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Influence of organic matter quality in the cleavage of polymers by marine bacterial communities

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The influence of the quality of organic matter on the hydrolysis of polymers by marine bacteria was investigated in microcosms containing aggregates created in rolling tanks. Two types of microcosms were analysed: microcosms type M1 from unaltered seawater and microcosms type M2 from phytoplankton cultures. Kinetics of aminopeptidase, α-glucosidase and β-glucosidase were measured in the ambient water and the aggregates in the two types of microcosms. Bacteria attached to aggregates expressed enzymes with K_m values higher than those of the bacteria in the ambient water for the three hydrolytic activities analysed in both types of microcosms. Attached bacteria showed higher rates of polymer hydrolysis than free-living bacteria only in microcosms M2 created from freshly produced phytoplanktonic material, while free-living bacteria were more active than attached bacteria in the microcosms M1 containing unaltered seawater. The ratio V_{max}/K_m, which describes the ability of enzymes to compete at low substrate concentration, shows that free-living bacteria are more efficient at dealing with low substrate concentrations in microcosms derived from natural seawater, where the liquid phase may be depleted of utilizable dissolved organic matter, than in the microcosms derived from phytoplankton cultures. Our data suggest that the hydrolytic activities of both attached and free-living bacteria are significantly influenced by the quality of the aggregates and the consequences of this influence are discussed.

INTRODUCTION

Most of the organic matter available to bacterioplankton in marine systems is found as high molecular weight compounds (Münster and Chróst, 1990). The concentration of low molecular weight organic compounds is low and it is not always sufficient to support microbial growth requirements (Sieburth, 1979). Additionally, an important fraction of this low molecular weight organic matter is resistant to microbial utilization (Amon and Benner, 1994, 1996). Since bacteria can only take up molecules <650 Da (Payne, 1980), macromolecules can only be utilized if they are first hydrolysed outside the cell. Thus, bacterial growth in marine systems is mainly supported by high molecular weight compounds and therefore relies on enzymatic hydrolysis and subsequent uptake of low molecular weight products (Hoppe *et al.*, 1988). As carbohydrates and polypeptides are considered the two main fractions of the identifiable polymeric organic matter in the sea, glucosidase and peptidase activities play a key role in bacterial growth in the ocean (Münster and Chróst, 1990).

Planktonic bacteria in marine systems can be found either free in the aqueous phase or attached to particles. These two bacterial communities have been comparatively characterized in terms of size (Iriberri *et al.*, 1987), growth rate (Iriberri *et al.*, 1990; Simon *et al.*, 1990), hydrolytic activities (Karner and Herndl, 1992; Smith *et al.*, 1992; Unanue *et al.*, 1993, 1998b; Martínez *et al.*, 1996; Agis *et al.*, 1998), incorporation of low molecular weight compounds (Unanue *et al.*, 1992; Turley and Stutt, 2000) and taxonomic composition (Delong *et al.*, 1993; Bidle and Fletcher, 1995; Rath *et al.*, 1998; Acinas *et al.*, 1999; Crump *et al.*, 1999; Phillips *et al.*, 1999; Schweitzer *et al.*, 2001). It has been reported that some taxonomic groups are selected in the particles (Delong *et al.*, 1993; Acinas *et al.*, 1999), and attached bacteria are often bigger and more active than free-living ones

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(Iriberri *et al.*, 1987; Simon *et al.*, 1990; Turley and Stutt, 2000). In spite of the abundance of comparative studies on the physiology and taxonomic composition of the attached and free-living bacterial communities, there are only a few data comparing their kinetics of polymer hydrolysis (Agis *et al.*, 1998).

A large fraction of the particulate matter present in marine systems consists of macroscopic aggregates also known as 'marine snow' (Alldredge and Silver, 1988). These amorphous aggregates are mainly of phytoplanktonic origin and may develop from aggregation of senescent diatom cells and/or coagulation of colloidal organic matter released by phytoplankton (Riebesell, 1992; Iriberri and Herndl, 1995). These particles are very fragile and are normally broken up by routine methods of collection, transport and storage. In addition, their availability is limited by their seasonal occurrence. Consequently, the use of an experimental system to create aggregates in the laboratory constitutes a useful alternative for in-depth analysis of the complex interactions between microbial communities and particulate matter (Kranck and Milligan, 1980; Shanks and Edmonson, 1989; Unanue *et al.*, 1998a).

