APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1988, p. 784–790
0099-2240/88/030784-07$02.00/0

Vol. 54, No. 3

Microbial Decomposition in Aquatic Environments: Combined Process of Extracellular Enzyme Activity and Substrate Uptake

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Received 20 July 1987/Accepted 11 November 1987

The aim of this study was to define a model for the coupling between extracellular enzyme activity and substrate uptake by bacterial populations in natural waters. The balance between uptake of leucine and extracellular hydrolytic production of leucine from a peptide model substrate was investigated in a combined fluorescence-radiotracer experiment with [^{3}H]leucine as a marker for the leucine pool and L-leucine-4 methyl-7-coumarinylamide (Leu-MCA) as a marker for the pool of dissolved peptide substrates. Results show that at low concentrations of the model substrate the input and uptake processes of leucine are nearly balanced, whereas at high concentrations of the model substrate much more leucine is liberated than taken up. In addition, samples from one polluted and one less polluted station in the Kiel Fjord were investigated for their extracellular enzymatic and uptake properties in an annual cycle. It was found that turnover rates of leucine (T_r, percent per hour) and hydrolysis rates of Leu-MCA (H_r, percent per hour), as well as the quotient T_r/H_r, reflect the impact of environmental conditions on decomposition processes at both sampling sites. The quotient T_r/H_r is interpreted as an indirect measurement of the pool size ratio (polymers/monomers), which may serve as an index of hydrolysis-uptake coupling in bacterial utilization of dissolved protein. Calculated on an annual average basis, turnover rates are ca. nine times higher than hydrolysis rates at the polluted station and ca. five times higher at the less polluted station. From the described model, this would mean that the relative fraction of polymers within the total dissolved organic carbon pool (with regard to the substrate combination dissolved protein-leucine) is about twice that at the polluted than at the less polluted station.

Contrary to primary production, decomposition is not a uniform process. Naturally synthesized polymers and refractile macromolecules have to undergo extracellular enzymatic decomposition before they are ready for incorporation by and final oxidation in bacteria. Once particulate organic substrates have been brought into solution, they are nearly exclusively reserved for bacterial nutrition. Therefore, extracellular enzyme activity is a very important feature in the ecology of water because it is—along with grazing—responsible for that part of detritus which is introduced into the food chain via bacterial heterotrophic activity. It is, however, an open question whether low-molecular-weight compounds, which result from extracellular decomposition, are immediately and completely taken up by bacteria (5, 9). In a well-balanced system an equilibrium would be expected between the enzymatic production of monomers and their incorporation by bacteria. During times when the system is unbalanced, more monomeric substrates may be produced via extracellular enzyme reactions than can be used up by the bacterial population. This will cause an increase of low-molecular-weight organic matter (LMOM) in the dissolved organic carbon pool of the water. On the other hand, during times of low hydrolysis capacities LMOM may be used by bacteria until threshold concentrations are reached. In this way, the balance between extracellular enzyme activity and heterotrophic substrate uptake activity has a bearing on the organic chemistry of natural waters. Determination of this balance may serve as an index for characterizing a water body from the viewpoint of decomposition, because input and output from the LMOM pool and bacterial development are considered in one and the same approach. Occasionally this approach may be hindered by the contribution of phytoplankton exudation. This may also directly supply the pool of monomers to a certain extent. Because the bulk of the excreted substances seem to consist of oligomeric molecules rather than monomers (3), this influence is regarded as low.

The theoretical considerations behind our investigations have been addressed by Saunders (19) for the case of balanced decomposition processes. Unbalanced decomposition is, however, also very likely to occur in natural waters. It has to be admitted that the methodologies which are available to solve this problem are not fully satisfactory, and therefore our experiments should be regarded as a preliminary approach. In practice, water samples from different sites in the Kiel Fjord were analyzed for their heterotrophic uptake kinetics of leucine and extracellular enzyme activities on L-leucine-methylcoumarinylamide (Leu-MCA), which is regarded as a model substrate for peptides. Relationships between the hydrolysis rate (H_r) of polymers and the turnover rate (T_r) of corresponding monomers are discussed with regard to their influence on organic carbon pool sizes in seawater. The influence of increasing dissolved polymer concentrations on extracellular enzyme activity, heterotrophic substrate uptake, and the pool of LMOM was investigated in a laboratory experiment with natural water samples from the Kiel Fjord area.

