Vol. 70: 273–282, 1991 | MARINE ECOLOGY PROGRESS SERIES
Mar. Ecol. Prog. Ser. | Published March 12

Simultaneous uptake and regeneration of ammonium by mixed assemblages of heterotrophic marine bacteria

Luis Tupas, Isao Koike

Ocean Research Institute, The University of Tokyo, 1-15-1 Minamidai, Nakano, Tokyo 164, Japan

ABSTRACT: Utilization of dissolved organic nitrogen (DON) and ammonium by natural assemblages of heterotrophic marine bacteria was studied in cultures of original seawater using ^{15}N-ammonium. Increase of ^{15}N in particulate organic matter with time, i.e. newly formed bacterial cells, signified active assimilation of ammonium; however, the ^{15}N content of ammonium in the media was continuously diluted, indicating simultaneous mineralization of DON. Ammonium supplied up to 80 % of nitrogen in new bacterial cells, yet more than half of the DON taken up, mostly in the form of dissolved combined amino acids, was converted to ammonium. This dual function of marine microbial populations is not in conformity with present ideas of microbial nitrogen metabolism and suggests that amino acid uptake is governed more by metabolic constraints than by substrate composition. These results give equal weight to the importance of bacterial heterotrophs as assimilators and regenerators of nitrogen in the marine environment.

INTRODUCTION

Heterotrophic bacteria are known to process a large fraction of dissolved nutrients in marine waters (Azam et al. 1983). Their ability to act as links between organic and inorganic nutrients as well as dissolved and particulate matter would influence the production of other organisms in the microbial food web and the general cycling of nutrients in the environment. Part of their metabolic functions would be the assimilation or regeneration of ammonium as a function of environmental and metabolic constraints, which are at present unclear.

A model of ammonium uptake and regeneration based on mass balancing of nitrogen and carbon in bacteria and their substrates has been described by Goldman et al. (1987) and Newell et al. (1988). They demonstrated that the C : N ratio of available substrates, rather than chemical forms, determines net uptake or excretion of ammonium. When organic nitrogen quantity is insufficient, ammonium is utilized as supplemental nitrogen to fulfill bacterial demands. Using current ideas of bacterial growth efficiency and C : N ratios of bacteria and dissolved organic matter, it has been concluded that bacterial heterotrophs are inefficient regenerators of ammonium (Goldman et al. 1987).

In contrast, other studies have reported that ammonium production from amino acid mineralization has no relationship to substrate C : N ratios (Hollibaugh 1978) and that ammonium production equals amino acid consumption (Gardner et al. 1987). It has been suggested that there may be preferential assimilation of carbon skeletons, resulting in release of ammonium after amino acid uptake (Zehr et al. 1985). Clearly, further understanding of the nitrogen metabolism of microbial heterotrophs is necessary, due to its large ecological impact.

Previous studies to characterize the function of marine bacteria in organic nitrogen cycling have mostly relied on the use of free amino acids (FAA) as substrates and tracers (Fuhrman 1987). Although dissolved free amino acids (DFAA) are important building blocks for the formation of protein, which comprises a large portion of bacterial biomass (Simon & Azam 1989), they constitute only a minor component of total available nitrogen in the pelagic environment (Coffin 1989). Results of such experiments provide information on microbial metabolism of specific amino acids, but extrapolation of these results to total nitrogen metabolism becomes difficult because of the great variations in substrate composition available to microbial populations. It is therefore necessary to examine the total

0171-8630/91/0070/0273/$ 03.00

nitrogen budgets of growing bacterial populations under minimum modifications of their nutritional conditions, to gain a better insight into their metabolisms and function in the microbial food web.

Taking this need into consideration, we examined ammonium uptake and regeneration by a natural assemblage of heterotrophic marine bacteria grown in a medium composed of their original seawater with ^{15}N-ammonium (constituting about 10 to 20 % of total ammonium in the water). Using standard tracer and isotope-dilution techniques, we observed simultaneous assimilation and production of ammonium by the bacterial population. This dual process has not been previously identified and would not be observable without the use of nitrogen tracers.