The aim of this study was to investigate the influence of the quality of the particulate and dissolved organic matter on the hydrolysis of polymers by attached and free-living bacteria. We created two types of microcosms with a similar quantity but different quality of organic matter, which simulated different natural growth conditions for marine bacterial communities. These two types of microcosms contained macroaggregates created in the laboratory in rolling tanks: microcosms type 1 (M1) from unaltered seawater, and microcosms type 2 (M2) from artificial seawater amended with phytoplanktonic material. These macroaggregates have been shown to be suitable models of natural marine macroaggregates (Unanue *et al.*, 1998a) and were expected to simulate those that appear under two different situations in marine systems: aggregates from the microcosms M2 would resemble those newly formed in association with a phytoplanktonic bloom, and aggregates from M1 would resemble the impoverished and probably more refractory aggregates that appear in natural seawater under appropriate hydrodynamic conditions. Three hydrolytic activities for the hydrolysis of peptides and carbohydrates, aminopeptidase, α-glucosidase and β-glucosidase, were analysed in the aggregates and the ambient water. Three kinetic parameters were estimated: the cell-specific maximum hydrolytic activity (V_{max}/cell), the Michaelis–Menten constant (K_m) and the ratio cell-specific V_{max}/K_m, which is an indicator of the ability of bacteria to obtain high hydrolysis rates at low substrate concentrations (Healey, 1980).

METHOD

Design of microcosms type 1 (M1)

Five experiments with microcosms M1 were performed following basically the design proposed by Shanks and Edmonson (Shanks and Edmonson, 1989). Natural seawater was collected from coastal waters of the Bay of Biscay, 43°24.5′ N, 3°2.7′ W (North Spain), from January to May at a depth of 3 m. Samples were taken using a Niskin bottle (PWS Hydro-Bios), and processed in the laboratory within 2 h after collection. For each experiment five cylindrical polypropylene tanks (13 l capacity) were filled with 12 l of seawater and rolled at 2.5 r.p.m. in the dark at room temperature.

Aggregates with a volume of 1.3 ± 0.23 µl (mean ± SE) appeared in the tanks after 1–2 days. The number of aggregates obtained per tank was variable from one sample to another. In most experiments the number of aggregates per tank was very low. Thus it was necessary to collect the aggregates from several tanks, and put them together in one tank in order to obtain enough volume of particulate material to carry out the chemical and microbial analysis (50–300 aggregates l^{-1}). The aggregates were collected with a glass pipette with a plastic tube 0.5 cm in diameter and 15 cm long at one end. Samples of particles and ambient water were collected from the rolling tanks 24 h later, to analyse the chemical composition, bacterial abundance and the kinetics of aminopeptidase, α-glucosidase and β-glucosidase activities.

Aggregates were carefully transferred from the tanks to Petri dishes. Precise volumes of particles without ambient water were taken with a digital micropipette, placing the tip directly on the aggregates. Subsamples of ambient water were taken from the tanks after sedimentation of the aggregates.

Design of microcosms type 2 (M2)

Four experiments with microcosms M2 were performed following the experimental design of Shanks and Edmonson (Shanks and Edmonson, 1989). In these microcosms, aggregates were formed in rolling tanks from a mixture of four species of diatoms (*Skeletonema costatum*, CCAP 1077/1B; *Navicula hanseni*, CCAP 1050/8; *Chaetoceros muelleri*, CCAP 1010/3; and *Nitzschia epithemoides*, CCAP 1052/18) commonly found in marine aggregates, (Beers *et al.*, 1986; Revelante and Gilmartin, 1991) and in the Bay of Biscay (Orive, 1989). Batch cultures of each diatom were grown on Guillard's medium for diatoms (f/2 + Si) (Guillard and Ryther, 1962) at 15°C on an orbital shaker (100 r.p.m.) with aeration and light (125 µE $m^{-2}s^{-1}$, 16 h light:8 h dark). Growth was

followed by algal cell counts and analysis of dissolved organic carbon (DOC). In order to measure algal abundance, the samples were filtered on 0.2 μm pore-size black polycarbonate filters and counted under epifluorescence microscopy at a magnification of ×1250. DOC was measured with a TOC analyser (Shimadzu TOC-5000/Shimadzu ASI-5000) following the high-temperature catalytic oxidation method (Sugimura and Suzuki, 1988). The cultures were harvested after 25–30 days of incubation, when they had reached the stationary phase. Cultures were centrifuged at 2100 *g* for 20 min in order to separate algal cells from the dissolved extracellular products. The pellets were resuspended in sterile artificial seawater (Sigma) and the supernatant was passed through 0.2 μm pore-size cellulose acetate filters in order to eliminate any particulate material present. The cells and extracellular products were mixed to obtain an equal contribution of each species to the final algal abundance and DOC concentration in the mixture.

