Vol. 23: 107–115, 1985 | MARINE ECOLOGY – PROGRESS SERIES Mar. Ecol. Prog. Ser. | Published May 16

Bacterial biomass production and ammonium regeneration in Mediterranean sea water supplemented with amino acids. 1. Correlations between bacterial biomass, bacterial activities and environmental parameters

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ABSTRACT: Batch systems were used to establish the nitrogen balance of a marine coastal water in order to quantify bacterial responses to organic nitrogen (amino acid) enrichment in summer and winter. During both seasons, the amino-acid enriched batch clearly showed 'sollicitation' and 'growth' phases in bacterial communities. Maximum growth rates were 0.38 h^{-1} at 25 °C and 0.07 h^{-1} at 10 °C in summer and winter experiments as estimated from direct counts. Short sampling time (1 h) and parameters (direct counts, plate counts, biovolumes measurements, ATP, chlorophyll, mineral salts, ^{14}C uptake, mineralization) describing bacterial growth and activity were used. Parameter associations determined via principal component analysis, discarding the problem of quantity with standardized variables, showed analogous responses in the blank and in enriched batches in summer. High mineralization rates of carbon (40 %) and nitrogen (regeneration ratio 0.65 in Jun, 0.5 in Jan) were observed in conditions of substrate excess.

INTRODUCTION

In natural eutrophic waters, mostly coastal waters of economic interest, heterotrophic bacterial communities participate in remineralization processes (Hollibaugh et al. 1980, Kirchman et al. 1982, Azam et al. 1983) and contribute to the food chain since they are a source of food for micro-predators (Sorokin et al. 1970, Fenchel 1982, Linley et al. 1983).

In order to establish mathematical models for trophic relationships between components of the nano- and microplankton ecosystems, growth rates, changes in biomass and activity measurements are of prime importance. Conducting studies in natural environments creates many problems; hence it is useful, initially, to work with laboratory systems such as batch experiments (Slater 1979, Parkes 1982).

In order to estimate bacterial reactivity of natural communities in eutrophic sea water (Arcachon lagoon, France), samples were subjected to high organic enrichments (560 µg-at Nl^{-1} amino acid: Van Wambeke et al. 1982). In autumn, with water temperatures of ca 20 °C, the first step of a dystrophic crisis is simulated and the bacterial communities are able to utilize such high organic enrichments in less than 24 h. This was not surprising as bacteria are supposed to adapt to such organic carbon concentrations.

However, extensive ecological work in oligotrophic waters has demonstrated the presence of bacteria adapted to low concentrations of organic substrates. When such oligotrophic waters are submitted to natural (phytoplankonic blooms) or artificial organic enrichments in coastal areas, the bacterial community may not be able to regulate the eutrophication processes. To assess the response of oligotrophic sea-water bacterial communities, similar experimental conditions were applied to samples from the Gulf of Marseilles. Two experiments were carried out during 2 entirely different seasonal situations (Jun and Jan). Mineralization processes and bacterial biomass growth responses were related by a nitrogen balance.

In this first paper, only bacterial-organic matter interactions are being considered. The influence of prey-predator interactions, limiting bacterial numbers

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3 to 10 d after initial enrichment in January, is not taken in account here, but will be considered in the second paper (Van Wambeke & Bianchi 1985).

MATERIAL AND METHODS

Two experiments were carried out: the first in June 1981 (zero time: 12 noon) of 48 h duration, the second in January 1982 (zero time, 10 am) of 10 d duration. Surface sea water was sampled on the 'Prado beach', Marseilles (France) and brought back to the laboratory within 1 h. The water volume was divided into 5 outdoor batches, of 350 l each (75 cm diameter, 80 cm height). The sea-water temperatures were 24 and 10.5 °C in June and January, respectively.

Batches were randomly prepared as follows:

- 2 batches with 100 µg-at N l^{-1} NH_4Cl in June 1981, 30 µg-at N l^{-1} in January 1982; these were called 'control batches'.
- 2 batches with 100 µg-at N l^{-1} amino acid DIFCO (casein hydrolysate) in June 1981, 30 µg-at in January 1982; these were called 'enriched batches'.
- 1 blank batch, with no enrichment.

After enrichment, sea water in the tanks was aerated continuously in January 1982 and occasionally in June 1981, in open air conditions.