MATERIALS AND METHODS

Sampling area. Surface water samples were taken from two sites of the brackish-water Kiel Fjord (Baltic Sea). The first was station Hauptpost, an enclosed harbor area with strong seasonal variability in salinity (8 to 24‰), occasional small-scale upwelling, and pollution. The average annual bacterial numbers per milliliter were 70,520 saprophytes and 3.6×10^6 total bacteria; the average chlorophyll *a* concen-

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tration was 9.3 μg liter^{-1}. The second was station Feuerschiff, which was situated in front of the fjord mouth, was less polluted than station Hauptpost, and was temporarily influenced by water masses from the inner fjord or from more distant regions. The average annual bacterial numbers per milliliter were 3,100 saprophytes and 1.5×10^6 total bacteria; the average chlorophyll *a* concentration was 3.5 μg liter^{-1}. The average water temperature of the fjord is about 10°C; in winter it is about 2°C, and in summer it may rise to 22°C at the surface (7).

Microbiological methods. A sterile surface-water sampler was used for sampling. Total numbers of bacteria and their biomass were determined by epifluorescence microscopical techniques (25). Saprophytes were detected on ZoBell agar of appropriate salinity. Heterotrophic uptake kinetics of natural bacterial populations were investigated with tritium-labeled leucine as described by Gocke (4) and Hoppe (6). Values of heterotrophic substrate uptake were compared with bacterial extracellular enzyme activities, which were determined by the hydrolysis of the model substrate Leu-MCA, which is known as a substrate for aminopeptidases. Fluorogenic MCA and methylumbelliferyl substrates have been used for extracellular enzyme studies of natural waters by Hoppe (8, 9), Somville (22), and Somville and Billen (23) and of marine sediments by King (12) and Meyer-Reil (13). Rego et al. (17) applied a fluorogenic model substrate for the determination of free and attached proteolytic activity. Further details of the use of fluorogenic substrates in ecological investigations were supplied by Pancholy and Lynd (14), Pettersson and Jansson (15) and recently by Kim and Hoppe (11) and Snyder et al. (21). Until now the usefulness of fluorogenic substrates as a tool for measuring enzyme activities has not been fully explored, and no commonly accepted standard procedure has been described for their application.

The method, as used throughout this study, is as follows. Stock solutions (5 mM and 0.5 mM) of the MCA substrate Leu-MCA (Fluka AG, Buchs, Switzerland) were prepared with Methyl Cellosolve (ethylene glycol monomethyl ether) as a solvent. Methyl Cellosolve is frequently used in biochemical studies; it does not significantly influence enzyme activities in the low concentrations used in our experiments. (Some MCA substrates are also soluble in water, and in this case water is preferred to the organic solvent.) For the experiments, each of six scintillation vials was filled with 20 ml of the water sample and supplemented with 4, 40, 200, or 400 μl of the 0.5 mM stock solution and 500 or 1,000 μl of the 5 mM stock solution. The resulting final MCA substrate concentrations ranged from 0.1 to 250 μmol liter^{-1}. A blank was prepared by boiling 20 ml of sample water in a water bath for 20 min, cooling, and then adding 200 μl of the 0.5 mM stock solution. The initial fluorescence was read immediately after addition of the MCA substrate (zero time reading). For this, 2.5-ml subsamples were taken from the scintillation vials and adjusted to pH 10 with 200 μl of pH 10 borate buffer (Merck). Fluorescence was measured with a spectrofluorimeter (Kontron SFM 25) at 364 nm excitation and 445 nm emission wavelengths. The fluorescence reading was repeated after 3 h of incubation at the in situ temperature.

An MCA calibration curve was established by dissolving MCA in Methyl Cellosolve and sterile water from the fjord to a concentration of 0.5 μmol liter^{-1}. Fluorescence readings for calibration were taken as described above.

The velocity of hydrolysis of MCA substrates by bacteria was calculated from the formula $V = (F_s \times 72)/F_c$, where V is the velocity of the enzymatic hydrolysis (in micrograms of carbon content of leucine [C_{Leu}] per liter per hour), F_s is the increase of fluorescence in the water sample per hour, 72 is a factor to convert micromolar concentration into micrograms of carbon (valid only for MCA substrates containing six carbon atoms in the amino acid component), and F_c is the slope of the MCA calibration curve (fluorescence per micromole of MCA). Values of V for the different MCA substrate concentrations used in the experiments were plotted as a Lineweaver-Burke transformation of the Michaelis-Menten equation. The final results are expressed in terms of maximal velocity of hydrolysis (V_m, in micrograms of C_{Leu} per liter per hour) and hydrolysis time or rate of hydrolysis (H_t, in hours; H_r, in percent per hour). Instead of using enzyme kinetics, it is possible to calculate H_t and H_r from experiments in which a very small amount of the model substrate was added to the water sample.