MATERIALS AND METHODS

Surface seawater was sampled from Ohtsuchi Bay, Japan (39° 20′ 30″ N, 141° 55′ 30″ E), in June 1988 (Culture 88-A), October 1988 (88-B), and June 1989 (89-B), and from Aburatsubo Bay (35° 09′ 20″ N, 139° 37′ 15″ E) in May 1989 (89-A). Water samples were collected with a clean bucket or Van Dorn bottle and filtered through a baked GF/C filter (Whatman) under gentle vacuum (<20 cm Hg). This filtrate contained essentially the bacterial population and eliminated most if not all chlorophyll *a*-containing organisms and bacterial grazers (see 'Results'). Filtered seawater (15 l) was placed in acid-washed polyethylene plastic bags, and ^{15}N-ammonium or -nitrate (98.5 %) was added, constituting 10 to 20 % of respective ammonium or nitrate concentrations. The seawater was incubated in the dark at ambient seawater temperature.

Increase in the bacterial population was monitored by direct counts using epifluorescence microscopy and DAPI (Porter & Feig 1980). Chlorophyll *a* (chl *a*) was measured according to the method of Suzuki & Ishimaru (1990). At various points during the different phases of growth, 1 l aliquots were filtered through baked GF/F filters (Whatman), and the filter and a portion of the filtrate were frozen for mass spectrometric analysis of nitrogen contents, nitrogen isotope ratios, and dissolved nutrients. Size distributions of bacterial populations were measured with a particle counter (Elzone 80XY, Particle Data Inc.) and showed that most of the growing bacteria were >0.6 μm in diameter, which is the nominal retention size of the GF/F filter (Kogure & Koike 1987).

Total dissolved nitrogen was determined using a Yanaco TN-7 nitrogen analyzer employing a high-temperature, catalyst-aided conversion of nitrogen to nitric oxide, and chemiluminescence detection. Inorganic nitrogen components (ammonium, nitrate, and nitrite) were measured on a Technicon autoanalyzer according to the methods of Strickland & Parsons (1972). Dissolved organic nitrogen (DON) concentrations were calculated as the difference between total dissolved nitrogen and inorganic nitrogen concentrations.

Total hydrolyzable amino acids (THAA) were measured using high-performance liquid chromatography (HPLC) after acid hydrolysis (Robertson et al. 1987). This analysis was a modification of the method of Lindroth & Mopper (1979), using methanol and acetonitrile as organic modifier and a 50 mM acetate buffer (Tupas & Koike 1990). Analysis of particulate organic nitrogen (PON) and isotope ratios was done by mass spectroscopy using the method of Ohtsuki et al. (1983). The ^{15}N content in ammonium or nitrate was determined after steam distillation of samples (Keeney & Nelson 1982).

A modification of the model described by Glibert et al. (1982) was used to estimate the amount of ammonium incorporated (p) after 1 set of growth and is given as:

$$p = \frac{(C_t \times R_t) - (C_0 \times R_0)}{(N_0 + N_t)/2}$$

where C_0 and C_t are the concentrations of PON at time 0 and time t of the experiment, and R_0 and R_t their respective ^{15}N contents. For the first-order approximation, the ^{15}N content in ammonium during growth can be expressed as the average of the ^{15}N content at times 0 (N_0) and t (N_t) (Glibert et al. 1982, Dugdale & Wilkerson 1986).

The isotope-dilution model of Blackburn (1979) was used to estimate the amount of ammonium mineralized (d), given as:

$$\ln(N_t - {}^{15}n) = \ln(N_0 - {}^{15}n) - \left[\frac{d}{d-i}\right] \times \left[\ln \frac{P_t}{P_0}\right]$$

$$P_t = P_0 + (d - i)$$

where P_t and P_0 are the concentration of ammonium at times t and 0 respectively and N_t and N_0 are as described above. ^{15}n is the natural abundance of ^{15}N, ca 0.37 %. This model also gives an estimate of ammonium consumed (i), and when compared with the estimate of ammonium uptake (p) indicates whether ammonium consumed from the medium is converted to biomass or metabolized to another form.

