Biphasic Extracellular Proteolytic Enzyme Activity in Benthic Water and Sediment in the Northwestern Mediterranean Sea

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In this study, we used the fact that bacteria are able to cleave a fluorogenic substrate analog (L-leucine-7-amido-4-methylcoumarin) to determine the maximal ectoproteolytic activities (V_m) and affinities (K_m) of natural benthic microbial communities by the multiconcentration kinetic method. This investigation was performed during the winter and summer of 1997 with a set of 36 samples of near-bottom water and sediment collected from a coastal area and an offshore area in the western part of the Gulf of Lions. The existence of biphasic microbial ectoproteolysis was statistically confirmed for both the near-bottom water and the sediment, regardless of the spatial and seasonal conditions. Globally, 72.2% of the entire set of bacterial consortia collected at the water-sediment boundary layer showed biphasic microbial kinetics. A specific estimator of the biphasicity indicated that deep benthic bacterial consortia responded better with episodic nutrient supplies than shallower benthic bacterial consortia responded.

In aquatic environments nutrients occur in sharp concentration gradients (3). To be competitive under these conditions, heterotrophic bacteria develop multiphasic enzymatic processes for the uptake of low-molecular-weight organic compounds (4, 21, 34, 42). Previous authors have clearly shown that in addition to the expected low- K_m transport systems (in the nanomolar range), assemblages of marine bacteria express transport systems with apparent K_m values of up to 10^{-4} M.

It is generally recognized that in sedimentary environments the benthic microbial remineralization response is directly related to the episodic supply of organic matter reaching the seafloor due to seasonal and regional pulses of ocean surface production (7, 22, 33, 52). The bulk (>95%) of the organic matter in marine sediments is composed of polymeric, high-molecular-weight compounds (HMWC) (8). Before heterotrophic bacteria can use these compounds, the compounds must be hydrolyzed to monomeric compounds (12). This hydrolysis, performed by bacterial ectoenzymes, is the rate-limiting step in the transformation of organic matter in both pelagic and benthic ecosystems (26). Furthermore, benthic environments are well-known for having microniches around HMWC-rich organic particles. Therefore, benthic microbial populations should be adapted to produce ectoenzymatic systems that are able to hydrolyze HMWC at a wide range of substrate concentrations. Nevertheless, it is only recently that such multiphasic kinetics has been described for the bacterial hydrolysis of organic polymers in the upper layer of Antarctic deep-sea sediment (50).

It is generally assumed that in natural ecosystems, enzymatic reactions follow Michaelis-Menten kinetics (32, 43), even if the Michaelis-Menten equation appears to be a tool with limited

capacity (30). According to this model, the reaction velocity (V) can be related to the substrate concentration (S) as follows: $V = V_m(S)/(K_m + S)$, where V_m is the maximal velocity of the enzyme reaction and K_m is the half-saturation constant (Michaelis constant). We used a nonlinear least-squares method to calculate V_m and K_m because the commonly used reciprocal transformations, such as Lineweaver-Burk plots ($1/V$ versus $1/S$), lead to erroneous estimates for kinetic parameters (13, 20, 27, 32, 41, 55).

Because of the considerations described above, we used only the linear transformation of a Lineweaver-Burk plot to display our data. In this way, we observed biphasic kinetics during ectoproteolysis in the overlying waters and marine sediments characteristic of the Mediterranean continental shelf. The aims of this study were to examine the actual significance of the kinetic parameters determined in different environments by using the most appropriate nonlinear algorithm from the Michaelis-Menten kinetic model and then to validate the occurrence of biphasic ectoproteolysis kinetics in the benthic boundary layer.

MATERIALS AND METHODS

Study area and sample collection. This study was part of the MATER and PNOG programs. The general objective of these programs was to estimate the energy and matter flow through the water column and the sediment in different margin zones of the northwestern part of the Mediterranean Sea. Samples were collected during the winter (February) and summer (August) of 1997 during the BBLL 2 and BBLL 4 cruises, respectively. During each of these cruises a shallow area (along the coast of Banyuls-sur-mer) and a deeper area (Lacaze-Duthiers Canyon) were investigated (Table 1).

In the coastal area we obtained samples at two sites, at depths of 35 and 86 m. The sediments in this area consisted of fine mud that was black up to the sediment surface, and the redox boundary occurred at depths between 0.3 and 2 cm. The organic matter contents were 2.5 to 3.2 and 2.4 to 2.8% in the surface layers (0 to 2 cm) and subjacent sediment layers (2 to 10 cm), respectively (37). In the Lacaze-Duthiers Canyon area samples were collected along the canyon axis and from the adjacent open slope at depths of 920 and 800 m, respectively. The sediments consisted of fine clays, and the redox boundary occurred at depths between 4 and 18 cm. The organic matter composition of the sediment (total and organic carbon, total nitrogen, amino acid, and sugar contents) and the distribution of meiobenthic organisms in the surface deposits of the northwestern

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TABLE 1. Locations and depths of the sampling stations in the northwestern Mediterranean Sea during the BBLL2 cruise (4 to 13 February 1997) and the BBLL4 cruise (8 to 12 August 1997)

Location	Winter sampling station	Summer sampling station(s)	Latitude ^a	Longitude ^a	Depth (m)
Banyuls-sur-mer coast	K1	K5	42° 29'.63 N	3° 9'.54 E	35
Banyuls-sur-mer coast	K2	K6, K7	42° 30'.59 N	3° 13'.4 E	86
Lacaze-Duthiers Canyon	K3	K8	42° 25'.64 N	3° 42'.18 E	800
Lacaze-Duthiers Canyon	K4	K9	42° 27'.60 N	3° 29'.84 E	920

Mediterranean margin have been described by Buscail and Germain (10) and de Bovée et al. (16), respectively. Samples were collected with a multiple corer (5). Cores disturbed during retrieval were discarded.