The detection limit of MCA and thus of C_{Leu} hydrolyzed from the complex molecule is in the nanomolar range. The reproducibility as calculated from sets of 10 parallel experiments is $s_m = 6.3\%$ at 0.1 μmol of Leu-MCA liter^{-1}, 4.4% at 10 μmol of Leu-MCA liter^{-1}, and 2.6% at 100 μmol of Leu-MCA liter^{-1}. Leu-MCA hydrolysis does not depend on the experimental volume of water.

Epifluorescence microscopical observations supported the assumption that hydrolysis of the model substrate occurred outside the cytoplasmic membrane, according to the definition of Priest (16). The extracellular enzyme activities presented here include both free and attached extracellular enzymes. Results of size fractionation of water samples were published by Hoppe (9).

Leucine uptake by microorganisms was traced by using [^{3}H]leucine and unlabeled leucine at concentrations between 0.007 and 50 μg of C_{Leu} liter^{-1}. V_m and T_t (derived from T_t: turnover rate [T_r] in percent per hour) were calculated from the Lineweaver-Burke regression line by the method of Wright and Hobbie (24). Conditions for incubation were the same as in the extracellular enzyme activity experiments (3 h at the in situ temperature). The method was described in detail by Gocke (4) and Hoppe (6).

The way in which turnover rates (of leucine) and hydrolysis rates (of Leu-MCA as a model substrate for peptides) were compared is explained below (see Results and Discussion). Despite the basic methodological problems, this approach may be at least of some theoretical value. The chlorophyll *a* content was determined by standard procedures (20).

Use of Leu-MCA as a model substrate for peptides. The model substrate (Leu-MCA) does not occur in nature; however, it is susceptible to hydrolysis by bacterial extracellular enzymes. The fluorigenic compound of the model substrate, in this case MCA, is inert to microbial decomposition, and thus its accumulation is related to the decomposition of the model substrate and of the natural peptide pool as well.

The quality of the model substrate as an analog substance for natural peptides is very important if the results with model substrate decomposition are to be extrapolated to the natural peptide pool. Obviously a model substrate for peptides can never be a universal one. Nevertheless, its use may be justified if it has some important properties in common with true peptides: (i) its enzymatic decomposition should be affected by peptidase inhibitors, (ii) its decomposition should be (competitively) inhibited by natural peptides, and (iii) its decomposition should also be representative of the decomposition of other amino acid MCAs.

Extracellular enzymes of natural bacteria populations involved in the hydrolysis of Leu-MCA are partly inhibited

TABLE 1. Relationship between hydrolytic decomposition of Leu-MCA and bacterial uptake of leucine[a]

Leucine concn (μg of C liter^{-1})[b]	Leucine dpm	Leucine V[c]	Leu-MCA concn (μg of C liter^{-1})[d]	Leu-MCA dpm	V_{uptake}[e]	$V_{Leu\text{-}MCA}$[f]	$V_{Leu\text{-}MCA}/V_{uptake}$
0.0051	393,529	0.0007	7.2	307,479	0.04	0.1213	3
0.0775	388,479	0.0112	36	230,943	0.065	0.4770	7.3
0.7295	234,707	0.0639	72	159,837	0.09	0.8206	9.1
3.6272	85,232	0.1154	360	75,091	0.115	2.4052	20.9
7.2443	47,213	0.1276	720	66,817	0.12	3.8927	32.4
36.2215	17,046	0.2304	7,200	12,000	0.29	10.8899	37.6

[a] Results are from a combined radiotracer-fluorescence tracer experiment (for further explanation, see the text).
[b] Concentration of leucine is always 0.0051 μg of C liter^{-1} for [^{3}H]leucine plus various amounts of unlabeled leucine.
[c] Velocity of leucine uptake (in micrograms of C per liter per hour).
[d] Combined experiment; concentrations of Leu-MCA are indicated in terms of micrograms of C_{Leu} per liter, and the concentration of [^{3}H]leucine is always 0.0051 μg of C liter^{-1}.
[e] Velocity of leucine uptake in the combined experiment (micrograms of C per liter per hour).
[f] Velocity of proteolytic activity (micrograms of C per liter per hour).

by a variety of peptidase inhibitors. Iodoacetamide and *p*-hydroxymercuribenzoate (thiol protease inhibitors), phenylmethylsulfonyl fluoride (a serine protease inhibitor), dithiothreitol and L-cysteine (metalloprotease inhibitors), and pepstatin (a carboxyl protease inhibitor) were used for these experiments at final concentrations of 1 to 10 mmol liter^{-1} as recommended in the literature (1, 2). Inhibition ratios of these inhibitors—all of them were effective—on Leu-MCA hydrolysis were between 10 and 68%.

