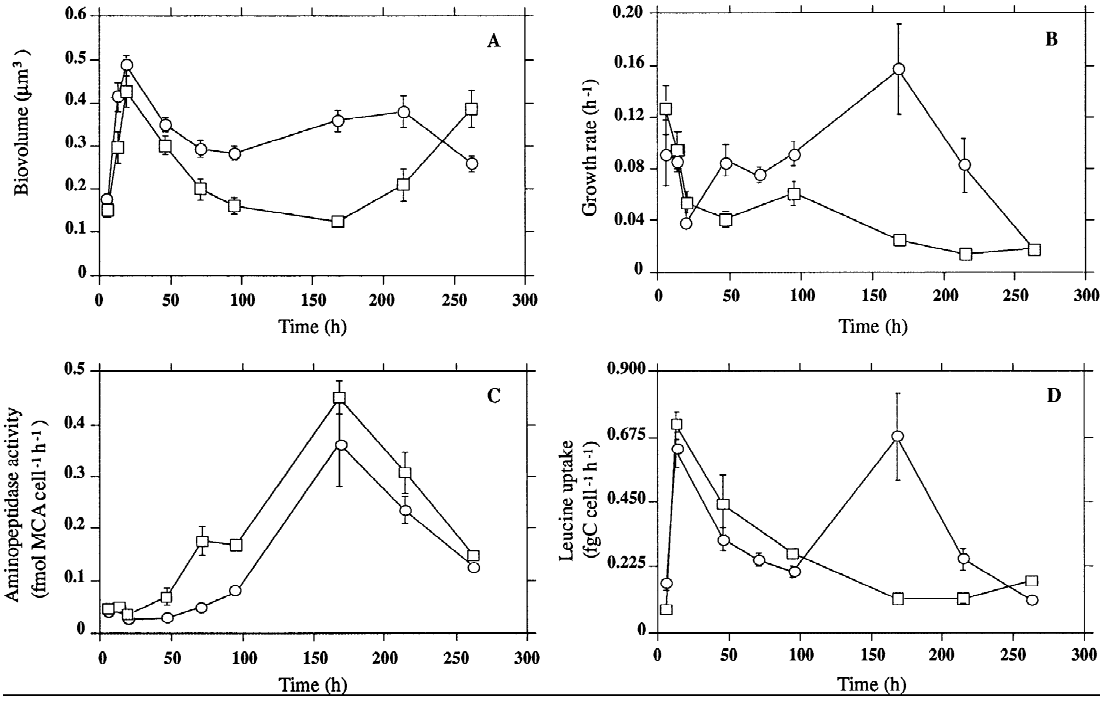

Fig. 5. Biovolume (A), growth rate (B), aminopeptidase activity per cell (C), and leucine uptake per cell (D) for free-living bacteria in the presence (○) and absence (□) of particles. Bars represent standard errors of three replicate subsamples.

In our experiments with phytoplankton-derived model particles, the number of attached bacteria rapidly increased to 10^9–10^{10} bacteria ml^{-1} of particle during the first 20 h, as a result of colonization and growth. The extremely high bacterial densities might have led to the accumulation of toxic compounds and the appearance of reducing microzones [36] which, in turn, could inhibit the growth of aerobic bacteria. It would explain why bacterial turnover rates in particles were similar or even lower than those of free-living bacteria. It has been widely reported that bacterial abundance in natural aggregates can be very high; attached bacteria, though, do not grow faster than free-living bacteria, in spite of the nutritional advantage of the particulate environment over the ambient water [4, 20, 28, 38]. These results have been obtained from natural samples with particles in different stages of decomposition. Azam et al. [6] suggested that aggregates might be sites of active bacterial growth when they are originally colonized as "source" particles. In our case, we did not detect higher turnover rates in attached bacteria as compared to their free-living counterparts, although the study was performed from the first stage of colonization of particles through different stages of their decomposition process. It has to be considered that, during the first 12 hours of our experiments, the extraordinary fragility of