Samples were immediately processed on the research vessel. For each core, the near-bottom water (NBW) was carefully and aseptically collected by siphoning it into a polycarbonate container. The last few milliliters was discarded to prevent introduction of mud particles into the seawater sample. The core was then extruded and sliced (0 to 2, 2 to 4, and 4 to 6 cm) with sterile cutters. Each sediment slice was transferred into a sterile 180-ml plastic vial and suspended in its own NBW (previously filtered with a 0.2- μ m-pore-size filter) so that it was diluted 1/4 (vol/vol) and constituted a sediment slurry.

Bacterial abundance. (i) **Sediment.** Sediment slurries were fixed with filtered (pore size, 0.2 μ m) formaldehyde (final concentration, 4%). Fixed samples were stored at 5°C until they were processed in the laboratory on land. In order to avoid biased counts, we varied the sample dilution depending on the sediment type and the bacterial abundance, as described by Yamamoto and Lopez (57). Therefore, a series of dilutions prepared with filtered (pore size, 0.2 μ m) seawater was used to obtain a final dilution of 1/1,600. Sediment slurries were sonicated for two periods consisting of 30 s of sonication separated by a 1-min cooling period by using a Vibra Cell 600 sonic probe equipped with a 5-mm microtip providing ~ 45 W cm^{-2} , and then they were stained with 4',6-diamidino-2-phenylindole (DAPI) (final concentration, 5 $\mu\text{g ml}^{-1}$). Each preparation was stained for at least 3 h in the dark at 5°C, and then 0.5 to 1.0 ml was filtered onto a black 0.2- μ m-pore-size Nuclepore filter. For each filter, the bacteria in 30 microscope fields were counted with an epifluorescence microscope (45).

(ii) **NBW samples.** NBW samples were immediately fixed by adding filtered (pore size, 0.2 μ m) formaldehyde (final concentration, 4%) and were stored at 5°C until they were processed in the laboratory on land. Subsamples (1 to 20 ml) were sonicated, stained, and filtered as described above. Bacterial counts were determined by epifluorescence microscopy by using an image analysis system (53).

Extracellular proteolytic activity. Protease activity in sediment slurries was measured by using the fluorogenic substrate analog L-leucine-7-amido-4-methylcoumarin (Leu-MCA) (Sigma Chemical Co.). Leu-MCA is commonly used for exoproteolytic activity assays (14, 24, 26, 48, 50). This compound is not a natural substrate, but justification for its use as an assay substrate has been discussed previously (25). Indeed, hydrolysis of Leu-MCA occurs at the same rate as uptake of labelled proteins occurs (6), and Leu-MCA competes well with easily degradable natural peptides (12), probably because exoproteases have a low level of specificity (11, 23). A stock solution of Leu-MCA (10 mM) was prepared in methylcellosolve (ethylene glycol monomethyl ether) and stored at -20°C . Previous experiments have shown that methylcellosolve at the concentration present in the samples does not have a significant effect on enzymatic activity measurements (49).

(i) **Sediment samples.** Sediment slurries (1/4, vol/vol) were distributed into sterile polycarbonate tubes (1 ml per tube). In order to determine the kinetic parameters (V_m and K_m), we used the multiconcentration kinetic method (56). Substrate was added at eight different final concentrations to the slurry samples (10, 25, 50, 100, 200, 333, 500, and 1,000 μM). The samples were incubated in the dark at the in situ temperature (usually $13 \pm 1^\circ\text{C}$, but $17 \pm 1^\circ\text{C}$ for shallow samples collected in the summer). Enzyme assays were performed in time course experiments for 1 h. For each sample and for each substrate concentration, three tubes containing sediment slurry were boiled for 30 min before the substrate was added in order to control abiotic cleavage of Leu-MCA. At the end of the 1-h incubation period the tubes were vortexed and then centrifuged (10 min, $2,680 \times g$). Fifty microliters of the overlying water was diluted with 3 ml of MilliQ water. Fluorescence was determined with a Hoefer model TKO 100 spectrofluorometer (emission wavelength, 445 nm). To quantify the 7-amido-4-methylcoumarin (MCA) liberated by bacterial attack on the peptide bond of MCA-Leu, it was necessary to construct a calibration curve under the same conditions in order to account for the adsorption of MCA onto the sediment particles (36, 39, 40). The contact between a substrate and an enzyme depends on the characteristics of the sediment (i.e., the size of the particles and the density and nature of the minerals) (29, 47). Therefore, a calibration curve was obtained for each sediment slice by using a range of MCA concentrations between 1 and 60 μM .

