MICROBIAL ECOLOGY

Microb Ecol (1999) 37:36–48

Ectoenzymatic Activity and Uptake of Monomers in Marine Bacterioplankton Described by a Biphasic Kinetic Model

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Received: 5 March 1998; Accepted: 31 July 1998

ABSTRACT

The kinetics of bacterial hydrolytic ectoenzymatic activity and the uptake of monomeric compounds were investigated in the Northwestern Mediterranean Sea. Aminopeptidase and α- and β-glucosidase activities were analyzed by using fluorogenic substrates at 15–22 concentrations ranging from 1 nM to 500 μM. Radiolabeled glucose and a mixture of amino acids were chosen as representatives of monomeric compounds, and the bacterial uptake rates (assimilation plus respiration) were determined over a wide range of substrate concentrations (from 0.2 nM to 3 μM). We found biphasic kinetics both for hydrolytic enzymes and uptake systems: high affinity enzymes at low concentrations of substrates (K_m values ranged from 48 nM to 2.7 μM for ectoenzymes and from 1.4 nM to 42 nM for uptake systems), and low affinity enzymes at high concentrations of substrates (K_m values ranged from 18 μM to 142 μM for ectoenzymes and from 0.1 μM to 1.3 μM for uptake systems). Transition between high and low affinity enzymes was observed at 10 μM for aminopeptidase and from 1 μM to 25 μM for glucosidases, and it was more variable and less pronounced for the uptake of glucose (40 nM–0.28 μM) and amino acids (10 nM–0.16 μM). Results showed that the potential rates of hydrolysis and uptake are tightly coupled only if the high affinity hydrolytic ectoenzymes and the low affinity uptake systems are operating simultaneously.

Introduction

Polymeric organic compounds constitute a high percentage of the total organic matter in aquatic systems [4, 10] and heterotrophic bacteria are mainly responsible for their hydrolysis [32]. Bacterial hydrolysis of high molecular weight compounds is the initial step in the microbial decomposition of organic matter in aquatic systems. Once polymeric compounds are transformed into monomers, they become available for bacterial uptake. If hydrolysis and uptake are tightly coupled, the low molecular weight compounds liberated by ectoenzymatic hydrolysis will be taken up. If uptake is faster than hydrolysis, the concentration of UDOM (utilizable dissolved organic matter) could limit growth. If hydrolysis is faster than uptake, low molecular weight com-

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pounds would accumulate, eventually increasing the downward flux of dissolved organic carbon (DOC). Therefore, the coupling between bacterial ectoenzymatic hydrolysis and the uptake of the hydrolysis products is of crucial importance to understanding the flux of carbon in the ocean.

Available methodologies do not allow the measurement of the actual hydrolysis of dissolved organic matter and uptake rates in natural systems. The methodologies rely on the use of radiolabeled and fluorogenic artificial substrates that are not representative of DOM [28, 32, 63, 68]. Moreover, usually the natural concentrations of these substrates are unknown, and therefore the choice of the tracer concentration for the experiments becomes a key factor for the realistic estimation of the bacterial activity. The measurement of microbial activities in aquatic systems have frequently been performed at saturating concentrations of substrate, but this situation is exceptional in natural systems where the availability of substrate normally limits bacterial growth. Thus, the examination of kinetics becomes essential to determine the appropriate substrate concentration according to the objectives of the study.

Bacteria respond to changing nutrient conditions by regulating the synthesis and activity of their enzymes [25]. Multiphasic kinetics for uptake of glucose have been reported in mixed bacterial communities as well as in isolates [2, 30, 52–54]. However, there is no such information with regard to bacterial ectoenzymatic activity, although the concentration and composition of polymeric compounds in marine systems are also subject to large fluctuations as a consequence of biological, chemical, and physical conditions. Ectoenzymatic activity as the initial response of the microbial community to changes in the nutritional conditions may exhibit an even more pronounced short-term variability than uptake activities [40]. Algae liberate a variety of monomeric and polymeric organic compounds [17, 38], and the decay of phytoplankton blooms constitutes a major source of polymeric compounds in aquatic systems. Moreover, the standing stock of polymers has frequently been reported to be more variable than that of monomers [5]. At high concentrations of polymeric compounds, ectoenzymatic activities have been found to increase, but it is largely unknown how these hydrolytic activities are regulated [10].

The aim of this study was to investigate the kinetics of two bacterial processes, hydrolysis of organic macromolecules and uptake of low molecular weight compounds in bacterial assemblages of the northwestern Mediterranean Sea. We analyzed hydrolysis of peptides and carbohydrates constituting a significant fraction of the polymeric organic matter in the ocean [51], by measuring the aminopeptidase and α- and β-glucosidase activities. Dissolved free amino acids and monosaccharides are labile products of enzymatic hydrolysis of peptides and polysaccharides, respectively, and they are common substrates efficiently taken up by bacteria. These compounds serve as carbon, nitrogen, and energy sources for microbial metabolism. Consequently, we used radiolabeled amino acids and glucose as monomeric-model substrates for the kinetic study of the uptake systems. Our data show that marine bacteria exhibit biphasic kinetics for both ectoenzymes and uptake systems. The balance between hydrolysis and uptake as a function of the concentration of the respective substrates, polymers and monomers, is also discussed.

Materials and Methods

Sampling

During November and December 1995, water samples were collected with 12-liter Niskin bottles at 20 and 50 m depth at two stations in the northwestern Mediterranean Sea from the R/V *Tethys II*. Station B was located at the entrance of the Bay of Villefranche-sur Mer (43°41′N–7°17′E), and Station 1 was 5.5 miles offshore (43°39′N–7°27′E). Previous studies have characterized this area [60, 66]. Samples were stored in combusted glass flasks at 4°C in the dark until analysis (generally within 6 h). Upon return to the lab, samples were brought to *in situ* temperature before measurements were started.