RESULTS

Initial GF/C filtrate conditions

In order to remove as much of the autotrophic and protozoan population as possible while retaining the greater portion of the bacterial population, seawater

Table 1. Effect of GF/C filtration on chlorophyll *a* (Chl *a*) content, bacterial number and protozoan number in seawater used for cultures

Culture	Chl *a* ($\mu g\ l^{-1}$)			Bacterial no. ($\times 10^6$ cells ml^{-1})			Protozoan no. (cells ml^{-1})
	Original	Filtered	% Removed	Original	Filtered	% Passed	Filtered
88-A	1.23	0.09	93	2.2	2.1	95	<2
88-B	1.73	0.07	96	1.9	1.8	95	<1
89-A	2.00	0.06	97	1.3	1.2	92	<2
89-B	5.56	0.07	99	2.1	2.0	95	<2

was filtered through a GF/C glass fiber filter (Whatman), which retains particles of ca 1.0 µm in diameter. Results of the filtration procedure are presented in Table 1. The filter was able to remove ca 96 % of the original chl *a* while allowing ca 94 % of the original bacterial population to pass through. Protozoan numbers in the filtrate were <2 ml^{-1}. Protozoan counts were not made in the original water samples because of inadequate preservation techniques. Protozoan numbers in Ohtsuchi and Aburatsubo Bays during the same time period ranged from 0.8 to 1.0 $\times$ 10^3 ml^{-1}.

Neither the filtration procedure nor the containment of water in plastic bags changed significantly the concentration of dissolved nitrogen components. In the filtrates used for the cultures, DON concentrations ranged from 13.8 to 15.0 µg-at. N l^{-1}, ammonium from 1.2 to 2.2 µg-at. N l^{-1}, and nitrate + nitrite from 1.8 to 5.0 µg-at. N l^{-1} in Ohtsuchi and 12.4 µg-at. N l^{-1} in Aburatsubo waters. THAA comprised ca 1.5 µg-at. N l^{-1}, which was ca 10 % of DON concentrations. FAA concentrations comprised ca 0.2 µg-at. N l^{-1}. Addition of ^{15}N-ammonium or -nitrate was limited to ca 10 to 20 % of ambient concentrations.

Changes in ^{15}N isotope concentrations

Experiments conducted in 1988 were aimed at determining the amount of ammonium incorporated and regenerated after 1 set of growth of the bacterial population. Biological and chemical parameters measured at the beginning, and their average after 1 growth phase, are presented in Table 2. In Culture 88-A rapid population growth started after 30 h of incubation, reached stationary phase after 60 h of incubation, and the experiment terminated after 110 h. In Culture 88-B

Table 2. Bacterial number, chlorophyll *a* (Chl *a*), protozoan number, dissolved organic nitrogen (DON), total hydrolyzable amino acids (THAA), NH_4^+, NO_3^- + NO_2^-, particulate organic nitrogen (PON), and ^{15}N-nitrogen measurements at initial and final phases of growth (average of 3 points after log phase, SD in parentheses)