(ii) **NBW samples.** When NBW samples were examined, seven different concentrations of Leu-MCA (10, 20, 50, 100, 200, 500, and 800 μM) were used to determine V_m and K_m . The time course experiments lasted 6 and 24 h for the coastal and deep sampling stations, respectively. For each incubation period,

subsamples were poured into a quartz cuvette and fluorescence was measured as described above. MilliQ water was used as a blank. A standard curve was drawn by using a range of MCA concentrations.

Data management. The basic hypothesis of this work was that protease activity in natural environments follows Michaelis-Menten kinetics. The least-squares method used to determine the optimal values of Michaelis-Menten parameters (i.e., parameter values that gave the best fit to the data) involved two steps. First, we defined a scalar function of V_m and K_m to measure the distance between the data and the fitted function (that is, the cost function). Then, we used the Levenberg-Marquardt algorithm (28) to determine parameter values that minimized this cost function. The cost function can be written as:

$$\text{Cost}(V_m, K_m) = \sum [(d - x)/w]^2 \quad (1)$$

where d is the experimental value, x is the corresponding value of the fitted function (i.e., Michaelis-Menten kinetics) evaluated at V_m and K_m , and w is a weighting factor which is generally the data standard error (included to give more weight to the more precise data). Assuming that the standard error was the same for every d value, we set w values at 1. A summation was performed for all of the experimental data for one sample (that is, the free MCA concentrations measured during the entire set of time course experiments corresponding to the range of MCA-Leu concentrations). The increases in MCA concentrations were fitted to the following function:

$$\frac{dx}{dt} = V_m \frac{S(t_0) - x}{K_m + S(t_0) - x} \quad (2)$$

where $S(t_0)$ is the substrate concentration added at the beginning of a time course experiment. The integrated form of equation 2 was used to calculate x values:

$$K_m \times \ln \left[\frac{(S(t_0) - x)}{(S(t_0) - x(t_0))} \right] + x(t_0) - x = -V_m \times t \quad (3)$$

where $x(t_0)$ is the autofluorescence measured at the beginning of each time course experiment and t is the time since the addition of the substrate. A logarithmic transformation was used to constrain parameter values to be positive. At the beginning of the minimization scheme, the initial values of V_m and K_m were estimated by assuming that each time course experiment followed linear kinetics and that the highest substrate concentration was the saturation concentration.

The optimized parameters corresponding to the minimization solution were designated $V_{m,\text{opt}}$ and $K_{m,\text{opt}}$. The systematic indetermination (S_{sys}) was written as follows:

$$S_{\text{sys}} = \sqrt{\frac{\text{Cost}(V_{m,\text{opt}}, K_{m,\text{opt}})}{ndf}} \quad (4)$$

where ndf is the number of degrees of freedom of the regression (that is, the number of data points minus the number of parameters) and S_{sys} measures the distance between the data and the fitting function. S_{sys} is an indicator of the goodness of fit. In our study (i.e., when w was 1 in equation 1), S_{sys} also provided an estimate of the standard deviation when the Michaelis-Menten kinetics (depending on the sample and the monophasic or biphasic kinetics) correctly described the nonrandom processes contained in the data.

The correlation factor r :cfr ($0 < \text{cfr} < 1$) and parameter indetermination values were obtained from the variance-covariance matrix. These indetermination values were multiplied by the S_{sys} value to obtain linear approximations of the standard errors of the parameters (49).

To determine whether there was actually a biphasic mechanism, we performed three minimizations for each sample corresponding to the following three types of Michaelis-Menten kinetics: global kinetics, in which all substrate concentrations were used, and low and high kinetics, in which the low and high substrate concentrations defined by the Lineweaver-Burk plots were used. The objective was to determine whether two-phase kinetics gave a better fit for the entire data set than single-phase kinetics gave. To ascertain whether two additional parameters improved the fit significantly, we calculated the variance (Var):

FIG. 1. Lineweaver-Burk plot for NBW samples collected during the winter. Lines A and B are the regression lines that represent two levels of high extracellular enzyme reactions for sample K3 and for pooled data for samples K2 and K4, respectively, while lines A1 and B1 are the corresponding regression lines for low extracellular enzyme reactions.

$$Var(\text{additional parameters}) = \frac{Cost(\text{global}) - (Cost(\text{low}) + Cost(\text{high}))}{2} \quad (5)$$

where the cost function values correspond to the three minimization solutions and the denominator represents the number of additional parameters. We compared this variance to the variance of the biphasic kinetics, which was used as a reference:

$$Var(\text{biphasic}) = \frac{Cost(\text{low}) + Cost(\text{high})}{n - 4} \quad (6)$$

where n is the number of data in the entire data set and 4 is the number of parameters of the biphasic kinetics ($V_{m\text{low}}$, $K_{m\text{low}}$, $V_{m\text{high}}$, $K_{m\text{high}}$). Following this, we used the F test of Fisher-Snedecor:

$$F_{2,n-4} = \frac{Var(\text{additional parameters})}{Var(\text{biphasic})} \quad (7)$$