Bacterial Abundance

Seawater subsamples were fixed with buffered formalin (2% v/v final concentration), stained with acridine orange (0.01% w/v final concentration) for 2 min, and filtered onto 0.2 μm pore-size black polycarbonate filters [29]. The wet filters were placed on microscope slides and mounted in low-fluorescence immersion oil. Within 3 weeks after sampling the filters were examined under a Nikon epifluorescence microscope, at a magnification of 1,250×. Twenty to 30 bacteria were counted in at least 30 randomly selected fields.

Ectoenzymatic Activity

Aminopeptidase and α- and β-glucosidase activities were measured using fluorogenic substrate analogs [32]. The analogs were L-leucine-4-methyl-coumarinyl-7amide (Leu-MCA) for aminopeptidase activity, 4-methyl-umbelliferyl-α-D-glucopyranoside (α-MUF) for α-glucosidase activity, and 4-methyl-umbelliferyl-β-D-glucopyranoside (β-MUF) for β-glucosidase activity. These substrates mimic peptides and carbohydrates, the two main fractions of the organic matter in aquatic systems. Leu-MCA is hydrolyzed by a variety of ectoenzymes, not only by leucine-

aminopeptidases (E.C. 3.4.1.1). It can be considered as a model substrate for the hydrolysis of a great variety of peptides, because leu-MCA hydrolysis is competitively inhibited by a variety of dipeptides and oligopeptides involving amino acids other than leucine [34]. α-MUF and β-MUF are hydrolyzed by α-glucosidase (E.C. 3.2.1.20) and β-glucosidase (E.C. 3.2.1.21), respectively, which are broad-specificity enzymes [10].

Kinetic experiments were performed for each sample by measuring the ectoenzymatic activity at 15–22 different concentrations of substrate ranging from 1 nM to 500 μM. Substrates were added to triplicate 2-ml subsamples in precombusted glass tubes and incubated in the dark at *in situ* temperature (16°C) for 6–12 h. The long incubation periods were necessary to yield a significant increase in fluorescence. The increase in fluorescence caused by the enzymatic cleavage of the fluorogenic substrates was measured with a spectrofluorometer (JASCO 820-FP, Tokyo, Japan) at 360 nm excitation and 445 nm emission. The fluorescence increased linearly over the course of the incubation period. Relative fluorescence units were calibrated with 4-methylcoumarin (MCA) and 4-methylumbelliferone (MUF) standards of 25 nM final concentration. 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.

Uptake of Low Molecular Weight Organic Compounds

Amino acids and glucose are end products of the aminopeptidase activity and α- and β-glucosidase activities, respectively. Glucose was chosen as representative of monosaccharides because it has been reported to be the most abundant monosaccharide in seawater [48, 57]. We used a mixture of amino acids instead of a single amino acid since the uptake of a mixture provides a better index of dissolved free amino acids (DFAA) utilization by natural bacterial assemblages. The molar composition of the mixture reflects that of algal protein hydrolysate and is comparable to the molar composition of DFAA in seawater [16].

D-[U-^{14}C]Glucose (>230 mCi/mmol) or [U-^{14}C]protein hydrolysate (>50 mCi/milliatom Carbon, Amersham) were added to triplicate or duplicate subsamples (100 ml) at 15–16 different final concentrations ranging from 0.2 nM to 3 μM. Water samples did not get any treatment before the assays. The maximum concentrations of ^{14}C-glucose and ^{14}C-amino acids assayed for the measurement of uptake rates were two orders of magnitude lower than those of model substrates assayed for the measurement of hydrolysis rates. There are several reasons for this difference. First, most measurements of ectoenzymatic activity reported in marine systems including kinetic studies have been performed using concentrations higher than 10 μM [13, 14, 32, 33, 40]. In contrast, uptake rates have been measured using a wide range of concentrations, from <1 nM for actual estimates of bacterial activity, to >1 μM if the purpose of the study was to estimate potential activities. Second, the standing stock of monomers has been reported to be one to two orders of magnitude lower than that of polymers [3, 55]. Finally, the minimum concentrations of substrate assayed in this study were limited by the sensitivity of the techniques.

Substrates were added to 100-ml subsamples dispensed in polyethylene bottles washed with 1 N HCl for 12 h and well rinsed with deionized water, and incubated in the dark for 10–12 h at *in situ* temperature (16°C). The low activity of the samples made it necessary to use long incubations. In previous experiments we found that incorporation was linear during the incubation period. Unpowdered gloves were worn during all sample handling to avoid contamination. After incubation, H_2SO_4 (final concentration, 0.02 N) was added through the cap in order to stop the incorporation. Respired CO_2 was trapped in wicks containing phenethylamine (0.2 ml). After 12 h, the wicks were placed in scintillation vials and radioassayed by liquid scintillation counting. The subsamples were filtered through 0.2 μm pore size filters (Millipore MF), applying a vacuum pressure of 150 mm Hg. The filters were rinsed three times with 10 ml of distilled water, placed in scintillation vials, and radioassayed by liquid scintillation counting. These measurements represented the assimilated ^{14}C-substrate. Controls for each concentration were processed as above, but formaldehyde (final conc. 2% v/v) was added 1 h before the addition of the radiolabeled substrates. Total uptake rates for each substrate were obtained from the sum of the assimilation rate plus the respiration rate.

Kinetic Data Analysis

Experimental data were fitted to several kinetic models using nonlinear regression analysis (Enzfitter, Elsevier-Biosoft, UK) [44.]