Five-litre bottles were filled with sterile artificial seawater and a mixture of algal cells (10^9 cells l^{-1} final concentration) and extracellular products (5 mg DOC l^{-1} final concentration). The bottles were autoclaved and placed on a roller table at 2.5 r.p.m. in the dark. After 2–3 days, the roller table was stopped, the aggregates were allowed to sediment and the liquid was removed. Then, the sterile aggregates were carefully transferred from the bottles to polypropylene cylindrical tanks containing 12 l of sterile artificial seawater using a glass pipette as described above. The DOC concentration was adjusted to the levels observed in the Bay of Biscay (1.5–2 mg C l^{-1}) with algal extracellular products. Subsequently, the tanks rolled at 2.5 r.p.m. for 12 h to allow the flux to stabilize and the aggregates to reach a uniform size. The aggregates showed a mean volume of 3.0 ± 0.06 μl and their mean abundance was 185 ± 15 aggregates l^{-1}.

After the formation of the phytoplankton-derived aggregates, they were inoculated with a microbial assemblage concentrated by tangential-flow filtration (100 000 Da; Nominal Molecular Mass Limit, Millipore) from the sampling location in the Bay of Biscay. Bacterial abundance was adjusted to that found in natural seawater (0.8–2.5 × 10^9 bacteria l^{-1}). Microcosms were rolled at 2.5 r.p.m. in the dark at room temperature for 24 h, and thereafter samples of aggregates and ambient water were withdrawn to characterize the above mentioned chemical and bacterial parameters.

Chemical analysis of aggregates and ambient water

The concentration of total organic carbon (TOC), carbohydrates and amino acids was analysed in the aggregates (70 μl of aggregates in 100 ml of distilled water) and the ambient water (100 ml) (Unanue *et al.*, 1998a). TOC was measured with a TOC analyser (Shimadzu TOC-5000/Shimadzu ASI-5000) following the high-temperature catalytic oxidation method (Sugimura and Suzuki, 1988). Total amino acid and carbohydrate concentrations were determined according to Parsons *et al.* (Parsons *et al.*, 1984). Regarding amino acids, this technique actually measures the total concentration of primary amines and therefore the amino acid concentrations are overestimated since they include ammonium.

Bacterial counts

Three subsamples of aggregates (7 μl in 1 ml of sterile artificial seawater) and ambient water (10 ml) were fixed with formalin (2% v/v final concentration). Bacterial abundance was measured by acridine orange epifluorescence direct counting (AODC) (Hobbie *et al.*, 1977). Immediately before staining, samples of aggregates were sonicated (100 W, six pulses of 5 s) to disperse the bacteria attached to particles (Velji and Albright, 1986). Aliquots were stained with acridine orange (0.01% w/v final concentration) for 2 min, and filtered using 0.2 μm pore-size black polycarbonate filters (Millipore). The wet filters were placed on microscope slides and mounted in low-fluorescence immersion oil. The filters were examined under an epifluorescence microscope (Nikon), at a magnification of ×1250. Bacteria present in at least 30 randomly selected fields, with 20–30 bacteria per field, were counted.

Hydrolytic activity

Bacterial α-glucosidase, β-glucosidase and aminopeptidase activities were measured using the fluorogenic substrates 4-methyl-umbelliferyl-α-D-glucopyranoside, 4-methyl-umbelliferyl-β-D-glucopyranoside and L-leucyl 4-methylcoumarinyl-7-amide, respectively (Hoppe, 1983). Fluorogenic substrates were added to triplicate subsamples of aggregates (10.5 μl in 3 ml of sterile artificial seawater) and ambient water (3 ml) at different concentrations: 0.1, 1, 5, 20, 50, 100, 200 and 350 μM for glucosidases and 5, 20, 50, 100, 200, 350 and 500 μM for aminopeptidase.

Subsamples were incubated in the dark at room temperature on an orbital shaker (120 r.p.m.). Fluorescence of methyl-umbelliferone (MUF) and 7-amino-4-methylcoumarine (MCA) caused by the enzymatic cleavage of the substrates over 2–5 h for α-glucosidase and β-glucosidase activities and over 1–3 h for aminopeptidase activity was measured on a Perkin Elmer LS 50B spectrofluorometer, at 360 nm excitation and 445 nm emission. Relative fluorescence units were calibrated

with 100 nM MUF and 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 substrates was not significant.