RESULTS AND DISCUSSION

Statistical proof of the existence of a biphasic mode. Using linear transformation of data in Lineweaver-Burk plots ($1/V$ versus $1/S$), we observed biphasic kinetics in ectoproteolysis in the NBW and sediment samples collected from both coastal and pelagic areas during two cruises (Fig. 1 and 2). Globally, the first kinetic phase (Fig. 1 and 2, lines A1 and B1) corresponded to low substrate concentrations (ranges, 10 to 100 and 25 to 150 μM for the NBW and sediment samples, respectively), while the second phase (lines A and B) corresponded to high substrate concentrations (ranges, 200 to 800 and 200 to 1,000 μM for the NBW and sediment samples, respectively).

We calculated the variances (equations 5 and 6) by using these low and high substrate concentration ranges. When the F test (equation 7) showed that the variances were significant at a probability of 0.1, we assumed that the biphasic mode was meaningful enough to explain the kinetics of the entire data set (Tables 2 and 3). When the F test showed that there was not a significant difference at a probability of 0.1, we assumed that there was no statistical proof for the existence of a biphasic mode and that the low and high kinetics corresponded to the same Michaelis-Menten kinetics. In the latter case, the parameter values for the global kinetics were considered the best estimates of the actual kinetic parameter values. Finally, to

FIG. 2. Lineweaver-Burk plot for surface sediment samples (depth, 0 to 2 cm) collected during the winter. Lines A and B are the regression lines that represent two levels of high extracellular enzyme reactions for sample K4 and for pooled data for samples K1, K2, and K3, respectively, while lines A1 and B1 are the corresponding regression lines for low extracellular enzyme reactions.

assess the validity of the statistical conclusions, the data point positions were examined with respect to the response of the model (Fig. 3). Indeed, these conclusions were based on the hypothesis that the data were consistent with the Michaelis-Menten kinetics.

This method of comparison was chosen (18) because the parameter values for the high and low kinetics were highly correlated, and thus the Student t test appeared to be inappropriate. Furthermore, parameter indeterminations could not be used as standard deviations of a Gaussian distribution because the Michaelis-Menten model is nonlinear.

Ectoproteolytic measurements in the sediment. When both the low and high kinetic data were considered, the average S_{sys} value was 155 times higher for the sediment samples (Table 3) than for the NBW samples (Table 2) with respect to the Michaelis-Menten kinetics (equation 4); this discrepancy may reflect specific artifacts due to sediment microbial populations (51). It is generally accepted that the significance of enzymatic activity measurements in sediment samples is less obvious than the significance of enzymatic activity measurements in water samples. This is mainly due to the poor diffusion and rapid adsorption onto sediment particles of the labelled substrate added (44, 47). However, equally important are the drastic modifications of the natural environment that occur during sample processing, such as disruption of sediment particles and bacterial aggregates through dilution, vibration, and sonication. When the method involves the addition of a fluorogenic substrate, calibration with the fluorescent part of this compound permits subtraction of the error due to adsorption processes. However, adsorption also interferes with the actual availability of the introduced compound to bacteria (17, 47), and unfortunately it seems to be impossible to correct data for this bias. The multiconcentration method involves adding different concentrations of substrate to a series of subsamples; due to adsorption processes, however, the actual availability of the substrate to bacteria does not increase linearly as the substrate concentration increases. As expected, at low substrate concentrations adsorption onto particles is rapid, which leads to a drastic decrease in the actual availability of the substrate to bacteria. At high substrate concentrations, however, when all adsorption sites on the particles are saturated, substrate

TABLE 2. Microbiological and statistical parameters for NBW samples collected in the winter and the summer at four sampling stations in the northwestern Mediterranean Sea