Samples were taken every 3 h in June and every 6 h in January. Samples are noted Tx in the text, where x = time in hours after initial enrichment. Sea-water samples were preserved with 1 % formaldehyde. Bacteria were enumerated by acridine orange direct counts (AODC; method of Hobbie et al. 1977). The sample was stained with 0.01 % w/v final concentration of acridine orange, and filtered through 0.2 µm Nuclepore filters. A visual estimate of cell-size distribution was used to estimate the biovolume from photographs of fields obtained with the AODC technique. The volume was calculated using the length (l) and width (w) of the cell according to the Krambeck et al. (1981) equation

$$\text{vol} = 3.14\ (w^2 l/4 - w^3/12) \quad (1)$$

ATP was estimated using the Laborde (1972) technique. Samples were filtered through 3 µm and the filtrate through 0.2 µm Sartorius membrane filters. After filtration the filter was transferred immediately into a boiling Tris Buffer (0.02 M, pH 7.6), and extracted for 5 min. Thereafter the sample was stored in a freezer at −25 °C. The ATP level was measured with the luciferin-luciferase reaction in the Pico-ATP MUKA apparatus. AMP and ADP were determined according to the Romano & Daumas (1981) technique. Owing to the simultaneous presence of living and detrital organic matter in such experiments, and the problem of high global contents of AMP in cells

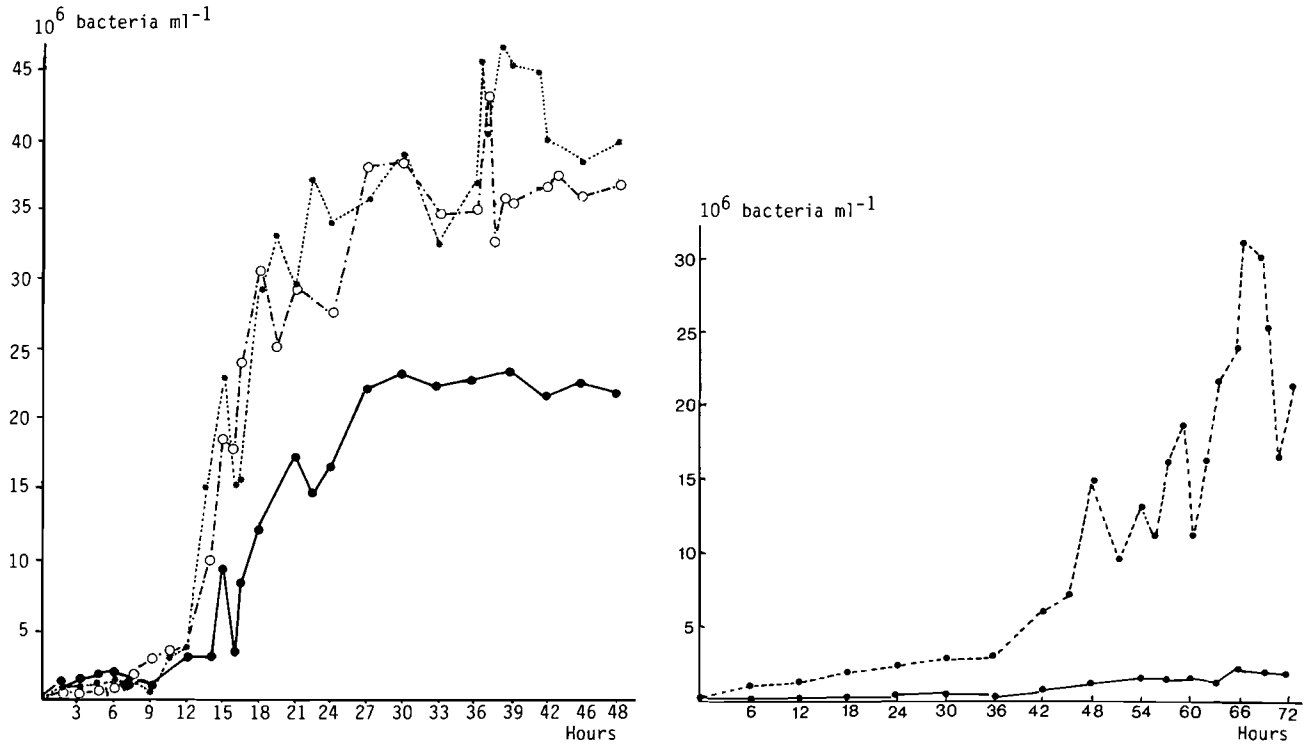

Fig. 1. Direct counts (AODC technique) for June (left) and January (right) experiments. Solid line: blank batch; broken lines: enriched batch(es)

Table 1. Maximum growth rates calculated from regression-analysis results of log (direct counts) = f (time) curves in both experiments

			Number of points analysed	Correlation coefficient	Maximum growth rates (h^{-1})	Generation time (h)
June experiment	Enriched batches 100 µg at N-AA l^{-1}	(1)	9	0.95	0.37	2.6
		(2)	9	0.98	0.39	2.5
	Blank batch		8	0.87	0.24	4.2
January experiment	Enriched batch 30 µg at N-AA l^{-1}		17	0.97	0.07	13.5
	Blank batch		14	0.96	0.06	15.7