The kinetic parameters were estimated from the hydrolysis values by means of non-linear regression using the Michaelis–Menten equation

$$V = V_{max} \times S/(K_m + S)$$

where V is the reaction rate at a given substrate concentration and S is the concentration of substrate added. The parameters estimated are V_{max} and K_m. V_{max} is the maximum velocity of the reaction, which is proportional to the amount of enzyme present, and K_m is the half-saturation constant.

K_m represents $K + S_n$. K is the affinity constant for that substrate, the lower the K the higher is the affinity; S_n is the concentration of natural substrate in the system. In aquatic systems the concentration of natural substrate is usually unknown and therefore we cannot determine the value of K. Consequently, $K + S_n$ is considered as the maximum estimation of K.

Cell-specific potential hydrolytic activity (V_{max}/cell) in aggregates and ambient water was estimated by dividing V_{max} by the bacterial abundance, and it assumes that all cells express the same amount of a given enzyme.

The ratio cell-specific V_{max}/K_m, which is the slope of the Michaelis–Menten equation at low substrate concentration, was also calculated for the free-living bacteria (Healey, 1980). This ratio can be considered as an indicator of the ability of bacteria to obtain a high hydrolysis rate at low substrate concentrations since organisms might compensate for a high K_m by a high V_{max}.

RESULTS

Chemical analysis

In both types of microcosms, the concentrations of total carbohydrates, total amino acids and TOC were two to three orders of magnitude higher in the aggregates than in the surrounding water (Table I). The average concentrations of carbohydrates, amino acids and TOC in both types of aggregates and in ambient water were not significantly different between the two types of microcosms M1 and M2 ($P > 0.05$, Mann–Whitney U-test).

Bacterial abundance

In microcosms type M1 the bacterial abundance in ambient water ranged from 0.84×10^6 to 5.69×10^6 cells ml^{-1} and in aggregates ranged from 1.34×10^9 to 6.15×10^9 cells ml^{-1}. In microcosms type M2 the bacterial abundance in ambient water ranged from 1.09×10^6 to 9.26×10^6 cells ml^{-1} and in aggregates ranged from 1.12×10^9 to 7.75×10^9 cells ml^{-1}.

Hydrolytic activities in microcosm type M1

The K_m values were higher in aggregates than in ambient water ($P < 0.05$, Wilcoxon signed-rank test) (Figure 1; Table II). The K_m values for aminopeptidase ranged from 191 to 1213 μM in aggregates and from 136 to 183 μM in ambient water. For α-glucosidase the range of the K_m was 3.7–19.2 μM in aggregates and 0.2–2.8 μM in ambient water. The K_m for β-glucosidase ranged from 1.1 to 63.8 μM in aggregates and from 0.03 to 6.1 μM in ambient water.

Cell-specific potential hydrolytic activity in aggregates was lower than in ambient water ($P < 0.05$, Wilcoxon signed-rank test) (Figure 2; Table III). For aminopeptidase

Table I: Chemical composition (carbohydrates, amino acids and TOC) of aggregates and ambient water in the two types of microcosms (M1 and M2) and enrichment factor (EF) in aggregates in relation to the ambient water (concentration in 1 ml of aggregates/concentration in 1 ml of ambient water)

	M1			M2		
	Aggregates	Ambient water	EF	Aggregates	Ambient water	EF
Carbohydrates	2.9–8.6	2.2×10^{-3}–6.0×10^{-3}	812–3819	1.2–14.6	1.8×10^{-3}–12.7×10^{-3}	120–5928
(mM glucose)	(6.4) (25)	(3.6×10^{-3}) (25)	(1944) (25)	(6.7) (66)	(6.1×10^{-3}) (66)	(1414) (66)
Amino acids	3.0–8.2	2.4×10^{-3}–5.4×10^{-3}	823–3224	1.2–12.4	1.6×10^{-3}–7.8×10^{-3}	495–5752
(mM glycine)	(5.1) (23)	(3.7×10^{-3}) (23)	(1448) (23)	(6.2) (33)	(3.9×10^{-3}) (33)	(1872) (33)
TOC	26–133	21.7×10^{-3}–62.3×10^{-3}	101–537	22–195	14.4×10^{-3}–32.7×10^{-3}	124–905
(mM C)	(63) (21)	(27.3×10^{-3}) (21)	(241) (21)	(73) (47)	(18.9×10^{-3}) (47)	(384) (47)

Data are given as variation range (mean value) (number of samples).