Season	Station	Kinetics	Depth (m)	Bacterial concn (10 ⁴ cells ml ⁻¹)	V_m (10 ⁻³ μ M h ⁻¹)	K_m (μ M)	V_m/K_m (10 ⁻⁵ h ⁻¹)	cfr	ndf	S_{sys} (10 ⁻²)
Winter	K1	Global	35	13.5	8.2 (5) ^a	26.5 (21)	0.31	0.69	19	2.89
		Low			6.0 (6)	9.9 (24)	0.60	0.84	10	1.57
		High			9.4 (9)	75.2 (53)	0.13	0.89	7	2.90
	K2 ^b	Global	86	5.3	8.0 (7)	14.1 (35)	0.57	0.65	26	2.52
		Low			6.7 (11)	7.5 (51)	0.90	0.82	14	1.95
		High ^c			8.9 (16)	45.4 (143)	0.20	0.88	10	3.14
	K3	Global	800	0.5	2.8 (4)	8.1 (26)	0.35	0.63	19	1.11
		Low			2.5 (6)	4.8 (35)	0.53	0.81	10	0.80
		High			3.7 (7)	115.0 (33)	0.032	0.91	7	0.80
	K4	Global	920	2.6	6.1 (7)	7.9 (35)	0.77	0.75	20	1.39
		Low			5.3 (5)	4.1 (26)	1.28	0.88	10	0.48
		High			8.4 (12)	74.8 (43)	0.11	0.83	8	1.49
Summer	K5	Global	35	47.3	22.8 (5)	35.5 (20)	0.64	0.79	26	3.46
		Low			16.6 (9)	15.6 (28)	1.07	0.97	14	2.62
		High			26.2 (17)	75.0 (79)	0.35	0.97	10	3.63
	K6	Global	86	16.5	30.3 (4)	30.6 (18)	0.99	0.77	26	4.33
		Low			21.8 (6)	11.8 (23)	1.85	0.85	14	2.84
		High			38.1 (14)	99.8 (53)	0.38	0.98	10	3.84
	K7	Global	86	23.2	22.7 (4)	34.1 (18)	0.67	0.78	26	3.14
		Low			17.2 (7)	16.1 (22)	1.07	0.87	14	2.10
		High			31.4 (17)	149.4 (49)	0.21	0.98	10	2.98
	K8 ^b	Global	800	2.2	13.2 (5)	7.3 (32)	1.82	0.65	26	3.04
		Low			12.7 (9)	6.3 (46)	2.03	0.82	14	2.99
		High ^c			13.5 (20)	9.5 (590)	1.43	0.96	10	3.36
	K9	Global	920	0.7	16.6 (4)	5.9 (27)	2.83	0.64	26	5.74
		Low			20.5 (4)	11.7 (15)	1.76	0.85	14	3.43
		High			15.1 (7)	AV ^d	AV	0.88	10	4.70

^a The values in parentheses are standard errors, expressed as percentages.

^b Biphasic extracellular proteolytic activity was not revealed by the *F* test at *P* < 0.1.

^c Kinetic parameters were not determined.

^d AV, aberrant value.

availability suddenly increases. With Leu-MCA, this nonlinear relationship between the actual concentrations available and the concentrations added (used to determine enzymatic kinetics) may alter the classic shape of Michaelis-Menten kinetics.

Relevance of the substrate concentration range. The range of substrate concentrations is the key factor in determining enzymatic kinetics. Usually, handling difficulties limit the number of substrate concentrations used. Furthermore, the lowest and highest concentrations used are often arbitrarily defined since the natural concentrations and the main characteristics of the microbial consortia frequently are not known before a new series of samples is processed. If the range of concentrations is ill-adapted, the kinetic parameters cannot be determined. Thus, parameter indeterminations and the correlation factor cfr are statistical tools which allow researchers to evaluate the adequacy of the range of substrate concentrations used (Tables 2 and 3). If the cfr value is greater than 0.98, then all of the substrate concentrations are in the linear part of Michaelis-Menten kinetics. In this case only the V_m/K_m ratio (i.e., the initial slope of the kinetics) can be determined, while each parameter value is meaningless and both parameter indeterminations are high. If parameter indeterminations show that V_m can be determined but K_m cannot be determined (the cfr value is acceptable), then all substrate concentrations are in the saturation part of Michaelis-Menten kinetics.

The mean cfr values for global kinetics for sediment and NBW samples were 0.92 and 0.71, respectively. Higher cfr values were obtained for sediment samples, in which the proteolytic activities were nearly 1,000-fold higher than the pro-

teolytic activities in NBW samples due to higher levels of bacterial abundance (Tables 2 and 3). As the concentrations of Leu-MCA added were in the same micromolar range for the water and sediment samples, it was more likely that this range covered the saturation part of Michaelis-Menten kinetics in the NBW samples than in the sediment samples. The concentrations of Leu-MCA used (10 to 1,000 μ M) were chosen in order to include the natural combined leucine concentrations, which were expected to vary between 50 and 300 μ M in surficial sediments (9), and therefore, we could obtain measurements under natural substrate concentration conditions. This raised the problem of choosing between the following two strategies for measuring enzymatic activity: (i) increase the number of measurements in order to include all enzymatic kinetics and ensure accurate determinations of both kinetic parameters and (ii) limit measurements to in situ substrate concentrations in order to focus on estimating in situ enzymatic activity rates while allowing some kinetic parameters to be undetermined.

For almost all of the samples, the parameter standard errors and the cfr values were higher for both the low and high kinetics than for the corresponding global kinetics (Tables 2 and 3). This resulted from using truncated ranges of Leu-MCA concentrations to determine the V_m and K_m values of each phase of the biphasic kinetics. However, as shown in Tables 2 and 3, 70% of the kinetic parameters were correctly determined (cfr < 0.98), which confirmed the relative suitability of the range of concentrations used.