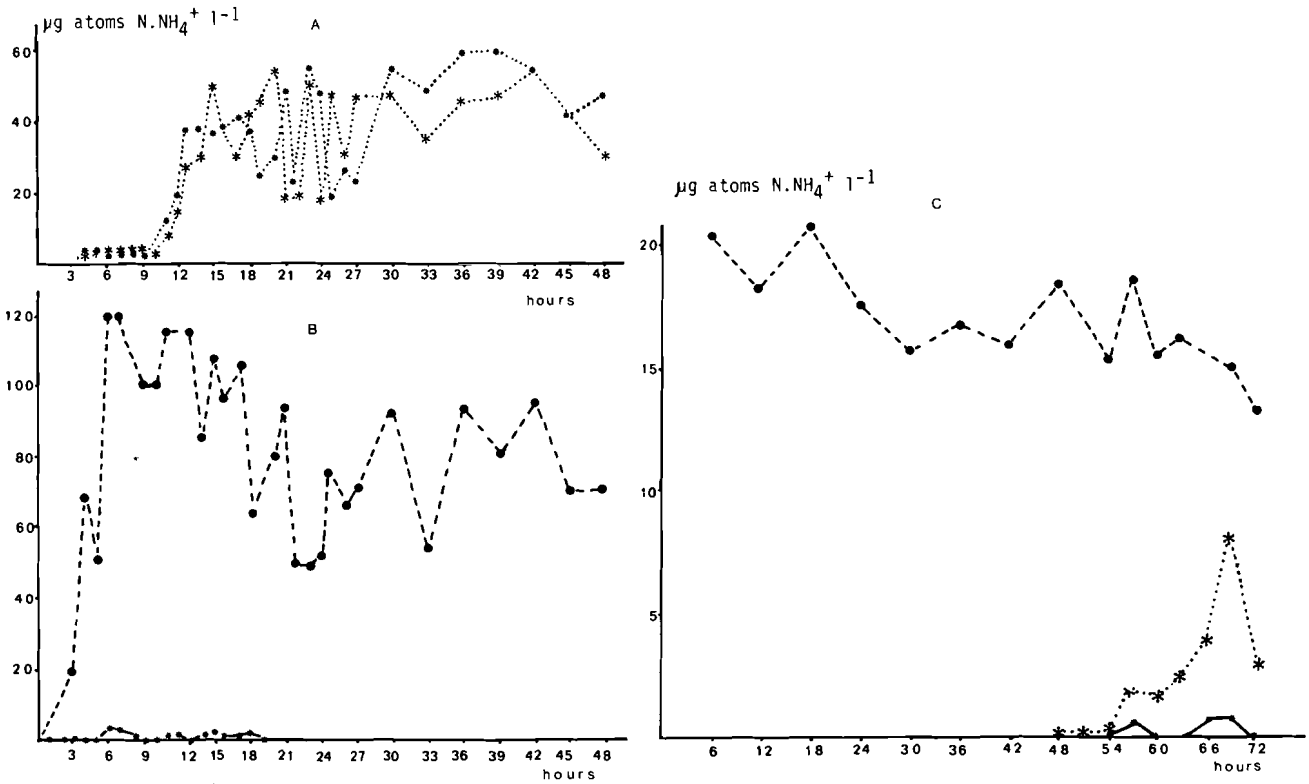

Fig. 2. Ammonium contents for June (left) and January (right) experiments. Solid line: blank batch; dotted line: enriched batch; dashed line: control batch

(Romano & Daumas 1981, Bianchi & Van Wambeke 1984), the energy charge ratio was not calculated; only the AMP/ATP ratio was calculated.

Heterotrophic activity was measured by determining the uptake and percentage mineralization of uniformly labelled glucose ^{14}C, specific activity 250 µCi.µM^{-1} (i.e. 42 µCi.µM C^{-1}) and mixed amino acids (CEA), specific activity 48 µCi.µM C^{-1}. A 15 ml sample was transferred to a flask and the labelled substrate was added. Final concentrations were 0.86 µg l^{-1} for labelled glucose and $1 \cdot 4 \times 10^{-2}$ µM C l^{-1} for labelled amino acids. Incubation time was 1 or 2 h, in the dark. After filtration on 0.2 µm Microporous filters, uptake was stopped by quick freezing at −25 °C. In the filtrate, ^{14}Carbon dioxide generated by substrate oxidation was fixed with NaOH granules. Then $^{14}CO_2$ and particulate ^{14}C were treated by using the Cahet & Jacques (1976) technique. Results were recorded in DPM for particulate ^{14}C and in percentage for mineralization using the following equation:

$$\% \text{ min} = (\text{DPM}^{14}CO_2)/(\text{DPM } ^{14}CO_2 + \text{DPM particulate } ^{14}C) \times 100 \quad (2)$$

Ammonium-salt concentration and chlorophyll *a* were measured using the Strickland & Parsons (1968) technique.