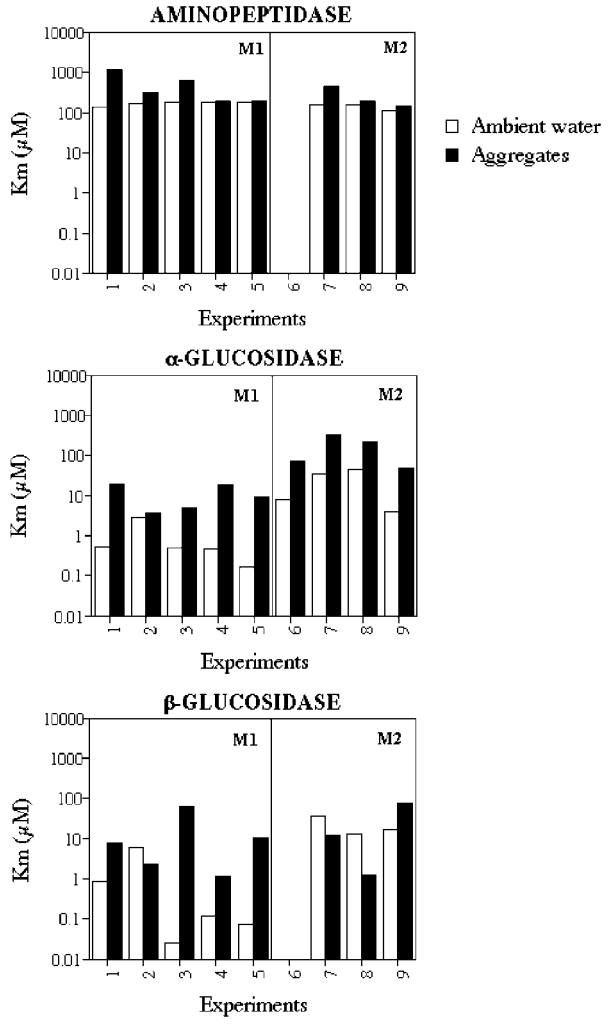

Fig. 1. K_m values for aminopeptidase, α-glucosidase and β-glucosidase in aggregates and ambient water of both types of microcosms M1 and M2.

the V_{max}/cell ranged from 20 to 580 amol cell^{-1} h^{-1} (where amol stands for attomoles) in aggregates and from 340 to 820 amol cell^{-1} h^{-1} in ambient water. In the case of α-glucosidase activity, the range of the V_{max}/cell in aggregates was 2.5–6.6 amol cell^{-1} h^{-1}, while bacteria in ambient water expressed slightly higher activities, 2.9–8.7 amol cell^{-1} h^{-1}. The V_{max}/cell for β-glucosidase ranged from 0.4 to 12.4 amol cell^{-1} h^{-1} in aggregates and from 2.3 to 20.2 amol cell^{-1} h^{-1} in ambient water.

Hydrolytic activities in microcosms type M2

In eight out of 10 samples the K_m values were higher in aggregates than in ambient water ($P < 0.05$, Wilcoxon signed-rank test) (Figure 1; Table II). The K_m values for aminopeptidase ranged from 150 to 458 μM in aggregates and from 115 to 163 μM in ambient water. For α-glucosidase the range of the K_m was 47.3–332 μM in aggregates and 3.9–45.6 μM in ambient water. The K_m for β-glucosidase ranged from 1.2 to 74.4 μM in aggregates and from 12.4 to 34.9 μM in ambient water.

Aggregate-attached bacteria expressed higher levels of the analysed ectoenzyme activities than those in the ambient water ($P < 0.05$, Wilcoxon signed-rank test) (Figure 2; Table III). The cell-specific aminopeptidase activity ranged from 410 to 1200 amol cell^{-1} h^{-1} in aggregates, while a narrower range, from 130 to 190 amol cell^{-1} h^{-1}, was observed in the ambient water. For α-glucosidase the range of V_{max}/cell was 6.0–18.1 amol cell^{-1} h^{-1} in the aggregates and 1.2–2.6 amol cell^{-1} h^{-1} in the ambient water. A similar trend was observed for β-glucosidase with activities ranging from 10.2 to 17.3 amol cell^{-1} h^{-1} in the aggregates and from 3.7 to 5.5 amol cell^{-1} h^{-1} in the ambient water.