Simple Kinetics. The mathematic model for a single kinetic is the usual Michaelis–Menten equation $V = V_{max} \times S/(K_m + S)$, where V is the rate of the reaction, V_{max} is the maximum velocity of the reaction obtained at saturating concentration of substrate, S is the concentration of substrate added, and K_m is the concentration of substrate needed to obtain half V_{max}. K_m represents $K + S_n$; K is the affinity constant, and the lower K is, the higher the affinity of the enzyme for that substrate. S_n is the concentration of natural substrate in the system. In aquatic systems the concentration of 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 [68]. The apparent turnover time, T_t, estimated for the utilization of the substrates was calculated from K_m and V_{max} values ($T_t = K_m/V_{max}$).

Double Kinetics. The mathematic model for double Michaelis–Menten kinetics is the sum of two Michaelis–Menten terms. This model represents two independent enzymes operating in the range of assayed substrate concentrations, $V = (V_{max1} \times S/(K_{m1} + S)) + (V_{max2} \times S/(K_{m2} + S))$.

Biphasic Kinetics. This model involves two enzymes operating at different ranges of substrate concentrations. The plot of the rate of the reaction versus substrate concentration is divided in two phases, each of which follows simple Michaelis–Menten kinetics. The mathematical model is $V = V_{maxn} \times S/(K_{mn} + S)$, where n denotes the phase number.

The goodness of the data fit to the kinetic models was statistically analyzed by comparing the residual mean sum of squares. We only considered those models that gave statistically significant es-

timates of the kinetic constants ($P < 0.05$). When a model provided kinetic constants estimates with a level of significance $P > 0.05$, the model was rejected.

Results

Bacterial abundance ranged from 4.58×10^8 to 6.14×10^8 cells liter^{-1}. The plots of substrate concentration versus bacterial activity, both hydrolysis and uptake, gave very good fits to a single Michaelis–Menten kinetic over the whole range of assayed concentrations ($R^2 > 85\%$, coefficient of multiple determination) (Figs. 1A and 2A). Saturating concentrations were very high (in most samples >200 μM for ectoenzymatic activity and >1 μM for uptake). However, detailed analysis of the plots in narrower concentration ranges showed that there was an initial saturation at very much lower concentrations (Fig. 1B and 2B), and a second saturation at the above mentioned concentrations (Fig. 1C and 2C). When the goodness of data fits to the different models are compared (Table 1), the residual mean sum of squares was mostly lower (only exception: glucose uptake at St 1, 30 Nov 95) when data were fitted to a biphasic model. Double models did not allow statistically significant estimations of the kinetic parameters, and therefore these models were rejected.

Ectoenzymatic Activity Kinetics

Figure 1 illustrates an example of one of the experiments. The kinetic constants V_{max}, K_m, and T_t for the analyzed activities are shown in Table 2. In all the samples and for all the assayed ectoenzymatic activities, we detected at least two phases. When the substrate concentrations were <10–25 μM we detected a first phase with high affinity ectoenzymes exhibiting low V_{max}, K_m, and T_t values. However, when the substrate concentrations were >10–25 μM ectoenzymes showed low affinity with higher kinetic constants. Only in one sample and for β-glucosidase activity the first phase was found in the very low range of concentrations 0.1–1 μM, and the second phase in the range of 1–500 μM.

The mean values of V_{max} for the α-glucosidase and β-glucosidase were lower than those of aminopeptidase, for both the low and high affinity systems, but the difference was more pronounced at low substrate concentrations. At concentrations lower than 10–25 μM, when the high affinity systems were operating, the mean value of V_{max} for aminopeptidase was 20 times higher than those for β- and α-glucosidase. At substrate concentrations higher than 10–25 μM, V_{max} for aminopeptidase activity was about 10 times higher than for glucosidases. The ratio α-/β-glucosidase was <1 in all samples with the exception of the sample collected on 2 Dec 95 at low concentrations of substrates, which showed a value of 1.8, due to the very low β-glucosidase activity of this sample.

Considering the high affinity system, aminopeptidase showed higher apparent K_m values than glucosidases and therefore, lower affinity to the substrate. However, for the low affinity system, aminopeptidases showed higher affinities than glucosidases. At higher concentrations of substrates, the K_m of aminopeptidase increased about 10 times while the K_m of glucosidases increased about 100 times (Table 2).

Turnover times were very long (47 d to 325 d for peptides and from 48 d to 11,748 d for carbohydrates); moreover, they were longer for the low affinity system than for the high affinity system. Generally, the low affinity system of glucosidases exhibited the highest turnover times, and they were always higher for α- than for β-glucosidase. Table 3 shows the cell-specific V_{max} for each sample with the low and high affinity systems.

Uptake Kinetics

Figure 2 shows an example of the plots of substrate concentrations versus total uptake rates (assimilation plus respiration). The biphasic model gave the best fit with only one exception (sample 30 Nov 95) (Table 1). For the uptake rates, the transition between phases was not as pronounced as for hydrolysis, and thus, some substrate concentrations fitted to both phases. The high affinity uptake systems worked at low concentrations in the range from 0.2 nM to 0.28 μM while the low affinity uptake systems were operating at concentrations higher than 40 nM for glucose and 10 nM for amino acids. The sample of 28 Nov 95 showed a good fit in the range of 2 nM–0.16 μM, but in the higher range of amino acid concentrations the estimates of the kinetic constants were not statistically significant.