Parameter	Culture 88-A		Culture 88-B	
	Initial	Final	Initial	Final
Bacterial no. ($\times 10^6$ cells ml^{-1})	2.1	6.9 (0.4)	1.8	7.5 (0.4)
Chl *a* ($\mu g\ l^{-1}$)	0.09	0.08	0.07	0.06
Protozoan no. (ml^{-1})	2	1	1	1
DON (µg-at. N l^{-1})	13.8	12.5 (0.7)	15.0	13.1 (0.4)
THAA (µg-at. N l^{-1})	1.54	0.66 (0.01)	1.55	0.38 (0.02)
NH_4^+[a] (µg-at. N l^{-1})	1.89	1.10 (0.26)	2.63	1.87 (0.03)
NO_3^- + NO_2^-[a] (µg-at. N l^{-1})	2.26	2.25 (0.13)	2.03	2.04 (0.02)
PON (µg-at. N l^{-1})	0.36	2.43 (0.03)	0.74	3.42 (0.24)
^{15}N-NH_4^+ experiments				
^{15}N in PON (%)	0.37	4.33	0.37	8.22
^{15}N in NH_4^+ (%)	8.87	5.12	16.8	8.50
^{15}N recovery (%)	100	98	100	96
^{15}N-NO_3^- experiments				
^{15}N in PON (%)	0.38	0.38	0.37	0.37
^{15}N in NO_3^- (%)	10.4	10.4	10.2	10.2
^{15}N recovery (%)	100	94	100	95

[a] Includes ^{15}N-isotope

rapid growth started after 5 h, reached stationary phase at ca 30 h and the experiment concluded after 72 h incubation. After log phase of both cultures, there was little variation in the values of the parameters measured, and they are therefore expressed as an average of 3 points after log phase, taken at intervals of 12 or 24 h. Bacterial number increased from ca 2×10^6 to ca 7×10^6 cells ml^{-1}. Chl *a* concentrations and protozoan number did not change during the 1 set of growth of the bacterial population.

DON concentration decreased slightly; however, THAA were reduced from one-half to one-fourth the initial concentrations. From 60 to 70 % of the decrease in DON can be accounted for by total amino acids in the medium. Ammonium concentrations also decreased slightly but nitrate + nitrite concentrations did not change.

PON concentration increased 5 to 7 times, which was attributed to the increase in bacterial biomass. For experiments using ^{15}N-ammonium as a tracer, an increase in the ^{15}N isotope in particulate matter, i.e. newly formed bacterial cells, was observed after 1 set of growth of the population, indicating the assimilation of ammonium by the bacteria. In experiments using ^{15}N-nitrate as a tracer, no change in the ^{15}N content of PON was observed after growth of the cultures, showing that nitrate is not used as a nitrogen source, at least in the presence of ammonium.

Analysis of ^{15}N-nitrogen in ammonium showed a decrease in ^{15}N content (isotope dilution), indicating production of unlabelled ammonium during the growth of the bacterial population. Microbial mineralization of DON in the seawater media was responsible for the regeneration of ammonium, because other biological sources, such as grazing, would have been insignificant (see 'Discussion'), and there was generally complete recovery of the ^{15}N-nitrogen isotope at the end of the experiments. No change was observed in the isotope content of nitrate in parallel experiments. This shows that there was no conversion of ammonium to nitrate.

Biological and chemical parameters were measured at different phases of growth for the 1989 cultures. In these experiments, only ^{15}N-ammonium was used as a tracer. In both cultures there was no observable lag phase (Fig. 1), and the bacterial population increased only 2-fold. Despite reaching a numerically stationary phase after ca 30 h, however, PON concentrations continued to increase. DON in both cases decreased with time by ca 2 µg-at. N l^{-1}. Ammonium concentrations fluctuated with time and decreased slightly at the end. Nitrate and nitrite concentrations exhibited no significant change.

^{15}N-nitrogen measurements are presented in Fig. 2. Recovery of ^{15}N-nitrogen from particulate and dissolved phases was > 95 % at all times. The increase in ^{15}N-nitrogen in particulate organic matter with time indicates active assimilation of ammonium throughout the growth of the population. Simultaneously, though,