A principal component analysis was employed to visualize the results, considering the high number of

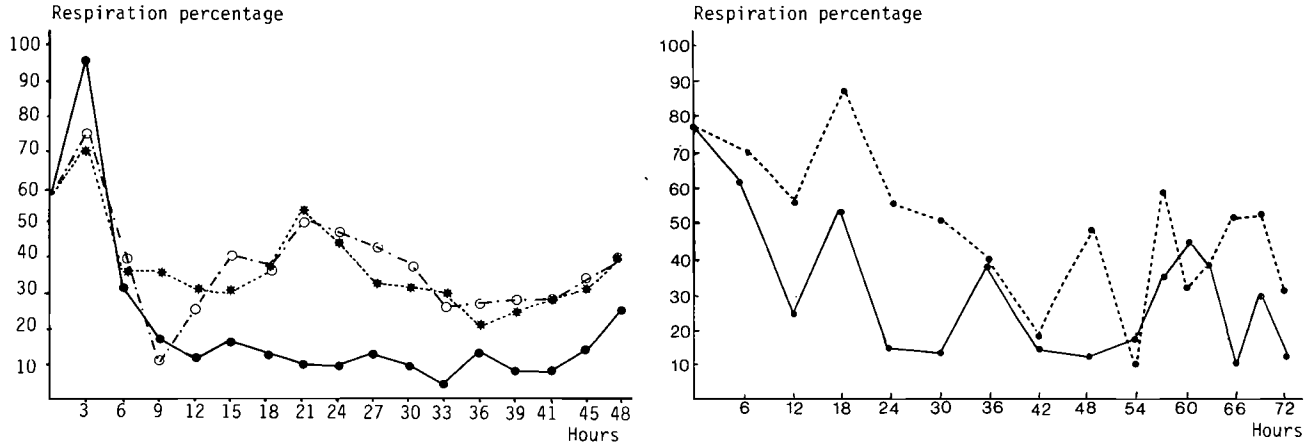

Fig. 3. Amino acid respiration expressed as percentage of total uptake for June (left) and January (right) experiments. Solid line: blank batch; broken lines: enriched batch(es)

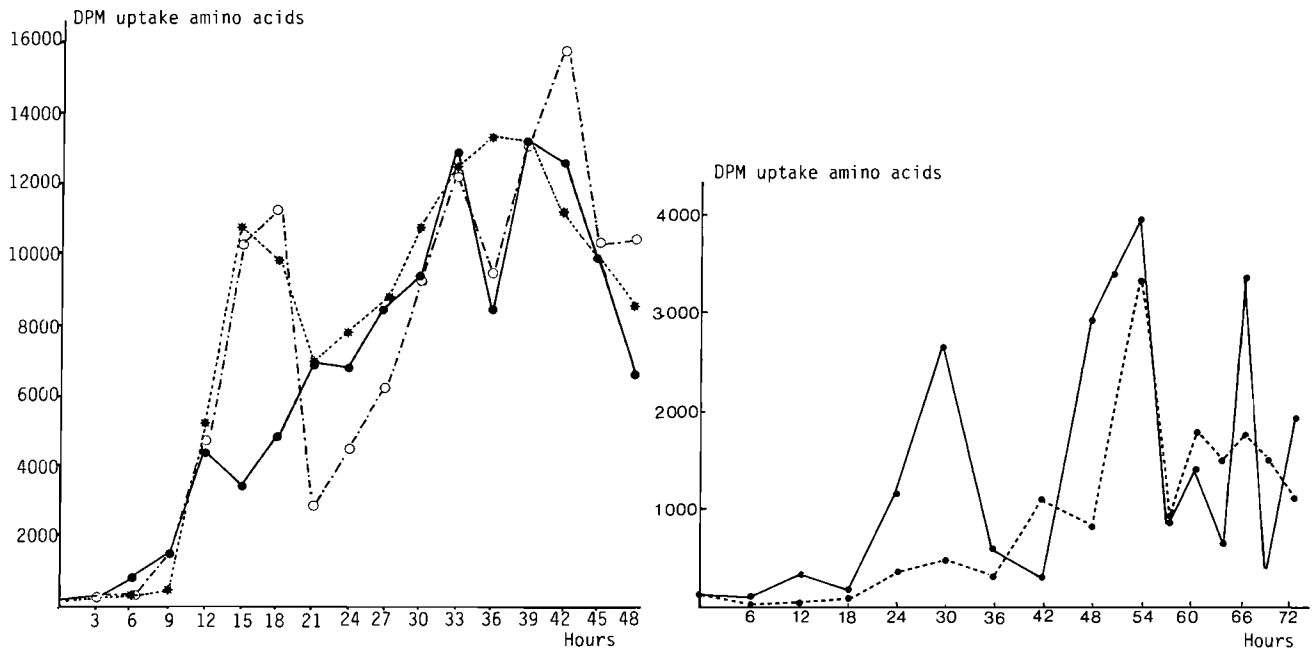

Fig. 4. Amino acid uptake in DPM of June (left) and January (right) experiments. Solid line: blank batch; broken lines: enriched batch(es)

variables studied. This analysis provided general information about the degree of similarity between responses in the different experiments, i.e. on the importance of the enrichment process (enriched batches) compared to the wall effect (blank batch). Relations between activity and biomass parameters varyied according to the time scale of the process compared to the sampling time interval.