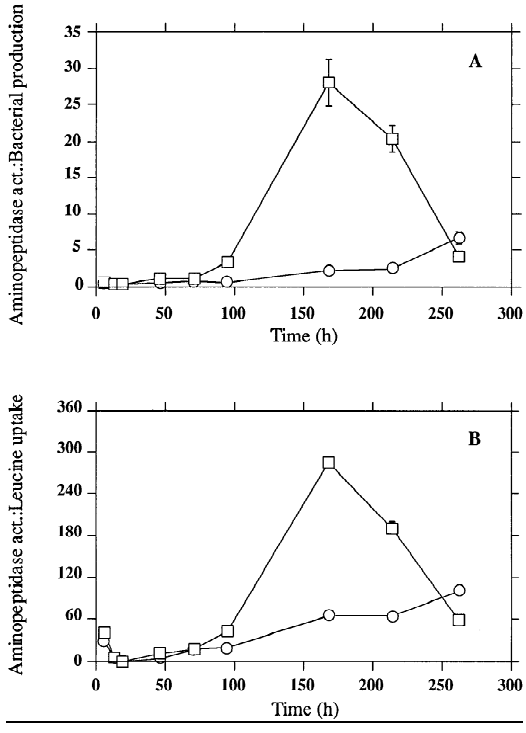

Fig. 6. Aminopeptidase activity:bacterial production (A) and aminopeptidase activity:leucine uptake ratio (B) for free-living bacteria in the presence (○) and absence (□) of particles. Bars represent standard errors of three replicate subsamples.

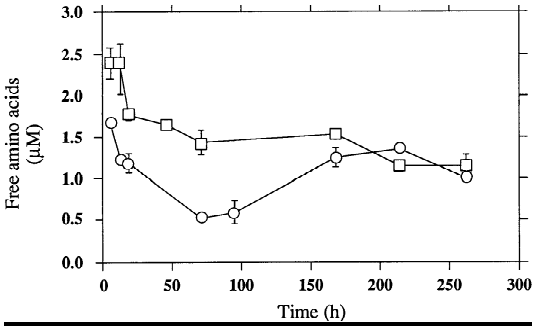

Fig. 7. Concentration of free amino acids in ambient water in the presence (○) and absence (□) of particles. Bars represent standard errors of three replicate subsamples.

the particles made their collection impossible. Thus, the first samples were taken at approximately 12 hours.

Extracellular and ectoenzymatic activities have been mainly associated with the bacterial community, but recently Karner et al. [22] showed evidence that flagellates also exhibit aminopeptidase activity in marine environments. In the microcosms analysed in this study, the aminopeptidase activity was positively correlated with bacterial abundance, but not with flagellate abundance. Therefore, we consider bacteria the main microorganisms responsible for the aminopeptidase activity measured in our experiments.

Attached bacteria did not show higher aminopeptidase activity per cell, than free-living bacteria. The activity throughout the decomposition process was very similar for both communities. Aminopeptidase activity on a cell-specific basis increased until 150 h, and decreased thereafter. This trend has also been observed by Middelboe et al. [27] and Smith et al. [40]. Müller-Niklas et al. [28], in natural aggregates of the Adriatic Sea also detected no differences in hydrolytic activity per cell between free-living and attached bacteria. However, Karner and Herndl [21] and Smith et al. [39] using natural aggregates, and Smith et al. [40] using a diatom bloom produced in a mesocosm, reported higher hydrolytic activities for attached bacteria. These results contrast, but it is possible that the observed pattern maybe influenced by the age and nutritional conditions of the particles and the ambient water. In the microcosms analysed in the present study, autoclaving the material could have enhanced the availability of free amino acids, which could affect both attached and free-living bacteria.

The concentration of FAA (end product of the aminopeptidase activity), both in ambient water and in particles, decreased significantly from the beginning of the experiments to ~100 h. This decrease might stimulate the aminopeptidase activity and/or cause inducible enzyme production [9]. Because of the increase in the aminopeptidase activity, per cell, the availability of FAA for both bacterial communities should be higher, and both communities should exhibit enhanced leucine uptake rates. Our results indicate that the increase of the aminopeptidase activity per cell caused an increase in the uptake of leucine per cell and turnover rates, both in the ambient water (~100 h) and in the particles (~70 h). In the free-living bacterial community, hydrolysis rates and uptake rates per cell, as well as turnover rates, showed the same trend from ~100 h to the end of the experiments. However, after ~100 h, particle-attached bacteria significantly reduced their leucine uptake per cell (Figs. 2 and 4), and turnover rates, while their aminopeptidase activity remained constant until ~200 h. Attached bacteria were obviously not able to take advantage of their enhanced aminopeptidase activity. This decrease in the leucine uptake rates in the particle-attached bacteria could be explained by the adsorption of the monomers to particles that could reduce the availability of amino acids to attached bacteria. Another possible explanation could be that the FAA originated from hydrolytic activity of attached bacteria were released into the ambient water.