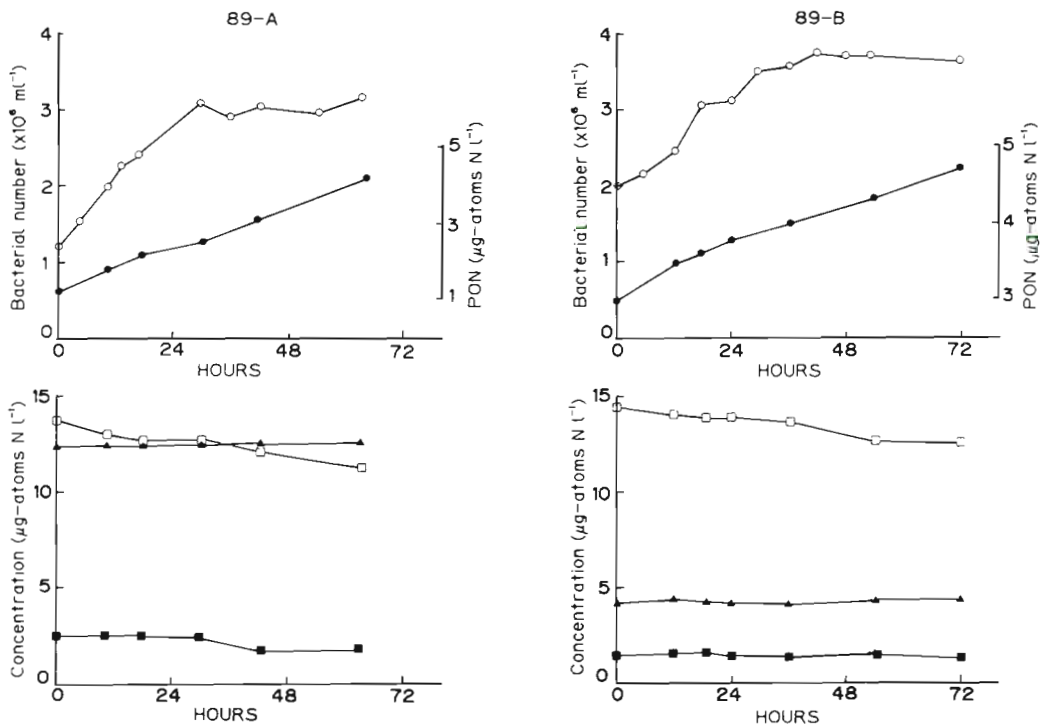

Fig. 1. Increase in bacterial number (○) and particulate organic nitrogen (PON, ●) with time, and change in concentration in the medium of dissolved organic nitrogen (□), nitrate + nitrite (▲), and ammonium (■) with time, for Cultures 89-A and 89-B

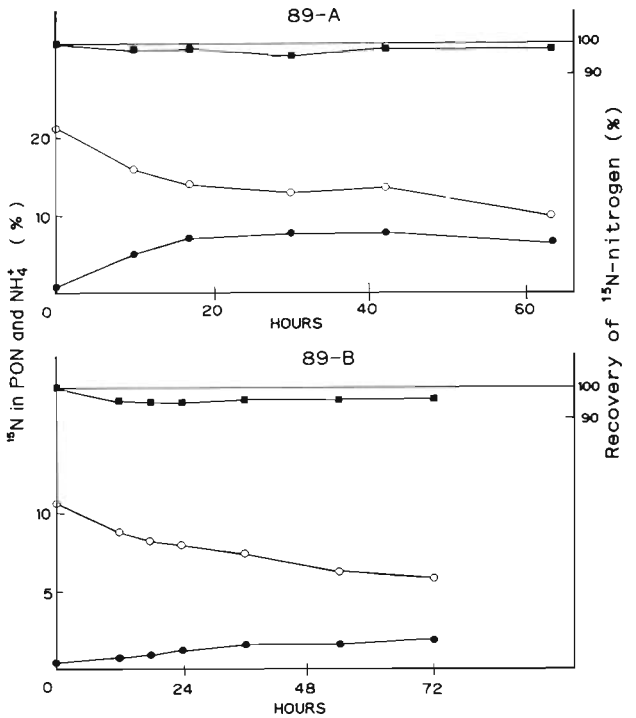

Fig. 2. Change in ^{15}N concentration in ammonium (○) and particulate organic nitrogen (PON, ●), and in recovery of ^{15}N-nitrogen (■), with time, for Cultures 89-A and 89-B

dilution of the ^{15}N isotope in ammonium was also observed, which shows that DON was being actively mineralized during the growth of the microbial population, and to a certain extent even after log phase.