The percentage of substrate respired was similar for glucose and amino acids and ranged from 14% to 47%. Potential uptake rates were always higher for amino acids than for glucose, both for the high and low affinity uptake systems (Table 4). The difference between V_{max} of both substrates was more pronounced at low concentrations (i.e., high affinity systems). For glucose, the low affinity uptake system showed four times higher V_{max} values than the high affinity

Table 1. Goodness of data fits to the simple and biphasic kinetic models; RMS = residual mean squares ($\times 10^{-4}$)

	Sample	Activity	Concentration range (μM)	RMS biphasic/ RMS simple
Ectoenzymatic activity	A (St 1, 30 Nov 95)	α-Glucosidase	0.001–10	
			25–250	1.44/1.67
			0.001–250	
		β-Glucosidase	0.01–10	
			10–250	3.5/5.6
			0.01–250	
	B (St 1, 2 Dec 95)	α-Glucosidase	0.6–25	
			25–500	1.88/8.6
			0.6–500	
		β-Glucosidase	0.1–1	
			1–500	8.1/9.68
			0.1–500	
	C (St 1, 4 Dec 95)	α-Glucosidase	0.1–10	
			10–500	3.6/11.5
			0.1–500	
		β-Glucosidase	0.1–10	
			10–500	6.6/11.25
			0.1–500	
	D (St B, 25 Nov 95)	Aminopeptidase	0.001–10	
			10–100	185/525
			0.001–100	
	E (St 1, 28 Nov 95)	Aminopeptidase	0.001–10	
			10–150	651/942
			0.001–150	
Uptake	A (St 1, 30 Nov 95)	Glucose	0.004–0.14	
			0.04–2.8	0.627/0.137
			0.004–2.8	
	B (St 1, 2 Dec 95)	Glucose	0.0014–0.04	
			0.04–2.8	0.155/0.519
			0.0014–2.8	
	C (St 1, 4 Dec 95)	Glucose	0.0007–0.28	
			0.04–2.8	0.251/0.529
			0.0007–2.8	
	D (St B, 25 Nov 95)	Amino acids	0.0002–0.16	
			0.01–3.2	3.2/4.1
			0.0002–3.2	
	E (St 1, 28 Nov 95)	Amino acids	0.002–0.16	1.5[a]
			0.002–1.12	3.3[b]

[a] RMS corresponding to the range 0.002–0.16
[b] RMS corresponding to the whole range 0.002–1.12

system. For amino acids, the V_{max} of the low affinity system was only two times higher than that of the high affinity system.

The high affinity uptake systems showed higher apparent K_m for amino acids than for glucose, while the low affinity uptake system showed lower K_m for amino acids than for glucose. The K_m values of the low affinity uptake system for glucose were two orders of magnitude higher than those of the high affinity system. In the case of amino acids, the K_m values of the low affinity systems were only five times higher.

The apparent turnover times were higher for glucose (ranging from 4 d to 625 d) than for amino acids (8 d–29 d, Table 4). The low affinity uptake system for glucose showed the highest turnover times (116 d–625 d). Cell-specific V_{max} values for uptake of low molecular weight compounds with the low and high affinity uptake systems are shown in Table 3.

Relationship between Ectoenzymatic Activity and Uptake

The relationship between the potential activities of hydrolysis and uptake was analyzed by dividing the calculated V_{max}

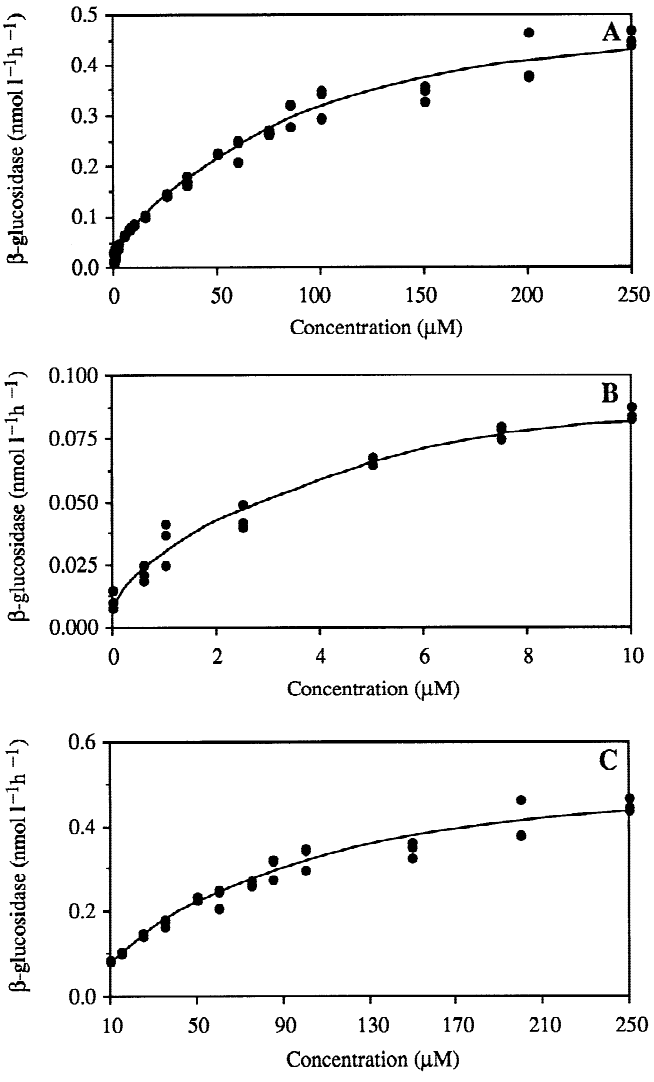

Fig. 1. Kinetics of β-glucosidase activity (30 Nov 95). (A) Curve represents the best fit of data to the simple model. (B) Curve represents the best fit of data to the biphasic model in the range of substrate concentrations 10 nM–10 μM. (C) Curve represents the best fit of data to the biphasic model in the range of substrate concentrations 10–250 μM.

of hydrolysis and the calculated V_{max} of uptake for the high affinity enzymes and low affinity enzymes, respectively. Data shown in Table 5 indicate that there was a very good balance of potential hydrolytic rates and potential uptake rates when the hydrolytic enzymes were operating in the low range of assayed concentrations while the uptake systems were working in the high range of the assayed concentrations (ratio V_{max} hydrolysis/V_{max} uptake close to 1; see Table 5).