Principal component analysis was used to compare changes in parameters during the 2 experiments for the same phase of bacterial growth. The series studied consist of ordered, metric and continuous variables. Variable variance is heterogeneous (e.g. 10^6 bacteria ml^{-1} against 0.01 µg ATP l^{-1}), hence standardized variables were used. PCA was calculated from a correlation matrix composed of these standardized variables.

RESULTS

In June, temperature fluctuations reflected the diel rhythm throughout the entire 48 h experiment with a slight increase, from 20 to 24 °C at TO to 22.5 to 25 °C, at T48. Cold January air reduced the batch temperature from 10.5 to 8 °C.

In spite of the different temperature conditions (10 C° higher in Jun) and the initial concentrations of amino acids (3 fold higher in Jun), maximum numbers of

bacteria (Fig. 1) were almost identical in both experiments (30×10^6 ml^{-1} in Jan; 40×10^6 ml^{-1} in Jun). However, at lower temperatures (Jan), the lag phase was longer and growth rates were smaller (Table 1).

The increase in bacterial numbers, as observed by the direct counts, resulted in the real increase in the number of bacteria minus bacterial consumption or mortality. The number, calculated from a regression analysis of log (Direct Count) = f(t) was considered a minimum theorical growth rate.

In June, the exponential phase lasted only 9 h, owing to prevailing temperatures and the fact that bacterial growth started during the night, thus limiting any phytoplanktonic inhibition caused by photosynthesis. During such a short period, the number of disappearing bacterial cells could be neglected, compared to the increase in the number of cells. In this case the 'calculated' growth rates approximated the true ones. This could explain (in the enriched batches) why growth rates (0.37 and 0.39; Table 1) were very high compared to those calculated by other methods (Hagstrom et al. 1979, Karl 1981, Christian et al. 1982). Newell & Christian (1981) observed in situ generation times of 8 to 30 h, whilst Hollibaugh et al. (1980) calculated bacterial doubling d^{-1} of 0.3 to 3.

During both experiments (Jun and Jan), NH_4^+ salts were well mixed into the batches after 6 h (Fig. 2a, b). In the control batches (NH_4^+ enriched) ammonium decrease occurred after exponential growth in June (12 h) and before the growth phase in January (66 h). From the organic nitrogen (amino acids) NH_4^+ appeared immediately after the growth phase in June (9 to 12 h), whilst in January the NH_4^+ increase started only after the number of cells began to increase.

In a first step, during the lag phase of bacterial growth, respiration percentages of amino acids (Fig. 3) and glucose were very high. In a second step, biomass and activity (Fig. 4) parameters developed simultaneously. This process explained the high initial information of Axis 1 and 2 in the PCA (Table 2). In all batches of both experiments, PCA gave similar results, and only 2 examples – one each of the enriched batches in June (Fig. 5) and in January (Fig. 6) – are given.

The first factor was heavily loaded with amino acids and glucose respiration (RAA, RGL). Both variables were projected in the same direction as the initial observation vectors (beginning of experiment).

The parameters describing heterotrophic activity and energy status of the bacterial communities showed a close correlation in both enriched batches of the June experiment (more than 0.8; Table 3). Amino acid uptake (UAA), glucose uptake (UGL), ADP, ATP as measured on 0.2 µm filters and the sum of adenylates on 3 µm filters (SI3) were plotted together and separated from the direct count (DIC) variable. Only AMP was plotted near direct counts (correlation coefficient 0.83 and 0.84 in the 2 duplicates; Table 3). In fact, AMP content of the cell is the most important adenylate in the AMP, ADP and ATP pool. AMP is present in active, inactive and dead cells (Romano & Daumas 1981), which are all counted in the AODC technique.

In January, slow bacterial growth and the number of samples taken and studied during the experiment permitted visualisation of the different steps of bacterial evolution. Energetic phase (adenylate pool), increase in biomass, numbers and activity (mineralization) were plotted separately in Fig. 6. For PCA, the first axis accounted only for 44 % of the initial information (Table 2). AMP was correlated with the cytoplasm volume ($r = 0.58$; Table 4); it accumulated independently of the activity state of the cell (Fig. 6). DIC, PLC (plate counts) and UAA show that exponential growth could be described by plate counts and/or amino acid uptake, in spite of (1) the selective effect of a ZoBell medium when counting viable cells and (2) the presence of natural cold amino acids in sea water, competing with the ^{14}C compounds for activity measurements (Gillespie et al. 1976).