Substrate inhibition experiments on Leu-MCA hydrolysis were carried out with a variety of dimeric and oligomeric peptides (Leu-Leu, Gly-Leu, Leu-Gly, Leu-Leu-Leu, Ala-Val-Leu, Leu-Leu-Leu-Phe-OMe, angiotensin [10 amino acids], and tryptone). The inhibition constant (K_i) and the type of inhibition were not determined, but only the degree of inhibition at certain peptide and Leu-MCA concentrations. For this, Leu-MCA hydrolysis by natural microbial populations was tested in parallel experiments (i) with a certain molar concentration of Leu-MCA, (ii) with double the Leu-MCA concentration as used in experiment (i), and (iii) with the concentration of Leu-MCA used in experiment (i) plus the same molar concentration of the peptide in question. Inhibition was expressed as a percentage and was 0% if the peptide did not influence Leu-MCA hydrolysis and 100% if the Leu-MCA hydrolysis in experiment (iii) was exactly half of that obtained in experiment (ii).

Dimeric peptides had a relatively weak effect on Leu-MCA hydrolysis by natural bacterial populations; probably they do not need to undergo splitting before incorporation. Inhibition by Leu-Leu was only 28%. With the tripeptide Ala-Val-Leu, inhibition of Leu-MCA hydrolysis was 43%, and a similar value was obtained with tryptone (from Sephadex column chromatography, an average molecular weight of 655 was used). Leu-Leu-Leu-Phe-OMe and angiotensin gave inhibition values of 100% or even more in the sense defined here, which would suggest that they are split with at least the same velocity than Leu-MCA.

Hydrolysis of Leu-MCA by natural bacterial populations is in some cases very similar to the hydrolysis of other amino acid-MCA combinations (Phe-MCA, Ala-MCA, Tyr-MCA). This would mean that Leu-MCA is also representative of other possible peptide model substrates. On the basis of these preliminary experiments, a definition of the meaning of H_r (hydrolysis rate of polymers) may be given: H_r represents the turnover rate of the part of the natural peptide pool which is susceptible to extracellular enzymes which also hydrolyze the model substrate. Because Leu-MCA is not hydrolyzed exclusively by Leu-peptidases but also by other peptidases, we claim that its decomposition is closely related to the turnover of a considerable part of the natural peptide pool. This must be kept in mind for the evaluation of the following experiments and considerations.

RESULTS

The first aim of the study was to investigate the relationship between bacterial extracellular enzyme activity and heterotrophic substrate uptake of compounds resulting from extracellular enzymatic decomposition. This was done in a combined radiotracer-fluorescence tracer experiment with [^{3}H]leucine as a tracer for heterotrophic bacterial uptake and Leu-MCA as a tracer for leucine production from a peptide compound. At first, [^{3}H]leucine incorporation in a natural brackish water sample was measured at increasing concentrations of leucine (Table 1, columns 1, 2, and 3). Parallel to this, the incorporation of [^{3}H]leucine was measured at increasing concentrations of Leu-MCA (column 4). In both experiments, only one concentration of [^{3}H]leucine (0.0051 μg of C liter^{-1}) was used; the rest was unlabeled leucine. The resulting ^{3}H uptake counts in the presence of Leu-MCA are shown in column 5. From the data in columns 2 and 3 and in column 5, the uptake velocity of [^{3}H]leucine during Leu-MCA hydrolysis can be calculated (V_{uptake}, column 6) in the following way. The disintegrations per minute and V values from columns 2 and 3 are plotted against each other. The resulting calibration curve is then used for readings of V_{uptake} by insertion of the disintegrations per minute from column 5. Leu-MCA hydrolysis must be measured in a third parallel experiment, with the same Leu-MCA concentrations as in the combined experiment. Values for the velocity of leucine liberation (in micrograms of C per liter per hour) from the model peptide substrate Leu-MCA are listed in column 7. The final result of this complex experiment, the relationship between the velocity of leucine production via extracellular enzymatic hydrolysis of the model peptide substrate ($V_{Leu\text{-}MCA}$) and the bacterial uptake of leucine during this process at increasing concentrations of Leu-MCA (V_{uptake}) is presented in column 8.