Discussion

The kinetic analysis of metabolic processes in mixed bacterial communities by using the Michaelis–Menten model has frequently been criticized. In particular, multiphasic kinetics for uptake of glucose and amino acids have been questioned and explained by simple diffusion models [46]. We have found evidence of biphasic kinetics not only for uptake of monomers, but also for ectoenzymatic activities. A simple physical model of chemical diffusion across a permeable membrane cannot be applied to ectoenzymatic activities, because the hydrolysis of the model substrates is extracellular and the fluorescent products are not transported across a membrane. Our results for ectoenzymatic activity fit well to a Michaelis–Menten equation over the range of substrate concentrations assayed.

There are only a few reports available on kinetic constants for ectoenzymes in marine systems. Table 6 summarizes K_m values, as well as the substrate concentrations used for the determination of the kinetic constants of different marine environments. Concentrations of proteins and polysaccharides in marine systems are usually lower than 5 μM [15, 41, 42, 55], but most of the reported hydrolytic activities and even the kinetic experiments have been performed by using higher concentrations of fluorogenic substrates. We found that the hydrolysis of macromolecules exhibited biphasic kinetics if a wide range of concentrations are used (from 1 nM to 500 μM). Our apparent K_m values for the low affinity system are in the upper range of the reported values. However, the values of K_m for the high affinity system are in the range of the lowest reported values. Most of our samples showed a transition between both phases at 10 μM for proteins, and for carbohydrates between 1 and 25 μM.

These results suggest that the determination of the apparent kinetic parameters for ectoenzymes should be made over a wide range of concentrations, covering the fluctuating concentrations of macromolecules in marine systems. Our K_m values estimated for the high affinity ectoenzymes ranged from 48 nM to 2.734 μM for glucosidases and from 2.205 μM to 2.478 μM for aminopeptidases. The values are consistent with concentrations of polymeric substrates, dissolved combined carbohydrates, and dissolved combined amino acids in marine systems. Therefore, under natural conditions bacteria should be adapted to use the high affinity systems operating at concentrations below 5 μM. Higher concentrations of polymers have been reported in enriched

Table 2. Summary of kinetic parameters (V_{max}, K_m, T_t) for α-glucosidase, β-glucosidase, and aminopeptidase activity

Ectoenzymatic activity	Sample	Concentration range (μM)	V_{max} (nmol l^{-1} h^{-1})	K_m (μm)	T_t (d)
α-Glucosidase high affinity	A (St 1, 30 Nov 95)	0.001–10	0.042	1.693	1,668
	B (St 1, 2 Dec 95)	0.6–25	0.076	1.691	927
	C (St 1, 4 Dec 95)	0.1–10	0.073	0.140	80
	Mean ± SE		0.064 ± 0.011	1.175 ± 0.517	892 ± 459
α-Glucosidase low affinity	A (St 1, 30 Nov 95)	25–250	0.472	114.0	10,063
	B (St 1, 2 Dec 95)	25–500	0.502	141.5	11,748
	C (St 1, 4 Dec 95)	10–500	0.553	130.7	9,851
	Mean ± SE		0.509 ± 0.024	128.8 ± 8.0	10,554 ± 1,040
β-Glucosidase high affinity	A (St 1, 30 Nov 95)	0.01–10	0.105	2.734	1,085
	B (St 1, 2 Dec 95)	0.1–1	0.042	0.048	48
	C (St 1, 4 Dec 95)	0.1–10	0.129	1.033	334
	Mean ± SE		0.092 ± 0.026	1.272 ± 0.785	489 ± 309
β-Glucosidase low affinity	A (St 1, 30 Nov 95)	10–250	0.567	77.1	5,665
	B (St 1, 2 Dec 95)	1–500	0.636	98.9	6,476
	C (St 1, 4 Dec 95)	10–500	0.644	61.4	3,970
	Mean ± SE		0.616 ± 0.024	79.1 ± 10.9	5,370 ± 1,279
Aminopeptidase high affinity	D (St B, 25 Nov 95)	0.001–10	1.949	2.205	47
	E (St 1, 28 Nov 95)	0.001–10	1.476	2.478	70
	Mean ± SE		1.713 ± 0.237	2.342 ± 0.137	59 ± 11
Aminopeptidase low affinity	D (St B, 25 Nov 95)	10–100	4.206	17.8	177
	E (St 1, 28 Nov 95)	10–150	4.547	35.5	325
	Mean ± SE		4.377 ± 0.171	26.7 ± 8.8	251 ± 74

microzones such as marine snow or fecal pellets, and consequently, the measurements of hydrolytic activities at very high concentrations of the model substrates might reflect the hydrolytic activity of bacteria living under exceptional nutritional conditions.

A review of the reported K_m values for uptake of low molecular weight compounds in marine systems indicates a relationship of the estimated K_m values with the maximum concentrations of substrates (amino acids and glucose) used in the kinetic study (Fig. 3). Data obtained from marine systems with different nutritional conditions and by using different methodological approaches show that $K + S_n$ depends on the range of concentrations used: the higher the substrate concentration, the higher the $K + S_n$ values. This is consistent with the results of Azam and Hodson [2] demonstrating that bacterioplankton assemblages can have several uptake systems operating at different ranges of substrate concentration.