Evaluation of the biphasic mode. For the entire set of samples which we studied, biphasic kinetics appeared to be the

TABLE 3. Microbiological and statistical parameters for sediment samples collected in the winter and the summer at four sampling stations in the northwestern Mediterranean Sea

Season	Station	Slice (cm)	Kinetics	Depth (m)	Bacterial concn (10^8 cells ml^{-1})	V_m ($\mu\text{M h}^{-1}$)	K_m (μM)	V_m/K_m (h^{-1})	cfr	ndf	S_{sys} (10^{-2})
Winter	K1	0-2	Global	35	10.1	455.1 (22) ^a	445.1 (17)	1.02	0.95	6	281
			Low			112.4 (12)	39.8 (28)	2.82	0.95	2	85.2
			High			487.6 (6)	542.3 (11)	0.90	0.96	2	158
		2-4 ^b	Global ^c		11.2	8.2×10^3 (2×10^4)	145.0×10^3 (2×10^3)	0.06	1	6	60.8
			Low ^c			24.0 (44)	166.6 (63)	0.14	0.99	2	14.7
			High ^c			1.3×10^{11} (9×10^7)	2.3×10^{12} (9×10^7)	0.06	1	2	62.9
		4-6 ^b	Global ^c		9.8	43.5 (18)	2,017.3 (24)	0.02	0.99	6	9.5
			Low ^c			7.9×10^7 (6×10^8)	3.56×10^9 (6×10^8)	0.02	1	2	5.4
			High ^c			44.6 (31)	2,092.7 (42)	0.02	0.99	2	15.3
	K2	0-2	Global	86	19.4	432.2 (5)	278.0 (12)	1.55	0.92	6	234
			Low			122.3 (7)	38.3 (16)	3.20	0.95	2	55.2
			High			427.2 (4)	266.7 (10)	1.60	0.94	2	169
		2-4 ^b	Global		10.6	62.2 (49)	1,145.2 (76)	0.05	0.98	6	85.9
			Low			4.5 (12)	6.3 (65)	0.72	0.82	2	13.1
			High ^c			64.9 (85)	1,231.9 (131)	0.05	0.99	2	132
		4-6 ^b	Global		8.4	18.1 (9)	298.1 (21)	0.06	0.92	6	18.5
			Low ^c			16.7 (54)	269.7 (69)	0.06	0.99	2	6.1
			High			18.1 (18)	298.0 (46)	0.06	0.94	2	31.5
	K3	0-2	Global	800	11.2	164.9 (8)	154.8 (25)	1.07	0.87	6	246
			Low			82.7 (1)	37.3 (3)	2.22	0.94	2	8.6
			High			179.5 (15)	214.5 (45)	0.84	0.93	2	321
		2-4	Global		9.3	207.8 (29)	628.9 (53)	0.33	0.96	6	334
			Low			142.7 (43)	195.6 (58)	0.73	0.99	2	59.3
			High			309.2 (45)	1,294.6 (69)	0.24	0.98	2	301
		4-6 ^b	Global		8.6	143.2 (6)	109.0 (21)	1.31	0.81	5	189
			Low ^c			481.1 (363)	561.6 (411)	0.86	0.99	2	228
			High			134.4 (0.1)	70.2 (0.6)	1.91	0.86	1	2.4
	K4	0-2	Global	920	3.5	56.0 (11)	139.0 (34)	0.40	0.86	22	225
			Low			23.2 (10)	8.4 (42)	2.76	0.83	10	85.0
			High			80.0 (19)	426.9 (42)	0.19	0.95	10	213
		2-4	Global		4.8	216.0 (13)	386.5 (29)	0.56	0.94	22	478
			Low			52.5 (12)	16.1 (40)	3.26	0.88	10	160
			High			258.3 (21)	590.5 (42)	0.44	0.96	10	557
		4-6	Global		6.9	92.6 (9)	161.3 (28)	0.57	0.88	22	286
			Low			45.2 (7)	22.9 (40)	1.97	0.91	10	65.1
			High			133.2 (15)	477.0 (42)	0.28	0.96	10	255
Summer	K5	0-2 ^b	Global	35	9.1	202.4 (10)	602.9 (18)	0.34	0.96	6	65.2
			Low			80.8 (16)	148.0 (27)	0.55	0.98	2	18.2
			High			219.4 (17)	715.3 (31)	0.31	0.97	2	85.1
		2-4 ^b	Global		10.0	77.0 (30)	727.6 (51)	0.11	0.97	6	60.3
			Low ^c			3.1×10^{23} (6×10^8)	4.3×10^{24} (6×10^8)	0.07	1	2	10.6
			High			68.4 (46)	561.0 (93)	0.12	0.96	2	98.8
		4-6	Global		14.0	32.8 (12)	593.0 (22)	0.06	0.96	6	26.3
			Low			10.6 (14)	85.5 (30)	0.12	0.97	2	8.9
			High			37.8 (12)	798.8 (21)	0.05	0.98	2	18.6
	K6	0-2 ^b	Global	86	11.1	233.2 (11)	367.2 (23)	0.64	0.93	6	250
			Low			84.2 (4)	52.4 (9)	1.61	0.95	2	26.4
			High			260.9 (14)	487.4 (30)	0.54	0.96	2	245
		2-4 ^b	Global		8.0	27.6 (4)	404.7 (8)	0.07	0.94	6	9.9
			Low ^c			138.4 (452)	2,477.9 (474)	0.06	0.99	2	14.6
			High			27.7 (0.7)	407.6 (2)	0.07	0.95	2	1.5
		4-6 ^b	Global		10.5	15.2 (13)	244.7 (31)	0.06	0.91	6	27.5
			Low ^c			2.2×10^{16} (1×10^8)	5.9×10^{17} (1×10^8)	0.04	1	2	3.4
			High			13.6 (21)	160.3 (73)	0.09	0.92	2	39.3
	K7	0-2	Global	10.7	10.7	104.3 (14)	310.2 (32)	0.34	0.92	6	172
			Low			45.1 (12)	46.0 (33)	0.98	0.94	2	55.3
			High			133.4 (6)	578.7 (13)	0.23	0.96	2	49.0
		2-4 ^b	Global		10.3	32.5 (10)	663.8 (18)	0.05	0.96	6	19.3
			Low			11.5 (34)	130.3 (61)	0.09	0.98	2	14.0
			high			36.4 (9)	833.6 (16)	0.04	0.98	2	13.1
		4-6 ^b	Global		7.1	20.2 (14)	534.8 (26)	0.04	0.95	6	20.4
			Low			14.2 (29)	292.0 (40)	0.05	0.95	2	4.5
			High			22.7 (28)	689.5 (52)	0.03	0.97	2	31.1
	K8	0-2	Global	800	10.9	139.0 (11)	110.9 (36)	1.25	0.86	6	344
			Low			75.6 (4)	15.5 (19)	4.87	0.86	2	61.0