In order to investigate whether aminopeptidase activity and leucine uptake were coupled processes during the decomposition of phytoplankton-derived model particles, two ratios were analyzed—aminopeptidase activity:bacterial production and aminopeptidase activity:leucine uptake. The aminopeptidase activity:bacterial production ratio indicates that, during the first 100 h, both attached and free-living bacteria took advantage of the low molecular weight compounds released by their ectoenzymatic activity. However, after ~100 h, attached bacteria exhibited higher aminopeptidase activity:bacterial production ratio than free-living bacteria. This ratio has been interpreted by other authors as suggesting the existence of a coupling between the hydrolytic activity and the uptake of low molecular weight compounds [17, 28]. However, it is possible that both bacterial communities are physiologically different [23]. The synthesis of extracellular polymers for attachment and the synthesis of hydrolytic enzymes require energy. Therefore, attached bacteria would have lower growth efficiency than free-living bacteria [26, 27, 43]. On the other hand, attached bacteria have been reported to be larger than free-living bacteria [1, 3, 19, 38]. Consequently, a higher aminopeptidase activity:

bacterial production ratio for attached bacteria can only reflect this size difference when bacterial size is not accounted for in the measurements of bacterial production.

We believe that the aminopeptidase activity:leucine uptake ratio is the most appropriate index for assessing the uncoupling between hydrolysis of organic matter and uptake of low molecular weight compounds during the decomposition of particles. When comparing this ratio in attached and free-living bacteria, we found similar values in both bacterial communities during the first ~100 h, and higher values in attached bacteria during the period from 100 h to 200 h (Fig. 3). It has to be taken into account that we analyzed only potential activities. However, these results clearly show that, during the bacterial decomposition of phytoplankton-derived model particles, aminopeptidase activity and uptake of the hydrolysate may be uncoupled processes when particles are largely degraded.

It is an open question whether the uncoupling between hydrolysis and uptake in attached bacteria results in a significant release of FAA into the ambient water, and whether the free-living bacteria take advantage of this release. In order to address this question, we designed microcosms lacking particles. Over the first 100 h, the presence of particles did not significantly stimulate the growth of free-living bacteria, and the aminopeptidase activity:bacterial production and aminopeptidase activity:leucine uptake ratios were not significantly different in the presence or absence of particles (Figs. 5 and 6). After ~100 h, when hydrolysis and uptake began to uncouple in the attached bacterial community, the free-living bacterial community growing in the absence of particles showed lower turnover rates, biovolume, and uptake rates per cell than in the presence of particles, but similar aminopeptidase activity. Moreover, after ~100 h, the concentration of FAA increased in the ambient water in the microcosms with particles, but we could not detect such an increase in the absence of particles. These results suggest that bacteria in ambient water benefit from the presence of phytoplankton-derived particles. Their uptake rates and turnover rates are enhanced, possibly due to an input of FAA. Herndl [13] also found that marine snow stimulates microbial activity in the surrounding water. The aminopeptidase activity:bacterial production and aminopeptidase activity:leucine uptake ratios were higher in the absence of particles, indicating that, in particle-free microcosms, free-living bacteria need to express higher aminopeptidase activity to maintain their growth and uptake rates.

In summary, during the first 100 h of the decomposition of phytoplankton-derived model particles, aminopeptidase activity and uptake of leucine were coupled processes both in attached and free-living bacteria; consequently, both bacterial communities took advantage of the low molecular weight compounds released by their hydrolytic activity. However, at ~100 h, when the laboratory-generated particles were already largely degraded, attached bacteria were not able to benefit from their hydrolytic activity. A possible explanation for this could be the appearance of inhibitory factors, such as the accumulation of metals released from organic complexes by hydrolytic enzymes, changes of pH, or oxygen depletion caused by the high bacterial densities [6], which seem to affect the transport systems first. The results obtained in the present study with phytoplankton-derived particles demonstrate that the relationship between hydrolysis and uptake processes varies throughout the decomposition of the particles; further, different patterns of variation exist for attached and free-living bacteria.