Table 3. Activity on a cell basis for the high and low affinity enzymes

	Sample	Activity	High affinity (amol $cell^{-1}$ h^{-1})	Low affinity (amol $cell^{-1}$ h^{-1})
Ectoenzymatic activity	A (St 1, 30 Nov 95)	α-Glucosidase	0.076	0.855
		β-Glucosidase	0.190	1.028
	B (St 1, 2 Dec 95)	α-Glucosidase	0.146	0.962
		β-Glucosidase	0.081	1.219
	C (St 1, 4 Dec 95)	α-Glucosidase	0.119	0.901
		β-Glucosidase	0.210	1.049
	D (St B, 25 Nov 95)	Aminopeptidase	4.253	9.177
	E (St 1, 28 Nov 95)	Aminopeptidase	2.913	8.974
Uptake	A (St 1, 30 Nov 95)	Glucose	0.025	0.096
	B (St 1, 2 Dec 95)	Glucose	0.029	0.082
	C (St 1, 4 Dec 95)	Glucose	0.029	0.139
	D (St B, 25 Nov 95)	Amino acids	0.286	0.532
	E (St 1, 28 Nov 95)	Amino acids	0.276	0.533

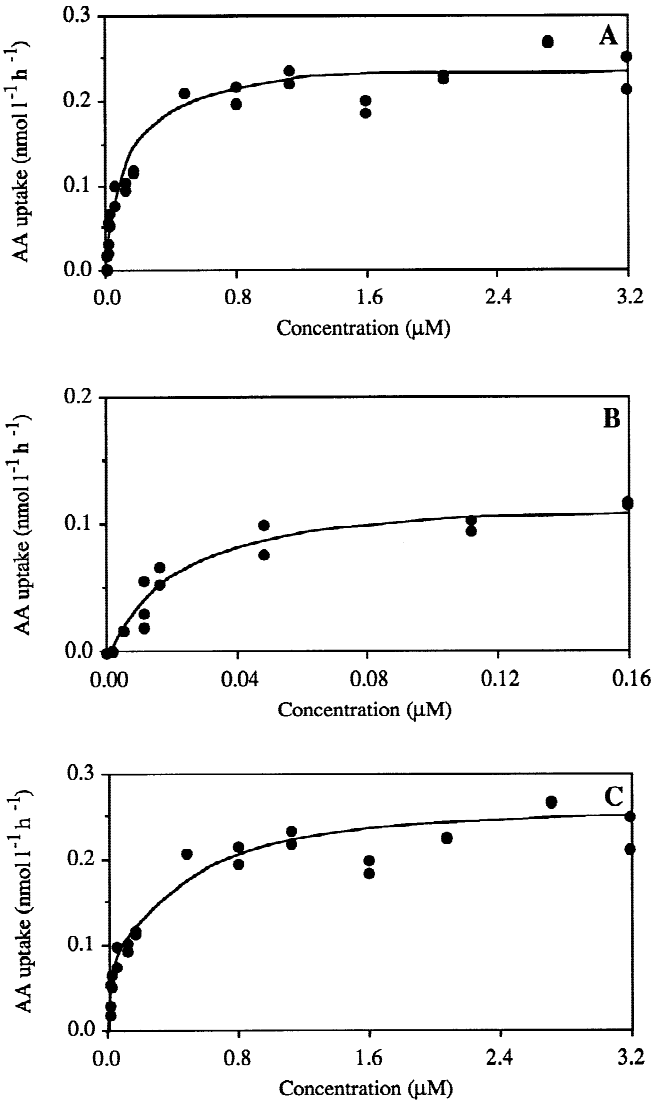

Fig. 2. Kinetics of uptake (assimilation plus respiration) of a mixture of amino acids (25 Nov 95). (A) Curve represents the best fit of data to the simple model. (B) Curve represents the best fit of data to the biphasic model in the range of substrate concentrations 0.2 nM–0.16 µM. (C) Curve represents the best fit of data to the biphasic model in the range of substrate concentrations 10 nM–3.2 µM.

We found two kinetic systems were working at different concentrations of substrate for uptake as well as for the hydrolysis of polymeric compounds: a high affinity uptake system working at low concentrations of substrate, and a low affinity uptake system working at high concentrations of substrate. The use of a higher number of concentrations in the same range, or the use of a wider range of concentrations [2, 52], would probably have shown the existence of more uptake systems specific for each range. Multiphasic uptake systems might be a strategy for changing nutrient conditions since they provide metabolic flexibility for bacteria and allow enhanced rates of substrate uptake over a broad range of ambient substrate concentrations. For the uptake systems, the transition between high and low affinity systems is not as pronounced as for the ectoenzymes, and there is a range of concentration which might be included in both phases. The apparent mean K_m for the high affinity system is 6.4 ± 2.6 nM for glucose and 33.6 ± 8 nM for amino acids, while for the low affinity system the mean value is 0.578 ± 0.353 µM for glucose and 0.152 ± 0.037 µM for amino acids. Nissen et al. [52] found that uptake kinetics of glucose show transitions between phases at glucose concentrations of 1.6 µM, 10 µM, and 200 µM; they used, however, a wider range of substrate concentrations and different calculation methods. The concentration of DFAA in seawater has been reported to range from 10 nM to 1.9 µM [6, 39, 42, 43, 49, 50] and that of dissolved monosaccharides from 20 nM to 1.6 µM [7, 36, 43, 45, 48, 55, 57, 58]. The apparent K_m values estimated for the high and low affinity uptake systems were in the range of the reported concentrations of DFAA and monosaccharides.

The existence of two phases could be due to different bacterial communities. Each phase would correspond to a different bacterial community growing at different nutritional conditions, for example, attached and free-living bacteria. Free-living bacteria growing in the bulk seawater should be adapted to low concentrations of carbohydrates and proteins, and therefore should exhibit high affinity uptake systems and high affinity ectoenzymes, very efficient in scavenging nutrients. In contrast, attached bacteria growing on organic-rich particles should exhibit low affinity enzyme systems suitable to operate efficiently at very high substrate concentrations. Kinetic studies performed on natural particles as well as on artificial aggregates derived from phytoplankton cultures have shown that indeed attached bacteria exhibit lower affinity uptake systems than free-living bacteria [67].