Continued on following page

TABLE 3—Continued

Season	Station	Slice (cm)	Kinetics	Depth (m)	Bacterial concn (10^8 cells ml^{-1})	V_m (μM h^{-1})	K_m (μM)	V_m/K_m (h^{-1})	cfr	ndf	S_{sys} (10^{-2})
		2-4	High			174.2 (7)	259.4 (18)	0.67	0.93	2	125
			Global		9.5	166.7 (11)	180.0 (30)	0.93	0.89	6	310
			Low			76.1 (9)	21.9 (32)	3.47	0.88	2	103
		4-6	High			197.4 (4)	313.3 (10)	0.63	0.94	2	76.1
			Global		7.4	151.7 (14)	170.6 (38)	0.89	0.89	6	358
			Low			77.4 (6)	26.9 (20)	2.88	0.90	2	63.7
			High			195.0 (18)	375.6 (42)	0.52	0.95	2	294
	K9	0-2	Global	920	12.6	38.9 (27)	172.3 (73)	0.23	0.89	6	345
			Low			16.5 (4)	4.1 (48)	4.03	0.82	2	35.1
			High			79.0 (56)	948.0 (94)	0.08	0.98	2	275
		2-4	Global		8.9	31.5 (21)	120.8 (64)	0.26	0.86	6	176
			Low			17.2 (15)	10.7 (87)	1.61	0.85	2	69.2
			High			49.6 (27)	498.1 (57)	0.10	0.96	2	112
		4-6 ^b	Global		8.8	609.0 (14)	322.4 (31)	1.89	0.93	6	1,046
			Low ^c			1.5×10^9 (2×10^8)	1.3×10^9 (2×10^8)	1.14	1	2	129
			High			565.2 (19)	254.3 (51)	2.22	0.92	2	1,492

^a The values in parentheses are standard errors, expressed as percentages.

^b Biphasic extracellular proteolytic activity was not revealed by the *F* test at $P < 0.1$.

^c Kinetic parameters were not determined.

most common kinetic mode for ectoproteolysis, mainly at the water-sediment interface. The highest percentages of occurrence of microbial biphasic ectoproteolysis were found in the NBW and superficial layer of sediment, where 77.8 and 66.7%, respectively, of the samples studied were characterized by a biphasic mode. It is possible to assume that detection of a

biphasic mode in sediment samples is an artifact resulting from adsorption processes, as mentioned above. However, detection of biphasic kinetics in NBW samples as well as in sediment samples suggests that the biphasic mode cannot be an experimental artifact resulting from adsorption processes. Conversely, real biphasic kinetics may remain undiscovered due to missing points at high concentrations. This may occur in sediment samples in which the ranges of Leu-MCA concentrations used may be inadequate considering the high proteolytic activities present (compared to NBW samples). Note that detection of the biphasic mode was not altered when the kinetics was underdetermined. Indeed, the difference in concentration between the low and high kinetic data implies that only some of the kinetics was detected.

Even though the variance test (*F* test) can result in validation of the actual existence of biphasic kinetics, this test is relatively tedious, and it is not appropriate for comparing the importance of the biphasic mode in different samples. For this reason, we defined the biphasic indicator as follows:

$$Ib = (K_{m\text{high}}/V_{m\text{high}})/(K_{m\text{low}}/V_{m\text{low}}) \quad (8)$$

where K_m/V_m values correspond to the slopes of the lines for high and low kinetics in the Lineweaver-Burk representation (Fig. 1) and *Ib* is a measure of the angle between the two lines. It is interesting that globally the highest *Ib* values were obtained for samples exhibiting microbial biphasic kinetics, as revealed by the *F* test at $P < 0.1$. This result validates the statistical conclusion that a biphasic mode actually exists in microbial populations. *Ib* can also be interpreted as the ratio between the initial slopes (i.e., V_m/K_m) of high and low Michaelis-Menten kinetics. Therefore, *Ib* measures the importance of the biphasic mode only in the linear part of Michaelis-Menten kinetics, not in the saturation part. Fortunately, most of our data allowed us to correctly determine both low and high V_m/K_m ratios. For some NBW samples (samples K2, K8, and K9), the standard error for K_m was at least 100 times greater than the standard error for V_m (Table 2). For these samples, only $V_{m\text{high}}$ was constrained and $K_{m\text{high}}$ was not constrained; therefore, *Ib* was not calculated as not significant.