We assume that the experimental conditions used in the present study are different from natural conditions, and therefore, cannot be directly translated to natural marine snow. However, our results support the hypothesis proposed by Cho and Azam [8], who suggested the role of the aggregates as enzyme reactors releasing DOM during their descent through the water column. The confirmation of this hypothesis could have important implications in understanding the flux of carbon in the ocean, and, it would explain the relatively high growth rates displayed by free-living bacteria in marine systems [6]. Our experiments showed that the uncoupled solubilization of amino acids might not be due to higher hydrolytic activity of attached bacteria, but to lower uptake rates.

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References

1. Alldredge AL (1989) The significance of suspended detrital aggregates of marine snow as microhabitats in the pelagic zone

of the ocean. In: Hattory T, Ishida Y, Maruyama Y, Morita RY, Uchida A (eds) Recent Advances in Microbial Ecology. Japan Scientific Societies Press, Tokyo, pp 108–112
2. Alldredge AL, Silver MW (1988) Characteristics, dynamics and significance of marine snow. Prog Oceanogr 20:41–82
3. Alldredge AL, Youngbluth MJ (1985) The significance of macroscopic aggregates (marine snow) as sites for heterotrophic bacterial production in the mesopelagic zone of the subtropical Atlantic. Deep-Sea Res 32:1445–1446
4. Alldredge AL, Cole JJ, Caron DA (1986) Production of heterotrophic bacteria inhabiting macroscopic organic aggregates (marine snow) from surface waters. Limnol Oceanogr 31:68–78
5. Asper VL (1987) Measuring the flux and sinking speed of marine snow aggregates. Deep-Sea Res 34:1–17
6. Azam F, Martinez J, Smith DC (1993) Bacteria-organic matter coupling on marine aggregates. In: Guerrero R, Pedrós-Alió C (eds) Trends in Microbial Ecology. Spanish Society for Microbiology, pp 410–414
7. Biddanda BA, Pomeroy LR (1988) Microbial aggregation and degradation of phytoplankton-derived detritus in seawater. Mar Ecol Prog Ser 42:241–251
8. Cho BC, Azam F (1988) Major role of bacteria in biogeochemical fluxes in the ocean's interior. Nature 332:441–443
9. Chróst RJ (1991) Environmental control of the synthesis and activity of aquatic microbial ectoenzymes. In: Chróst RJ (ed) Microbial enzymes in aquatic environments. Springer-Verlag, Berlin, pp 29–59
10. Fowler SW, Knauer GA (1986) Role of large particles in the transport of elements and organic compounds through the oceanic water column. Prog Oceanog 16:147–194
11. Fuhrman JA, Azam F (1980) Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica and California. Appl Environ Microbiol 39:1085–1095
12. Guillard RRL, Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Denotula confervacea* (Cleve) Gran. Can J Microbiol 8:229–239
13. Herndl GJ (1988) Ecology of amorphous aggregations (marine snow) in the Northern Adriatic Sea. II. Microbial density and activity in marine snow and its implication to overall pelagic processes. Mar Ecol Prog Ser 48:265–275
14. Herndl GJ (1992) Marine snow in the Northern Adriatic Sea: Possible causes and consequences for a shallow ecosystem. Mar Microb Food Webs 6:149–172
15. Hobbie JE, Daley RJ, Jasper S (1977) Use of Nuclepore filters for counting bacteria by epifluorescence microscopy. Appl Environ Microbiol 33:1225–1228
16. Hoppe H-G (1983) Significance of exoenzymatic activities in the ecology of brackish water: Measurements by means of methylumbelliferyl-substrates. Mar Ecol Prog Ser 11:299–308
17. Hoppe H-G, Ducklow H, Karrasch B (1993) Evidence for dependency of bacterial growth on enzymatic hydrolysis of particulate organic matter in the mesopelagic ocean. Mar Ecol Prog Ser 93:277–283
18. Iriberri J, Herndl GJ (1995) Formation and microbial utilization of amorphous aggregates in the sea: Ecological significance. Microbiología SEM 11:309–322
19. Iriberri J, Unanue M, Barcina I, Egea L (1987) Seasonal variation in population density and heterotrophic activity of the attached and free-living bacteria in coastal waters. Appl Environ Microbiol 53:2308–2314
20. Karl DM, Knauer GA, Martin JH (1988) Downward flux of particulate organic matter in the ocean: A particle decomposition paradox. Nature 332:438–441
21. Karner M, Herndl GJ (1992) Extracellular enzymatic activity and secondary production in free-living and marine snow associated bacteria. Mar Biol 113:341–347
22. Karner M, Ferrier-Pagès C, Rassoulzadegan F (1994) Phagotrophic nanoflagellates contribute to occurrence of α-glucosidase and aminopeptidase in marine environments. Mar Ecol Prog Ser 114:237–244
23. Kirchman DL (1993) Particulate detritus and bacteria in marine environments. In: Ford TE (ed) Aquatic Microbiology. Blackwell Scientific Publications, Boston, pp 321–341
24. Kranck K, Milligan T (1980) Macroflocs: Production of marine snow in the laboratory. Mar Ecol Prog Ser 3:19–24
25. Lee S, Furhman JA (1987) Relationships between biovolume and biomass of naturally derived marine bacterioplankton. Appl Environ Microbiol 53:1298–1303
26. Middelboe M, Søndergaard M (1993) Bacterioplankton growth yield: Seasonal variations and coupling to substrate lability and β-glucosidase activity. Appl Environ Microbiol 59:3916–3921
27. Middelboe M, Søndergaard M, Letarte Y, Borch NH (1995) Attached and free-living bacteria: Production and polymer hydrolysis during a diatom bloom. Microb Ecol 29:231–248
28. Müller-Niklas G, Schuster S, Kaltenböck E, Herndl GJ (1994) Organic content and bacterial metabolism in amorphous aggregations of the Northern Adriatic Sea. Limnol Oceanogr 39:58–68
29. Orive E (1989) Differences in phytoplankton abundance and distribution between the Abra of Bilbao and the adjacent shelf waters. Hydrobiologia 182:121–135
30. Parsons T, Maita T, Lalli C (1984) A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon Press, Oxford
31. Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. Limnol Oceanogr 25:943–948
32. Prézelin BB, Alldredge AL (1983) Primary production of marine snow during and after an upwelling event. Limnol Oceanogr 28:1156–1167
33. Riebesell U (1992) The formation of large marine snow and its sustained residence in surface water. Limnol Oceanogr 37:63–76
34. Riemann B, Bjornsen PK, Newell S, Fallon R (1987) Calculation of cell production of coastal marine bacteria based on measured incorporation of ^{3}H-thymidine. Limnol Oceanogr 32:471–476
35. Shanks AL, Edmondson EW (1989) Laboratory-made artificial