Moreover, the existence of two uptake phases in a single strain has been reported by Nissen et al. [52] who found multiphasic uptake by an oligotrophic marine isolate. Höfle [30], working with *Cytophaga johnsonae*, found that uptake was mediated by a multiphasic system. Therefore, biphasic kinetics in the natural bacterial community could be attributable to two different ectoenzyme and uptake systems, possibly present in the same bacterial species. The constant synthesis of both high and low affinity enzymes would be a rather inefficient energy expenditure, and consequently these enzymes are likely to be inducible. Nissen et al. [52]

Table 4. Summary of kinetic parameters (V_{max}, K_m, and T_t) for uptake of glucose and amino acids

Substrate	Sample	Concentration range (μM)	V_{max} (nmol l^{-1} h^{-1})	K_m (μM)	T_t (d)
Glucose high affinity	A (St 1, 30 Nov 95)	0.004–0.14	0.014	0.0101	30
	B (St 1, 2 Dec 95)	0.0014–0.04	0.015	0.0014	4
	C (St 1, 4 Dec 95)	0.0007–0.28	0.018	0.0078	18
	Mean ± SE		(0.016 ± 0.001)	(0.0064 ± 0.0026)	(17 ± 13)
Glucose low affinity	A (St 1, 30 Nov 95)	0.04–2.8	0.053	0.347	270
	B (St 1, 2 Dec 95)	0.04–2.8	0.043	0.112	116
	C (St 1, 4 Dec 95)	0.04–2.8	0.085	1.271	625
	Mean ± SE		(0.060 ± 0.013)	(0.578 ± 0.353)	(337 ± 151)
Amino acids high affinity	D (St B, 25 Nov 95)	0.0002–0.16	0.131	0.0256	8
	E (St 1, 28 Nov 95)	0.002–0.16	0.140	0.0416	12
	Mean ± SE		(0.136 ± 0.005)	(0.0336 ± 0.0080)	(10 ± 3)
Amino acids low affinity	D (St B, 25 Nov 95)	0.01–3.2	0.244	0.115	20
	E (St 1, 28 Nov 95)	0.002–1.12	0.270	0.189	29
	Mean ± SE		(0.257 ± 0.013)	(0.152 ± 0.037)	(24 ± 5)

suggested that biphasic kinetics could be due to a single enzyme that alters its conformation at threshold concentrations, and therefore exhibits different kinetic constants. This would be an economical and rapid strategy for bacteria adapted to fluctuating substrate concentration.

From our results we deduce that the use of very high substrate concentrations at the level of saturation of the low affinity systems keeps these systems in an active state, and that these measurements are representative of the potential activity of bacteria living in nutrient-enriched microenvironments. Likewise, the use of a small range and number of substrate concentrations for the kinetic assays can hide the existence of different levels of saturation.

For our study area, apparent turnover times are very slow, both for uptake and hydrolysis. The turnover times of polymers are one or two orders of magnitude higher than those of monomers. However, the calculated turnover times depended on two factors, the V_{max} and K_m values. Ectoenzymes with low affinity and low V_{max} would lead to long turnover times of the substrates as observed in this study for the low affinity glucosidases with turnover times >10 years. The low affinity aminopeptidases showed higher V_{max} and higher affinity for the model substrate, Leu-MCA, and thus provided turnover times much less than 1 year. The fastest turnover times were found for the high affinity aminopeptidases with turnover times of approximately 50 d. In the present study the estimated turnover times of glucose and amino acids for the high affinity transport systems were similar to the maximum estimates of Suttle et al. [64] in the

Table 5. Relationship between the potential activities of hydrolysis (V_{max} H) and uptake (V_{max} U) combining high affinity and low affinity enzymes

Sample	Activities ratio	V_{max} H_h/V_{max} U_h[a]	V_{max} H_l/V_{max} U_l[b]	V_{max} H_h/V_{max} U_l[c]	V_{max} H_l/V_{max} U_h[d]
A (St 1, 30 Nov 95)	α-Glu/G uptake	5.1	11.7	1.8	33.5
	β-Glu/G uptake	2.8	14.8	1.0	42.4
B (St 1, 2 Dec 95)	α-Glu/G uptake	3.0	8.9	0.8	33.7
	β-Glu/G uptake	7.5	10.7	2.0	40.5
C (St 1, 4 Dec 95)	α-Glu/G uptake	4.1	6.5	0.9	30.7
	β-Glu/G uptake	7.2	7.6	1.5	35.8
D (St B, 25 Nov 95)	Ampase/AA uptake	14.9	17.2	8.0	32.1
E (St 1, 28 Nov 95)	Ampase/AA uptake	10.5	16.8	5.5	32.5

[a]Ratio V_{max} of high affinity hydrolytic enzymes/V_{max} of high affinity uptake enzymes
[b]Ratio V_{max} low affinity hydrolytic enzymes/V_{max} of low affinity uptake enzymes
[c]Ratio V_{max} high affinity hydrolytic enzymes/V_{max} of low affinity uptake enzymes
[d]Ratio V_{max} low affinity hydrolytic enzymes/V_{max} of low affinity uptake enzymes

Table 6. Review of K_m values for ectoenzymatic activities reported in marine systems

Aquatic system	Activity	Substrate concentration (μM)	K_m (μM)	Reference
NW Mediterranean Sea	α-Glucosidase	0.001–10	1.7	This study
		0.6–25	1.7	
		0.1–10	0.14	
		25–250	114.0	
		25–500	141.5	
		10–500	130.7	
	β-Glucosidase	0.6–10	2.7	
		0.1–1	0.05	
		0.1–10	1.03	
		10–250	77.1	
		1–500	98.9	
		10–500	61.4	
	Aminopeptidase	0.001–10	2.2	
		0.001–10	2.5	
		10–100	17.8	
		10–150	35.5	
Eutrophic fjord	α-Glucosidase	0.5–40	0.2–30	[32]
	β-Glucosidase	0.5–40	0.3–4.8	
Coastal area	α-Glucosidase	<0.05–0.3	0.01	[62]
Baltic Sea	β-Glucosidase	12.5–200	20.34	[14]
	Aminopeptidase	12.5–200	206	
Caribbean Sea				
Oligotrophic	Aminopeptidase	0.1–25	0.6	[56]
Eutrophic	Aminopeptidase	0.1–25	47.6	