The values in column 8 show a clear tendency concerning the $V_{Leu\text{-}MCA}/V_{uptake}$ relationship. Although there is a relatively close relationship between the parameters at low concentrations of the model peptide (leucine supplier), an increasing discrepancy becomes obvious at higher Leu-MCA concentrations. This means, from the ecological viewpoint, that liberation of leucine increases much faster than its uptake at increasing concentrations of the model peptide substrate. At low concentrations of the model substrate, production is nearly balanced by the bacterial uptake of the

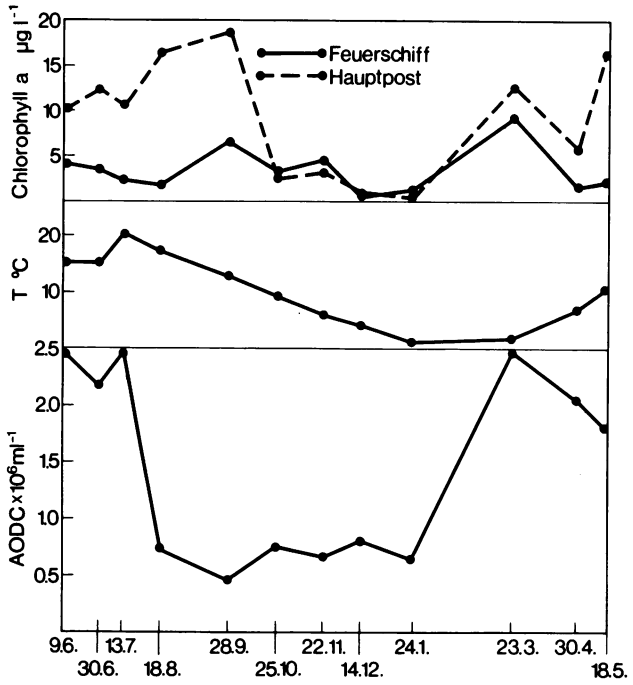

FIG. 1. Annual variations of chlorophyll *a* concentrations, water temperature, and total bacterial numbers in the sampling area. The shape of the curves for water temperature (T) and acridine orange direct counts (AODC) are at station Hauptpost similar to those for station Feuerschiff. Numbers along the abscissa represent dates (day.month.).

hydrolysis product. At high concentrations a considerable amount of the hydrolysis product is not immediately incorporated, but is temporarily introduced into the dissolved organic carbon pool of LMOM. By extrapolating the results to the even lower concentrations of the model substrate than we used, a nearly complete balance between hydrolytic production of a compound and its bacterial incorporation can be expected. Balanced and unbalanced growth with respect to these parameters may occur in natural situations as well. The ecological implications are discussed below.

Subsequently, the balance between the turnover rate of leucine (T_r), as an example of an easily incorporable monomer, and the hydrolysis (H_r) of peptides, as a process of leucine supply, was studied in an annual cycle at two different sites in the Kiel Fjord. Background data are provided in the first paragraph of Materials and Methods and in Fig. 1. The quotient T_r/H_r is regarded as a new approach to the characterization of microbial decomposition activities in aquatic ecosystems (Table 2). During the investigation, turnover rates (T_r) of leucine varied at the inner fjord station Hauptpost between 0.29 and 12.13% h^{-1} with a mean value of 4.40% h^{-1}. The hydrolysis rate (H_r) of peptides (as far as they are represented by the model substrate Leu-MCA) ranged from 0.07 to 2.19% h^{-1}, with a mean value of 0.78% h^{-1}. At station Feuerschiff, where the conditions are temporarily influenced by the fjord or the open sea, turnover rates of leucine varied between 0.22 and 8.85% h^{-1}, with a mean value of 2.60% h^{-1}, and hydrolysis rates ranged from 0.08 to 17.79% h^{-1} with a mean value of 2.86% h^{-1}. The full set of data is listed in Table 2. Total bacterial numbers at station Hauptpost correlated significantly with T_r as well as with H_r ($r = 0.74$ and 0.67, respectively), whereas at station Feuerschiff only a weak correlation with T_r ($r = 0.59$) was found. T_r and H_r values correlated with each other at both stations (Hauptpost: $r = 0.74$; Feuerschiff: $r = 0.94$).