marine snow: A biological model of the real thing. Mar Biol 101:463–470
36. Shanks AL, Reeder ML (1993) Reducing microzones and sulfide production in marine snow. Mar Ecol Prog Ser 96:43–47
37. Sherr BF, Sherr EB, Rassoulzadegan F (1988) Rates of digestion of bacteria by marine phagotrophic protozoa: Temperature dependence. Appl Environ Microbiol 54:1091–1095
38. Simon M, Alldredge AL, Azam F (1990) Bacterial carbon dynamics on marine snow. Mar Ecol Prog Ser 65:205–211
39. Smith DC, Simon M, Alldredge AL, Azam F (1992) Intense hydrolytic enzyme activity on marine aggregates and implications for rapid particle dissolution. Nature 359:139–142
40. Smith DC, Steward GF, Long RA, Azam F (1995) Bacterial mediation of carbon fluxes during a diatom bloom in a mesocosm. Deep-Sea Res 42:75–97
41. Sugimura Y, Suzuki Y (1988) A high-temperature catalytic oxidation method for the determination of non-volatile dissolved organic carbon in seawater by direct injection of a liquid sample. Mar Chem 24:105–131
42. Trent JD, Shanks AL, Silver MW (1978) *In situ* and laboratory measurements on macroscopic aggregates in Monterey bay, California. Limnol Oceanogr 23:626–635
43. Vandevivere P, Kirchman DL (1993) Attachment stimulates exopolysaccharide synthesis by a bacterium. Appl Environ Microbiol 59:3280–3286
44. Velji MI, Albright LJ (1986) Microscopic enumeration of attached marine bacteria of seawater, marine sediment, fecal matter, and kelp blade samples following pyrophosphate and ultrasound treatments. Can J Microbiol 32:121–126
45. Weiss P, Schweitzer B, Amann R, Simon M (1996) Identification in situ and dynamics of bacteria on limnetic organic aggregates (lake snow). Appl Environ Microbiol 62:1998–2005