Table 2. June 1981 and January 1982 experiments. Eigenvalue percentages of the 2 principal latent roots in principal component analysis

		Eigen value percentage of the 2 first latent roots		Cumulative percentage
		Axis 1	Axis 2	Axis 1 + Axis 2
June experiment	Enriched batches	54.7	16.3	71.
	100 µg at N-AA l^{-1}	64.2	11.7	75.9
	Blank batch	46.7	12.2	59.
January experiment	Enriched batch 30 µg at N l^{-1}	44.3	15.8	60.1
	Blank batch	35.5	20.5	56.

Table 3. June 1981 experiment. Correlation matrix obtained from the 13 variables × 17 observations in one of the enriched batches. DIC: bacterial direct counts; UAA, UGL: amino acid and glucose uptake (DPM); RAA, RGL: amino acid and glucose respiration (% of total uptake); AT2, AD2, AM2: ATP, ADP and AMP values measured on 0.2 µm filters; SI3: ATP + ADP + AMP measured on 3 µm filters

AT2	1.00												
NH4	0.83	1.00											
AD2	0.9	0.8	1.00										
SI3	0.88	0.84	0.86	1.00									
UAA	0.85	0.9	0.74	0.86	1.00								
AM2	0.82	0.83	0.86	0.83	0.73	1.00							
UGL	0.82	0.8	0.87	0.65	0.69	0.78	1.00						
DIC	0.79	0.84	0.72	0.76	0.79	0.83	0.73	1.00					
TEM	0.42	0.34	0.29	0.38	0.24	0.45	0.26	0.47	1.00				
PH	−0.07	0.1	−0.17	0	0.15	−0.12	−0.24	−0.24	−0.1	1.00			
O_2	−0.26	−0.19	−0.29	−0.24	−0.22	−0.13	−0.29	0.21	0.25	−0.13	1.00		
RGL	−0.3	−0.23	−0.35	−0.23	−0.27	−0.05	−0.42	0.06	0.03	−0.1	0.6	1.00	
RAA	−0.44	−0.35	−0.26	−0.38	−0.47	−0.17	−0.29	−0.22	−0.2	−0.28	0.28	0.58	1.00
	AT2	NH4	AD2	SI3	UAA	AM2	UGL	DIC	TEM	PH	O_2	RGL	RAA

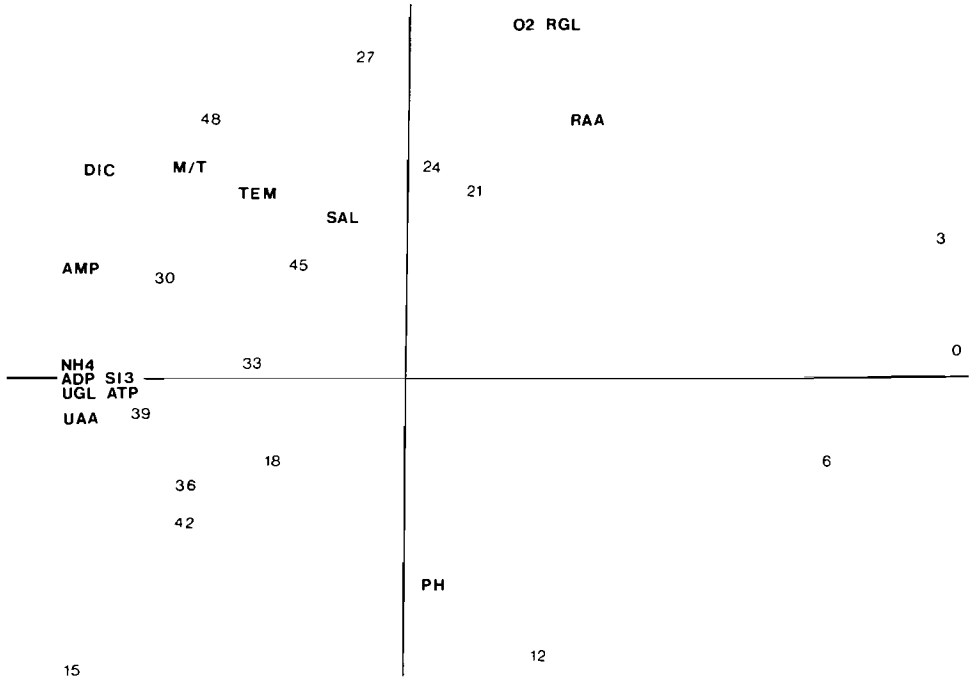

Fig. 5. Principal component analysis on one of the amino acid-enriched batches in June. DIC: direct counts; UAA, UGL: amino acid and glucose ^{14}C uptake; RAA, RGL: amino acid and glucose respiration; AT2, AD2, AM2: ATP, ADP, AMP values measured on 2 µm filters; SI3: sum of ATP+ADP+AMP, measured on 3 µm filters; TEM: temperature; PH: pH; O_2: dissolved oxigen; NH_4: ammonium. Numbers indicate time (h) after beginning of experiment (observation vector of PCA)

DISCUSSION

In the June experiment, growth of bacteria in the blank batch was too high to originate only from the wall effect (ZoBell 1943), even when considering the high temperature and the occurence of growth during the night. In previous experiments (Van Wambeke et al. 1982), batches with volume/surface ratios 4 times higher than in the January and June experiments were used. The bacterial response in the blank batch as noticed on the maximum direct count / T0 count ratio was 2, for that experiment, against 14 in the January, and 70 in the June experiment.