TABLE 2. Turnover rates (T_r) of leucine and rates of hydrolysis of Leu-MCA (H_r) at two stations in the Kiel Fjord area

Date (day/mo)	Station Hauptpost (inner fjord)		Station Feuerschiff (outer fjord)	
	T_r	H_r	T_r	H_r
9/6	3.9	0.55	4.1	0.31
30/6	5.1	2.19	8.5	13.50
13/7	12.1	1.89	8.9	17.79
18/8	7.2	1.84	1.5	1.06
28/9	5.2	1.13	1.3	0.33
25/10	2.3	0.11	2.2	0.19
22/11	2.0	0.08	0.6	0.11
14/12	0.3	0.07	0.2	0.08
24/1	0.7	0.08	0.5	0.09
23/3	5.9	0.57	1.6	0.41
30/4	1.0	0.33	0.5	0.21
18/5	7.1	0.53	1.3	0.17

The quotient of the leucine turnover rate and the rate of hydrolysis of peptides varied over a wide range over the year at both stations. The extreme values found were 2.3 and 24.4 at the inner fjord station Hauptpost and 0.5 and 13.1 at station Feuerschiff. The annual mean values were 9.19 and 4.93, respectively. The mean values used here are the arithmetical means, calculated from the T_r/H_r relationship of the single experiments. The difference in the annual mean values of the T_r/H_r relationship shows that the bacterial turnover of leucine compared with peptide hydrolysis at the inner fjord station is nearly double that at the offshore station and that peptide hydrolysis is faster at the offshore station than at the inner fjord station. The effect on microbial activity and substrate pool balance is discussed later in detail.

Bacterial substrate uptake and extracellular enzyme activity and thus also T_r/H_r values are certainly dependent on factors such as water temperature, primary production, bacterial numbers, and biomass and substrate supply. The range of this dependency, as it occurs in the North Sea and the Baltic Sea, has recently been reviewed by Rheinheimer (18). In the temperate zone, where the Baltic Sea is situated, these factors show seasonal changes, and it therefore appears reasonable to display T_r/H_r values seasonally (Table 3). T_r/H_r values were lowest in summer and highest in autumn and spring at both stations. They decreased during winter and increased during spring with increasing temperature and chlorophyll concentration. Lowest T_r/H_r values coincided with highest annual temperatures and bacterial

TABLE 3. Relationship between the turnover rate of leucine (T_r) and the rate of hydrolytic decomposition of Leu-MCA (H_r)[a]

Season	T_r/H_r for station:	
	Hauptpost (inner fjord)	Feuerschiff (outer fjord)
Spring	8.5	6.7
Summer	4.2	0.9
Autumn	16.7	7.2
Winter	6.8	4.0
Mean value	9.1	4.7

[a] Values are derived from an annual investigation in the Kiel Fjord (western Baltic Sea).

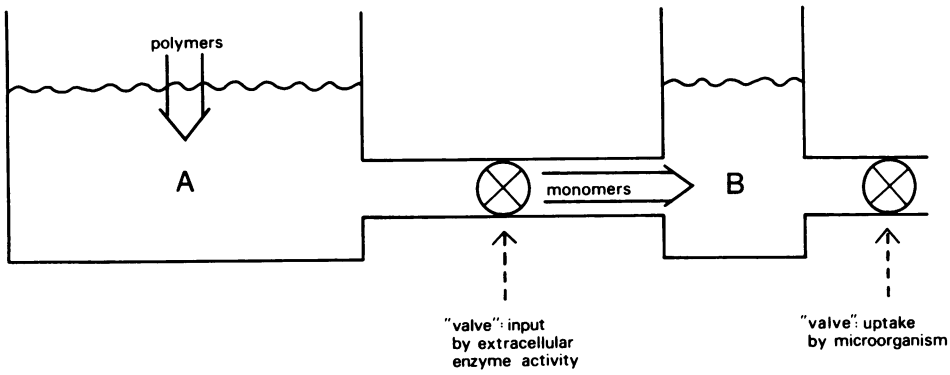

FIG. 2. Significance of the relationship T_r/H_r as a relative measurement of the pool size ratio: a theoretical example. T_r (turnover rate of leucine) and H_r (hydrolysis rate of proteins) are real measurements; the pool sizes of protein and of leucine are not known. A is the pool of polymers (protein) (H_r = 0.8% h^{-1}). B is the pool of monomers (leucine) (T_r = 4.8% h^{-1}). Our assumption is as follows. The actual pool of monomers (B) is fairly constant, which implies that the input from the polymer pool (A) is taken up by heterotrophic microorganisms within a short period. In the example, T_r/H_r = 6 (real measurements), B = 2 μg of C_{Leu} $liter^{-1}$ (hypothetical value), and A/B = ? (pool size ratio). The output from pool B = 2 × 4.8/100 = 0.096 μg of C_{Leu} $liter^{-1}$ h^{-1} = output from pool A. Pool size A = 0.096 ×100/0.8 = 12 μg of C_{leu} $liter^{-1}$. Therefore, A/B = 6. Consequently, the relationship between T_r and H_r equals the inverse relationship of the corresponding pool sizes.