Origin of the observed biphasic kinetics. Several authors have described the coupling between bacterial uptake of low-

FIG. 3. Data resulting from the model responses for two surface sediment samples (depth 0 to 2 cm) collected in coastal (A) and pelagic (B) areas during the winter (BBL2 cruise). The dashed and solid lines are the model responses for high and low substrate concentrations, respectively.

FIG. 4. Evolution of the kinetic mode through the sediment (indicator of biphasicity I_b) during the summer ($n = 5$) and the winter ($n = 4$) (A) and in the pelagic area ($n = 4$) and the coastal area ($n = 5$) (B).

molecular-weight compounds or bacterial biomass production and the seasonal fluctuations in organic matter input in benthic systems (2, 7, 15, 19, 22, 33, 38, 46, 52). Recently, in a study of deep sediments of the subantarctic Indian Ocean sector, Talbot and Bianchi (50) suggested that biphasic kinetics could be an adaptation of marine bacteria to the episodic supply of organic material reaching the seafloor and that the two types of kinetics play a role in a "hydrolysis relay" for the degradation of polymeric compounds (one type of kinetics for low concentrations and the other for high concentrations). The observation that the biphasic kinetics of ectoenzymatic processes occurred in both the NBW and sediment layers of the coastal and pelagic areas during the winter and summer sampling periods could support this hypothesis.

Probably because of the lack of sufficient data, the I_b values (equation 8) for the sediment layers did not reveal significant differences between the winter and summer periods (Fig. 4A). However, the major differences in the I_b values were the differences between the values for the coastal and pelagic surficial sediments (Fig. 4B). Furthermore, biphasicity in the sediment layers appeared to be more pronounced in pelagic sediments than in coastal sediments. As shown in Fig. 5, this more pronounced biphasicity in the deep sea was mainly due to higher $V_{m_{low}}/K_{m_{low}}$ values for coastal bacterial communities. It has been demonstrated that in the open ocean only about 10% of the primary production descends down to 150 m (1) and only 1% of the photosynthetically produced material reaches the sea bottom at depths of 4 to 5 km (54). Considering such poor organic material input, it is possible to assume that a high $V_{m_{low}}/K_{m_{low}}$ ratio is a fundamental adaptation of a bacterial enzymatic system which allows optimal survival in the deep sea.

FIG. 5. Comparison of the V_m/K_m ratios for low and high kinetics in the surficial sediment layer (depth, 0 to 2 cm) for the pelagic area ($n = 4$) and the coastal area ($n = 5$). The errors bars indicate standard deviations.

Even if our observations suggest that biphasic kinetics could reflect adaptation of bacterial communities to episodic food supplies, a hypothesis that could satisfy the ecological point of view, we should consider other mechanisms that may account for multiphasic kinetics measurements. Primarily, experimental data do not indicate the enzymatic activities of natural bacterial consortia. In multispecies consortia, each bacterial species may produce its own enzymatic system characterized by specific kinetic properties. Biphasic kinetics cannot be considered adaptive mechanisms but more likely are selective mechanisms which result in optimal efficiency in polymer degradation by the bacterial community. Furthermore, in natural bacterial communities, each bacterial species may produce several kinds of proteases which have broad specificity (11). Indeed, using a collection of 44 bacterial strains, Martinez et al. (35) demonstrated that cell-specific proteolytic activities varied over a very broad range. In fact, experimental data clearly show that benthic bacterial communities are able to efficiently degrade protein compounds at a wide range of substrate concentrations, expected to cover the natural variability in nutrient concentrations, but at this time we are not able to precisely determine which mechanism actually manages the multiphasic kinetics observed.

Conclusion. The accuracy of kinetic parameter values depends on the range of Leu-MCA concentrations used. This study shows that with sediment samples, a lack of preliminary information concerning the natural concentrations of organic biopolymers makes it difficult to determine the ideal range of concentrations for the substrate added. This range should be wide enough to avoid indetermination of some kinetic parameters. In this study most kinetic parameters were correctly determined, which confirmed the suitability of the range of concentrations used. Our results, therefore, provide further evidence that benthic bacterial populations use multiphasic ectoenzymatic processes to hydrolyze biopolymers. The highest percentages of biphasic kinetics versus monophasic kinetics occur in the NBW and in the topmost sediment pellicle, precisely where inputs of organic polymers fluctuate greatly depending on the seasonal flux of particles from the surface. Due to this microbial enzymatic process, it is essential to determine the maximal ectoproteolytic velocities by a multiconcentration method. This condition is enhanced in sediment environments in which microbial populations are known to be more diverse.

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