Sargasso Sea, Gocke et al. [24] in coastal Baltic waters, and Jørgensen and Søndergaard [37] in two marine stations. Very long turnover times might be attributed to contamination by DFAA, but this potential problem should be ruled out since great attention was paid to avoid contamination by using acid-washed bottles and wearing unpowdered gloves for sample handling. Other studies have provided very much shorter turnover times for glucose and amino acids in seawater; Rich et al. [57] estimated glucose turnover times <2 d in the equatorial Pacific, and Fuhrman [18] found extremely fast turnover times for DFAA, <0.5 h, in the Atlantic continental shelf. We found faster turnover times for DFAA than for glucose. The differences between the substrates were smaller at low concentrations (high affinity systems) but became more pronounced at higher concentrations (low affinity systems). The protein fraction is hydrolyzed and incorporated faster than the carbohydrate fraction, particularly at low substrate concentrations. These results suggest that bacteria are more efficient in utilizing proteins and peptides. This general trend has been repeatedly found in a number of studies performed in different aquatic environments [11, 39, 47, 61]. However, we have to take into account that the estimated rates and kinetic constants are apparent values since they are related to the artificial substrates and not to the natural substrates. Moreover, we do not know the concentration of the natural substrates. As a consequence, we did not estimate actual rates of hydrolysis and uptake, but potential rates.

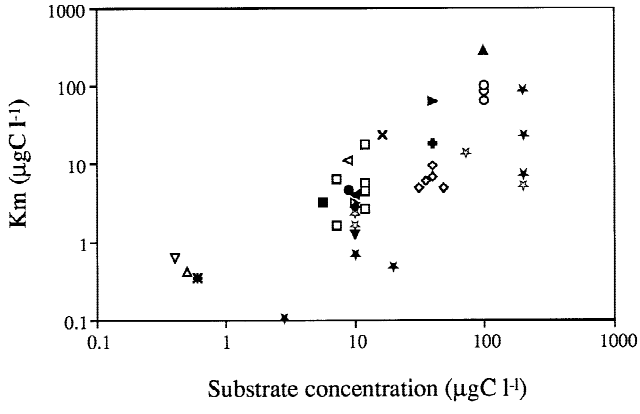

Fig. 3. Summary of reported K_m values for uptake of low molecular weight compounds in marine systems, related to the maximum concentration of substrate used in the kinetic study. If a range of K_m was given, the maximum value was plotted. Glucose: (●) [21]; (◀) [22]; (♦) [23]; (▼) [24]; (■) [26]; (▲) [27]; (✚) [59]; (▶) [65]; (★) [This study]. Amino acids: (▽) [16]; (✳) [19]; (◁) [21]; (▷) [22]; (○) [27]; (✕) [35]; (□) [37]; (◇) [59]; (△) [64]; (☆) [This study].

Hydrolysis of polymers is considered as the main source of monomers for marine bacteria, and numerous studies have demonstrated that enzymatic hydrolysis of dissolved organic polymers and the uptake of the low molecular weight compounds are tightly coupled processes [8, 9, 12, 20, 31, 34, 63]. In order to facilitate tight coupling between hydrolysis and uptake, polymers should be hydrolyzed on the cell surface by ectoenzymes and the hydrolysate should not diffuse into the bulk seawater [1]. However, the presence of free dissolved ectoenzymes in the Bay of Villefranche as found by Karner and Rassoulzadegan [40] might result in a loose hydrolysis–uptake coupling. We found a very good balance between both processes when comparing potential activities (V_{max}) of the low affinity uptake system and the high affinity hydrolysis system. According to the assumption that the hydrolysis of 1 mol of substrate releases 1 mol of monomers, uptake and hydrolysis are only balanced (monomers would not accumulate) if the polymer concentration is <10 µM and if bacteria take up the monomers released by their ectoenzymatic activity.

At very high concentrations of polymeric substrates (10–500 µM), bacteria are able to greatly increase their hydrolytic activity; however, they were unable to increase their uptake rates to a similar level (see Table 5). Presumably very high concentration of monomers would be taken up by transport systems with a very low affinity and very high V_{max}. Such conditions might be established in senescent stages of phytoplankton blooms when polymers are released from damaged and senescent algae. We have to point out that our experimental approach did not take into account the complex mechanisms of regulation of the synthesis and activity of ectoenzymes, such as the effect of polymer concentration on the uptake rate or the effect of the monomer concentrations on the hydrolysis rates, because two different methodologies were used to measure hydrolysis and uptake in different subsamples. However, following another experimental approach in brackish waters from the Kiel Fjord (Baltic Sea), Hoppe et al. [34] found that hydrolysis of the model substrate (Leu-MCA) and uptake of [^{3}H]leucine were coupled at concentrations of Leu-MCA below 0.1 µM. At higher concentrations of the model substrate, these authors found that much more leucine is liberated than is taken up.

In conclusion, in spite of the limitations of the experimental approach, the present study presents the first evidence of biphasic kinetics for hydrolytic ectoenzymes. Polymers are discontinuously distributed in the seawater, and consequently marine bacteria should be adapted to efficiently hydrolyze substrates over a broad range of concentrations. Further studies with bacterial isolates will be necessary to determine if different microorganisms are responsible for the kinetic diversity.

Acknowledgments

This research has been undertaken in the framework of the Mediterranean Targeted Project (MTP)-EMPS project. We acknowledge the support from the European Commission's Marine Science and Technology (MAST) Programme under contract MAS2-CT94-0090. This study was also funded by a research grant from the Spanish Government (AMB95-1217-CE) and by the Austrian Science Foundation (FWF grant #10497) to G.J.H. We are grateful to the captain and crew of RV *Thetys II* for excellent service at sea.

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