numbers and increasing chlorophyll concentrations in summer. Highest values for this parameter were obtained during times of decreasing temperature and chlorophyll content and low bacterial numbers (autumn) and to a certain extent also during times of increasing temperature and relatively high bacterial numbers (spring).

DISCUSSION

Water bodies of different water quality are frequently characterized by different concentrations of dissolved organic materials and nutrient supply. The impact of heterotrophic microorganisms in natural waters is twofold: on the one hand they are limited by the rate of nutrient supply, and on the other hand they are responsible for the actual nutrient concentration. Because most of the dissolved organic substances in the sea normally occur in the form of combined molecules, this mechanism of regulation can be fully understood only if microbial extracellular enzyme activities and uptake capacities are observed simultaneously. A simple proof of this may be derived from turnover time and $K_t + S_n$ (natural concentration of the organic compound in question) determinations in in vitro experiments. Anticipating a certain initial value of T_t and $K_t + S_n$ for leucine (e.g., 3 h and 12.1 μg of C $liter^{-1}$) in a natural water sample, one should expect specific changes of these values when the in vitro incubation of the water sample continues for several days. However, it turned out that these parameters were remarkably stable, at least for incubation periods three to five times as long as the turnover time. This finding can probably be explained by assuming a tight coupling between extracellular enzyme and uptake activities of the bacteria, which guarantees the stability of the monomer substrate pool for a certain period at the expense of the polymer pool. This implies that the majority of dissolved free amino acids are indeed free, as has been stated by Jorgensen and Sondergaard (10). Consequently, there should be an equilibrium between the parameters T_r and H_r as they are defined in this investigation, and the range and level of this equilibrium may be judged as an interesting index to classify water bodies from the viewpoint of microbial ecology and water chemistry.

When working with natural water samples, it is normally not possible to determine the actual rate of microbial substrate uptake, because substrate concentrations in the water are not known. The V_ms of substrate uptake and extracellular enzyme activity have a close relationship to bacterial biomass and environmental conditions. However, they are not suitable for comparisons of substrate uptake and substrate input, because V_ms of extracellular enzyme activity are obtained at substrate concentrations which are unrealistically high for natural waters. The turnover rate (T_r) and the rate of hydrolysis by extracellular enzymes (H_r) are directly dependent on the natural substrate pool sizes and microbial activity. For monomeric substrate uptake, the substrate pool is well defined. Experimentally, this pool is labeled with a monomeric radiotracer. The uptake and turnover of the tracer compound reflect microbial utilization of the corresponding unlabeled compound in the natural water sample. This is not the case for a substrate pool which has to be hydrolyzed by extracellular enzymes (e.g., cellulose and peptides). At present, it is possible to characterize only the part of the pool which is analogous to the model substrate (e.g., methylumbelliferyl-α-glucose, Leu-MCA).

The quotient T_r/H_r is a relative measurement of the influence of heterotrophic microbial activities on selected substrate pools in the natural environment. The theory behind this statement is demonstrated by an example (Fig. 2). The parameters which can be obtained experimentally are H_r (e.g., 0.8% h^{-1}) and T_r (e.g., 4.8% h^{-1}). If we assume that the concentration of free dissolved leucine within the natural dissolved organic carbon pool is 2 μg of C_{Leu} $liter^{-1}$, this would mean that 0.096 μg of C_{Leu} $liter^{-1}$ is taken up by the bacteria within 1 h. For this calculation, the assumption has to be made that the actual leucine pool is fairly constant over time, which implies that the input from the polymer pool is more or less immediately taken up by the microorganisms. Proof of this assumption is given by the results of the combined input-uptake experiments (Table 1), which suggest an equilibrium between input and uptake at least at low concentrations of the polymeric substrate.