Estimates of ammonium uptake and regeneration

Estimates of ammonium uptake and regeneration for each experiment were based on parameters measured at the beginning of the experiment, and on the average of 3 values when the culture was in a numerical steady state. Using a model based on the accumulation of ^{15}N-nitrogen into particulate organic matter (Glibert et al. 1982), it was calculated that ca 50 to 80 % of the nitrogen in the newly formed particulate matter originated from ammonium (Table 3). The estimates of ammonium consumption (i) derived from the isotope-dilution model (Blackburn 1979) were about the same as p; thus, the loss in ammonium from the medium was largely converted into bacterial biomass.

Applying the isotope-dilution model (Blackburn 1979), it was estimated that 50 to 70 % of the DON taken up was mineralized to ammonium (Table 3). Patterns of ammonium regeneration agreed well with that of ammonium uptake, i.e. low ammonium uptake with low ammonium regeneration. Since the recovery of labelled nitrogen as ammonium and as PON was

Table 3. NH_4^+ incorporation (p, in µg-at. N l^{-1}) and its contribution to particulate organic nitrogen (PON) increase (p/M), and dissolved organic nitrogen (DON) mineralization (d, in µg-at. N l^{-1}) and its proportion to DON consumed (d/C) from the media after 1 growth phase, for Cultures 88-A, 88-B, 89-A, and 89-B. Values are the average of 3 points at steady state; SD in parentheses. Calculations for p, i, d, and k are explained in the text. PON increase (M) and DON consumption (C) were calculated from their changes in concentration during the incubation

	88-A	88-B	89-A	89-B
p	1.42 (0.07)	2.19 (0.04)	1.55 (0.43)	0.69 (0.24)
i	1.48 (0.08)	2.04 (0.37)	1.68 (0.41)	0.80 (0.26)
M	1.92 (0.16)	2.80 (0.17)	2.20 (0.93)	1.41 (0.48)
p/M (%)	74	79	74	48
d	0.80 (0.08)	1.28 (0.35)	1.23 (0.32)	0.75 (0.20)
k	0.81 (0.12)	1.38 (0.54)	1.15 (0.23)	0.79 (0.23)
C	1.29 (0.16)	1.99 (0.34)	1.87 (0.72)	1.51 (0.46)
d/C (%)	62	64	68	47

nearly 100 % at all times, another estimate of DON mineralization based on mass balancing can be derived. The mass balance estimate (k) is the difference between total nitrogen uptake (ammonium plus DON consumption) and the increase in PON. The k estimates obtained in this manner agreed well with those from isotope dilution. This value confirms the previous estimate of ammonium production and indicates that all nitrogen pathways involved have been accounted for.

Ammonium uptake and regeneration with time

Increase in PON, cumulative estimates of ammonium incorporation, and proportion of ammonium to PON over time are shown in Fig. 3. Different patterns were observed in the 2 cultures. In 89-A (Aburatsubo Bay water) the contribution of ammonium to PON was ca 80 % at the beginning of the experiment and decreased to ca 60 % at the stationary phase. On the other hand, in 89-B (Ohtsuchi Bay water) the contribution of ammonium to PON was roughly constant, ca 40 to 45 %, throughout the growth of the microbial population. Several factors would have contributed to the varying proportion of ammonium in newly formed bacterial cells, such as species composition of the population, their physiological state, and dissolved nitrogen and carbon content of the media.

Estimates of DON consumption and the cumulative amounts and portion of DON mineralized with time are shown in Fig. 4. In both cultures, it was observed that a large portion of DON was converted to ammonium from the beginning until the end of 1 set of growth. Here again, high ammonium uptake corresponded to high DON mineralization.