During the June experiment, the small difference between AODC in the amino-acid enriched batch and in the blank batch, was enhanced when considering bacterial-cell carbon contents, due to the variations in average cell volumes in both batches. Cells of 0.01 to 0.05 μm^3 were observed in the blank batch while the average volume was 0.2 μm^3 in the enriched batches. This means 850 µg C l^{-1} in the enriched batch against 20 to 100 µg C l^{-1} in the blank (calculations with specific gravity 1.1 g C cm^{-3}, carbon / wet weight ratio 0.1; Linley et al. 1983).

Assuming that phytoplankton biomass was the major component of particulate carbon present at the begin-

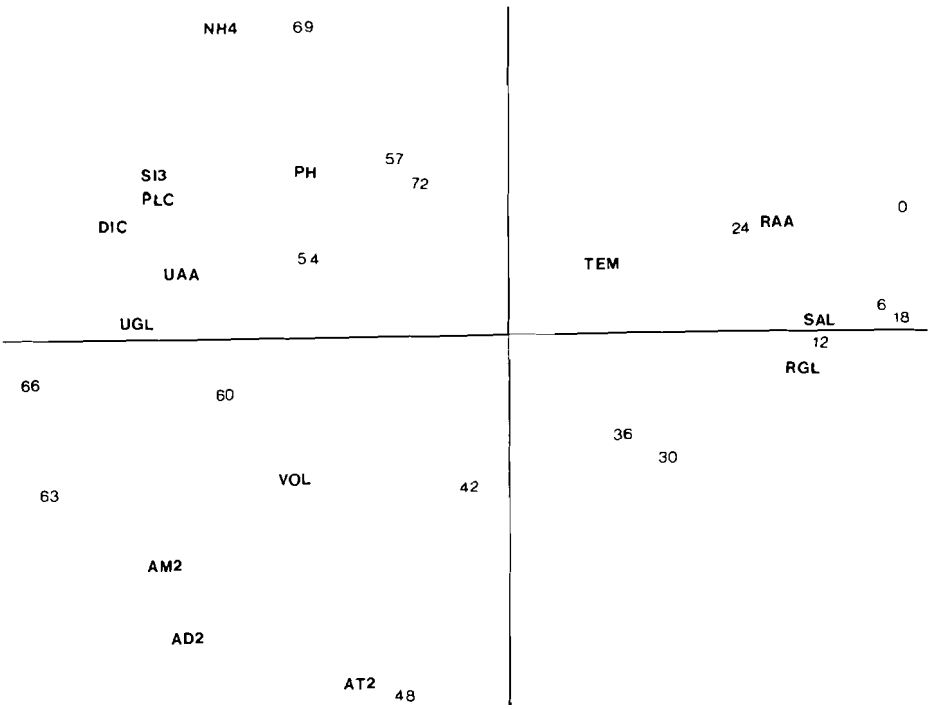

Fig. 6. Principal component analysis on the amino-acid enriched batch in January. VOL: average bacterial volume; PLC: plate counts; other symbols same as in Fig. 5

Table 4. January 1982 experiment. Correlation matrix obtained from the 14 variables × 16 observations in the enriched bath. TEM: temperature; SAL: salinity; DIC: bacterial direct counts; PLC: plate counts; RGL, RAA: glucose and amino acid respiration (% of total uptake); UAA, UGL: amino acid and glucose uptake (DPM); AT2, AD2, AM2: ATP, ADP, AMP values measured on 0.2 µm filters; SI3: AMP + ADP + ATP values measured on 3 µm filters; VOL: bacterial volume