Consequently, the input of leucine into the pool of free dissolved leucine would be 0.096 μg of C_{Leu} $liter^{-1}$ h^{-1}. The exclusive source for this supply is the pool of (dissolved) proteins if direct input into the leucine pool via exudation is assumed to be negligible. The turnover rate of the protein pool by extracellular enzymatic activity has been determined

to be 0.8% h^{-1}. Thus, the pool size of hydrolyzable leucine within the protein pool can be calculated; in the example (Fig. 2) it is 12 μg of C_{Leu} $liter^{-1}$. (The example would be clearer with the substrate combination glucose-cellulose, because the only monomer which can be produced from cellulose by enzymatic hydrolysis is glucose.) Of course, the calculation can also be made the other way round, when the polymer concentration is known or is assumed to have a certain value.

The pool size ratio between the monomer pool (leucine) and the polymer pool (protein) is 0.167, which is the inverse relation of T_r/H_r (= 6). In practice, pool sizes of monomers and polymers in natural waters are unknown. For the state of input-uptake equilibrium, measurement of T_r and H_r allow calculation of the pool size ratio $T_r/H_r = A/B$, where (Fig. 2) A is the concentration of the polymer pool and B is the concentration of the corresponding monomer pool. If this theoretical model is used to interpret results from field experiments, one should bear in mind that these may fulfill the assumption only insufficiently. Nevertheless, the attempt is made here to get at least an idea of the pool size ratio in relation to the microbial situation, which need not be identical with chemical determinations.

The two sampling sites are clearly different in their T_r/H_r pattern distributions (Table 3). The mean value of this quotient is nearly twice as high at the inner fjord station as at the offshore station. The mean values suggest that the polymer pool size, as it is defined here, is about nine times that of the corresponding monomer pool at the inner fjord station and about five times that of the monomer pool at the offshore station. This may be attributed to the higher load of polymeric materials introduced into the enclosed inner fjord area by a river, land drainage, and the polluted harbor area. The average H_r in this area (0.8% h^{-1}) is therefore considerably smaller than in the offshore region (2.8% h^{-1}), although the V_m of polymer degradation (7.4 μg of C_{Leu} $liter^{-1}$ h^{-1} for Leu-MCA is about 1.7 times higher than at the offshore station (4.5 μg of C_{Leu} $liter^{-1}$ h^{-1}).

If T_r/H_r values are grouped according to their seasonal distribution, a characteristic pattern, similar in both areas, becomes obvious. The same observation was also made by Kim (S.-J. Kim, Ph.D. dissertation, University of Kiel, Kiel, Federal Republic of Germany, 1985), who investigated this area 1 year later. The closest coupling between T_r and H_r is established during the summer months, when both parameters reach their maximum value and the input of organic materials may be expected to be low. Following our interpretation of T_r/H_r values, this would mean that pools of polymers and corresponding monomers are equal in size and that their hydrolysis and subsequent uptake by microorganisms are established during a similar period. The greatest discrepancy between T_r and H_r occurred during autumn, owing to a larger decrease of H_r than T_r. During these months, chlorophyll *a* values and temperature drastically dropped. It can be assumed that the concentration of dissolved organic materials, especially macromolecules increased during this time, because of autolysing and decaying living resources and a weather-dependent higher input from land and fresh waters.

In general, we received the impression that the response of microbial activities toward changing environmental conditions is not uniform: uptake mechanisms adapt to them more quickly, whereas extracellular enzyme activities react more slowly. Therefore, increasing polymer concentrations in the autumn are not counterbalanced by hydrolytic activity; decreasing temperatures may also have a bearing on this effect. During the winter, the discrepancy between T_r/H_r values from the two stations may be influenced by pollution effects at the inner fjord station, because autochthonous production of organic matter at both stations is low anyway. T_r and H_r both show their minima during this time, which is certainly due to the low water temperature. The possibly ambigious effect of temperature on the two microbial activities in question is an important point for further investigations. During the spring, values of T_r and H_r and the T_r/H_r quotient increase, along with bacterial numbers and chlorophyll concentrations. At this time of the year, values of T_r/H_r may be a result of increasing nutrient concentrations and water temperature, with a somewhat retarded response of extracellular enzyme activity.

A study such as the one presented here cannot be a perfect one, given our present state of limited knowledge of in situ process dynamics and still insufficient methodology. Publications dealing with the coupling of dynamic parameters in terms of rates and not prevailing concentrations are rare in the literature. Therefore, lacking any suitable scale, the approach as described here and the interpretation of the results necessarily remain preliminary.

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