Table II: K_m values (μM) in ambient water and aggregates for aminopeptidase, α-glucosidase and β-glucosidase activities in the two types of microcosms (M1 and M2)

	M1	M2
Aminopeptidase		
Aggregates	514 ± 194	269 ± 96
Ambient water	170 ± 10	147 ± 16
α-Glucosidase		
Aggregates*	10.9 ± 3.2	165.6 ± 66.5
Ambient water*	0.9 ± 0.5	22.7 ± 10.1
β-Glucosidase		
Aggregates	17.2 ± 11.8	29.3 ± 22.7
Ambient water*	1.4 ± 1.2	21.3 ± 6.9

Data are given as mean value ± SE.
*Statistically significant differences between M1 and M2 ($P < 0.05$, Mann–Whitney *U*-test).

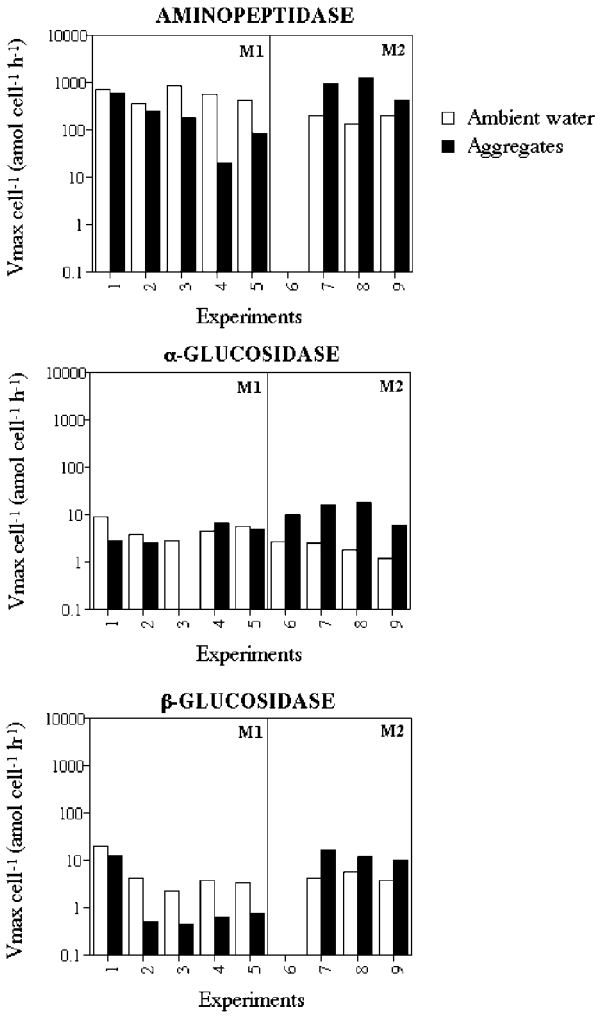

Fig. 2. Cell-specific V_{max} values for aminopeptidase, α-glucosidase and β-glucosidase in aggregates and ambient water of both types of microcosms M1 and M2.

Comparison of hydrolytic activities in M1 and M2

The average K_m values for aminopeptidase activity in the microcosms M1 were higher than in M2 for both the aggregates and the ambient water; however, no statistically significant differences were observed between the two types of microcosms ($P > 0.05$, Mann–Whitney *U*-test) (Table II). In the case of α-glucosidase activity, the observed K_m values were significantly higher in M2 than in M1 ($P < 0.05$, Mann–Whitney *U*-test), for both ambient water and aggregates (Table II). The average K_m value of β-glucosidase activity was also higher in the microcosms M2 as compared with M1 (Table II) for both the aggregates and the ambient water, however, this difference was only statistically significant in ambient water ($P < 0.05$, Mann–Whitney *U*-test).

Cell-specific hydrolytic activities showed significant differences between the two types of microcosms (Table III) in both the aggregates and the ambient water. Attached bacteria showed an enhanced hydrolytic response in terms of aminopeptidase and α-glucosidase activities ($P < 0.05$, Mann–Whitney *U*-test) in the aggregates of type M2 as compared with the possibly more refractory aggregates of type M1. Free-living bacteria, on the contrary, expressed significantly ($P < 0.05$, Mann–Whitney *U*-test) lower levels of aminopeptidase and α-glucosidase activity in the microcosms M2 than in M1.

The ratio cell-specific V_{max}/K_m in the ambient water was higher in the microcosms M1 ($P < 0.05$, Mann–Whitney *U*-test) than in M2 (Table IV). In the aggregates there were no statistically significant differences between the two types of microcosms.