DIC	1.00													
UGL	0.71	1.00												
SI3	0.81	0.73	1.00											
AM2	0.57	0.68	0.51	1.00										
PLC	0.82	0.51	0.47	0.46	1.00									
UAA	0.60	0.69	0.48	0.50	0.69	1.00								
AD2	0.60	0.49	0.33	0.85	0.51	0.42	1.00							
NH4	0.80	0.51	0.77	0.17	0.64	0.34	0.12	1.00						
VOL	0.27	0.40	0.28	0.58	0.36	0.28	0.42	0.07	1.00					
PH	0.42	0.02	0.36	0.14	0.40	0.30	0.18	0.42	0	1.00				
AT2	0.24	0.24	0.21	0.50	−0.07	−0.03	0.74	−0.06	0.15	0.01	1.00			
TEM	−0.25	0	−0.06	−0.08	−0.23	−0.01	−0.3	−0.22	0.07	−0.29	−0.32	1.00		
RAA	−0.34	−0.24	−0.17	−0.44	−0.54	−0.71	−0.52	−0.08	−0.27	−0.5	−0.2	0.26	1.00	
RGL	−0.43	−0.76	−0.68	−0.47	−0.22	−0.39	−0.2	−0.46	−0.36	−0.24	−0.2	0	0.34	1.00
	DIC	UGL	SI3	AM2	PLC	UAA	AD2	NH4	VOL	PH	AT2	TEM	RAA	RGL

ning of the experiment, the chlorophyll *a* measurement permits calculation of the particulate carbon available for bacteria as follows: 0.5 µg chl *a* × 60 = 30 µg C l^{-1}. Considering that 10 % were assimilated by bacteria (Newell et al. 1981, Linley et al. 1983), this would produce a bacterial biomass of only 3 µg C l^{-1}. In this case it would have to be supposed that other sources of organic carbon, such as dissolved combined amino acids (Amano et al. 1982) and/or dissolved combined carbohydrates (Gocke et al. 1981, Burney et al. 1981), were available for bacterial growth.

Lack of information on the quantity of available organic matter at the beginning of the experiment did not permit calculation of growth efficiency in the blank batch. Nevertheless, respiration percentages on amino acids and glucose ^{14}C were much lower in the blank than in the enriched batches: 10 to 15 % versus 40 % for amino acids, 25 to 40 % versus 30 to 60 % for glucose.

For the enriched batches, high respiration percentages of ^{14}C amino acids in both experiments show that nitrogen (NH_4^+) remineralization was not only due to the adjustment of the C/N ratio of amino-acid substrates (4) to the bacterial C/N ratio (6: Fenchel &

Jørgensen 1977, Hollibaugh 1978), but also to a reduction in the efficiency of substrate assimilation during growth when the substrate was in excess (Parsons et al. 1981). In June, ammonium accumulation occured immediately after the beginning of exponential growth (T9–T12), but in January it occured only after the bacterial increase (T26)..

NH_4^+ concentration curves were the result of both phytoplankton utilization of mineral nitrogen and of remineralization of amino acids by bacteria. Hollibaugh (1978) did not find much variation of NH_4^+ concentrations in the batch enriched only with NH_4^+, when these batches were kept in the dark. In June (Fig. 2a), fast NH_4^+ accumulation was enhanced by bacterial growth occuring during the night (Caperon et al. 1979). Amino acid uptake was very closely correlated (0.9 and 0.93 in the duplicates) with NH_4^+ as noticed on the correlation matrix in June. This was not the case in January (Table 3), when mineralization activity was separated from growth.

In order to determine NH_4^+ bacterial remineralization, as done by Hollibaugh (1978), NH_4^+concentrations had to be adjusted, taking into account the decrease in NH_4^+ in the NH_4^+ control batch between T0 and maximum ammonium release in the amino-acid enriched batch. Thus, the regeneration ratio (N-NH_4^+ released / N-AA introduced at the beginning) was corrected: N-NH_4^+ released (measured + consumed) / N-AA introduced at T0. In January, $(8 + 8)/30 = 53\%$ of organic nitrogen was regenerated, against $(45 + 20)/100 = 65\%$ for the casein hydrolysate tested in June. As with the number of bacteria, and in spite of the slow bacterial reaction in January, the sum of the resulting activity was almost the same in June.

In conditions favouring heterotrophic activity only, on substrates with high C/N ratios such as amino acids, a homeostatic mechanism occured rapidly and eliminated dissolved organic matter through bacterial biomass and mineralization. With substrate excess, most of the nitrogen was mineralized, thus providing the major source of regenerated nitrogen for primary producers.

The relative analogy of bacterial responses in the blank and the enriched batches suggest a rapid flux from combined to available organic matter for bacteria in natural sea water. A qualitative study of bacterial communities at the beginning of the experiment would yield the origin (chemical, biological; presence of exoenzymes ?) of this transformation.

In June, the steady-state plateau observed over 24 h was neither due to dilution (closed batches) nor to precipitation (bubbled batches) nor to a noticeable presence of bacterivorus, but was probably due to a lack of nutrients such as phosphorus. In January, bacterial growth (72 h) was immediately followed by a micro-predator increase (Wambeke & Bianchi 1985). Considering the organic eutrophication of our experimental system, lack of mineral nutrients and predation account for the low maximum numbers of bacteria observed.

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This paper was presented by Professor G. Rheinheimer; it was accepted for printing on February 11, 1985