Table III: V_{max}/cell values (amol cell^{-1} h^{-1}) in ambient water and aggregates for aminopeptidase, α-glucosidase and β-glucosidase activities in the two types of microcosms (M1 and M2)

	M1	M2
Aminopeptidase		
Aggregates*	220 ± 98	840 ± 231
Ambient water*	560 ± 89	170 ± 17
α-Glucosidase		
Aggregates*	4.2 ± 0.9	12.5 ± 5.6
Ambient water*	5.0 ± 1.0	2.0 ± 0.6
β-Glucosidase		
Aggregates	3.0 ± 2.4	13.0 ± 3.6
Ambient water	6.8 ± 3.4	4.4 ± 0.9

Data are given as mean value ± SE.
*Statistically significant differences between M1 and M2 ($P < 0.05$, Mann–Whitney *U*-test).

Table IV: Cell-specific V_{max}/K_m values (pl cell^{-1} h^{-1}) in ambient water for aminopeptidase, α-glucosidase and β-glucosidase activities in the two types of microcosms (M1 and M2)

	M1	M2
Aminopeptidase*	3.376 ± 0.609	1.206 ± 0.248
α-Glucosidase*	12.991 ± 5.406	0.189 ± 0.077
β-Glucosidase*	38.10 ± 12.073	0.261 ± 0.096

Data are given as mean value ± SE.
*Statistically significant differences between M1 and M2 ($P < 0.05$, Mann–Whitney *U* test)

DISCUSSION

The hydrolytic activity of bacteria showed significantly different patterns in the microcosms M1 as compared with M2. However, the chemical analyses showed no significant differences in the concentrations of carbohydrates, amino acids and TOC present in the two types of microcosms for either the aggregates or the ambient water (Table I). This different physiological behaviour may be attributable to differences in the quality of both the particulate and dissolved organic matter present in the two different types of microcosms. The microcosms M1 were created from unaltered seawater. The organic materials present in natural seawater include a large fraction of aged, partially altered materials, which are refractory to microbial attack. As shown by Keil and Kirchman (Keil and Kirchman, 1993, 1994) and Obernosterer *et al.* (Obernosterer *et al.*, 1999), labile materials such as proteins become refractory due to abiotic factors such as ultraviolet radiation in surface waters. On the contrary, microcosms of type M2 were created from freshly produced phytoplanktonic material and consequently, the organic matter in these microcosms presents an abundance of compounds that are readily available for bacteria. The microcosms of type M2 may resemble a situation of phytoplanktonic bloom where the ambient water and the aggregates are enriched in organic matter that is easily available to bacterioplankton.

In both types of microcosms we found that the K_m values of attached bacteria were higher than those of free-living bacteria for the three hydrolytic activities analysed. A high K_m value indicates the need for a high concentration of substrates to achieve the maximum reaction velocity and it is expected that these enzymes will be expressed at high substrate concentrations. Particles are highly enriched in carbohydrates and amino acids as compared with the ambient water (with concentrations two to three orders of magnitude higher). Therefore, the enzymes of attached bacteria can be adapted to work efficiently at concentrations of substrates higher than those experienced by free-living bacteria. The low K_m of the hydrolases expressed by free-living bacteria suggests that their enzymes are hydrolysing polysaccharides and proteins at low concentrations, thereby supplying microorganisms with a small but steady flow of hydrolysis products. Although the K_m values of attached and free-living bacteria are different, it still remains unknown whether these differences are due to species selection (Delong *et al.*, 1993; Rath *et al.*, 1998; Acinas *et al.*, 1999) or to regulation of the synthesis and activity of hydrolytic enzymes in the same bacterial species. When the attached bacteria become free-living, the substrate limitation conditions of the aqueous phase may induce the synthesis of new enzymes of higher affinity.

Despite the fact that fluorogenic substrate analogues may not completely represent the naturally occurring substrates, our results show how the kinetic parameters of the hydrolytic enzymes assayed are sensitive to the quality of the natural substrates present. The K_m values of glucosidase activities (α and β) were significantly higher in microcosms of type M2 than of type M1 for both attached and free-living bacteria, although the chemical analysis showed that the concentration of carbohydrates was similar. In spite of the methodological limitations explained above, we consider that higher K_m values in M2 reflect a higher concentration of available substrate. These results suggest that the aggregates and the ambient water of M2 are enriched in hydrolysable α and β carbohydrates compared with those of M1. Consequently, the differences in K_m may reflect differences in the quality of the organic compounds in both types of microcosms. On the contrary, although no statistically significant differences were found between the average K_m values of aminopeptidase activity for both types of microcosms, the average values were higher in M1 than in M2. This could be due to a higher average molecular

weight of the peptides available in fresh organic matter of phytoplanktonic origin as compared with natural seawater, resulting in a reduced number of amino-ends available for aminopeptidase activity. Additionally, the apparent peptide availability may be lowered in the microcosms of type M2, due to adsorption to phytoplankton-derived colloidal matter and polysaccharides such as dextran, as shown by Schuster *et al.* (Schuster *et al.*, 1998).

At low substrate concentrations such as those of the ambient water, microorganisms can compensate for a high K_m value by increasing the amount of enzyme (V_{max}). Consequently, the ability of bacteria to hydrolyse substrate at low concentrations is described in a more realistic manner by the quotient V_{max}/K_m, which is the initial slope of the Michaelis–Menten curve (Healey, 1980). In the ambient water where the substrate concentrations are low, the ratio cell-specific V_{max}/K_m was lower in microcosms created from phytoplankton cultures than in microcosms derived from natural seawater. In those microcosms that simulate the situation of a phytoplanktonic bloom, the free-living bacteria expressed enzymes with high K_m, but did not compensate for this high K_m by a high cell-specific V_{max}. If we assume that the requirements for low molecular weight compounds are similar in the free-living bacteria in both microcosms, these results suggest that in the microcosms of type M2 there is a higher availability of readily available low molecular weight dissolved organic matter (DOM). In this situation it is not necessary for the free-living bacteria to spend resources on the synthesis of hydrolytic enzymes in order to increase carbon uptake.

Surprisingly, attached bacteria living on aggregates constituted by abundant polymeric compounds were not always more active in the hydrolysis of polymers than free-living bacteria. Attached bacteria were more active only in microcosms created from freshly produced phytoplanktonic material, while free-living bacteria were more active than aggregate-attached bacteria in the microcosms containing unaltered seawater (Figure 2; Table III). It has been shown that in marine systems, high molecular weight DOM can be utilized by bacterioplankton more rapidly than the DOM fraction <1 kDa (Amon and Benner, 1994, 1996). These authors propose that a large proportion of the low molecular weight organic materials in seawater is refractory compared with the larger polymeric materials. Therefore, in the microcosms of type M1, free-living bacteria may depend mostly on the hydrolysis of polymeric material. On the other hand, previous data obtained from microcosms similar to M2 (Unanue *et al.*, 1998b) revealed an uncoupling between the high hydrolytic activity of attached bacteria and their uptake of the resulting products. This could lead to a release of part of these hydrolysis products to the ambient water, supporting most of the carbon and nitrogen demands of the free-living bacteria. These monomeric compounds would also repress the synthesis and inhibit the activity of the ectoenzymes and extracellular enzymes of the free-living bacteria in the ambient water (Chróst, 1991).

In summary, our results suggest that fresh aggregates of phytoplanktonic origin, like those formed during a phytoplanktonic bloom, are colonized by an active attached bacterial community, which might release low molecular weight compounds to the aqueous phase repressing the hydrolytic activity of free-living bacteria. Under these conditions, it seems likely that bacterial hydrolysis of polymers and uptake of low molecular weight compounds would be uncoupled processes (Cho and Azam, 1988; Smith *et al.*, 1992, 1995; Unanue *et al.*, 1998b; Schweitzer *et al.*, 2001), leading to rapid particle solubilization in the water column. Alternatively, aggregates composed of aged, refractory organic material seem to host a slowly-degrading attached bacterial community with low hydrolytic activity. In these systems with impoverished aggregates, there would not be a significant flux of low molecular weight compounds from the aggregates to the aqueous phase. The reduced availability of utilizable low molecular weight material in the aqueous phase of these systems may cause free-living bacteria to shift their carbon utilization patterns towards the more labile polymeric molecules. Free-living bacteria may exhibit higher hydrolytic activities than attached bacteria under these nutritional conditions. In this situation, the growth of free-living bacteria would be supported by their own hydrolytic activity and, therefore, the hydrolysis of polymers and the uptake of low molecular weight compounds might be coupled processes (Hollibaugh and Azam, 1983; Somville and Billen, 1983; Azam and Cho, 1987; Hoppe *et al.*, 1988; Chróst, 1989; Müller-Niklas *et al.*, 1994). Further research is needed, but this study leads to the suggestion that the relationship between polymer hydrolysis and subsequent uptake in marine bacteria will be indirectly influenced by the quality of the particulate matter in the marine system.

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