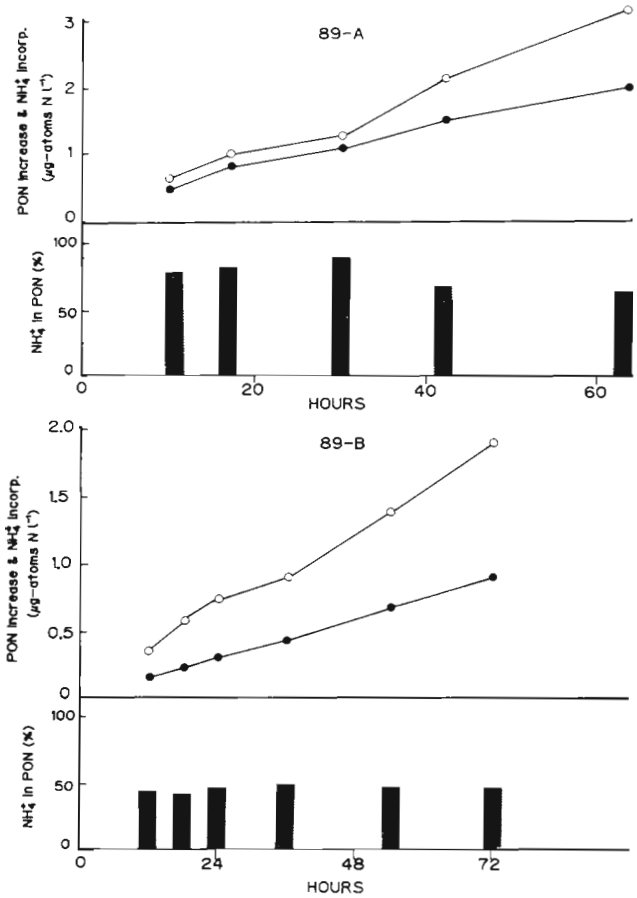

Fig. 3. Increase in particulate organice nitrogen concentration (PON, ○) and ammonium incorporation (●), and accumulated proportion of ammonium incorporation to PON increase, over time, for Cultures 89-A and 89-B

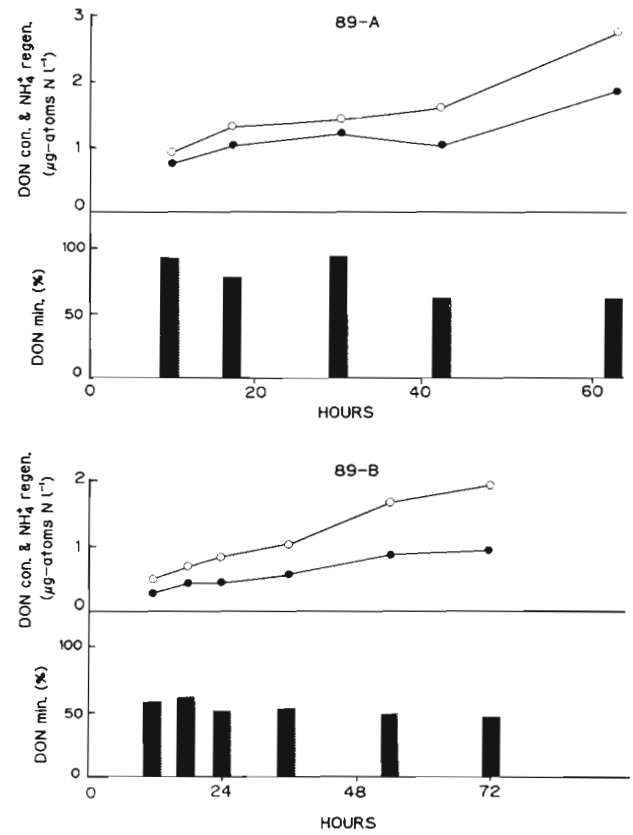

Fig. 4. Consumption of dissolved organic nitrogen (DON, ○), amount of ammonium regenerated (●), and the proportion of ammonium regenerated to DON consumed, with time, for Cultures 89-A and 89-B

Amino acid utilization by bacterial cultures

One objective of these experiments was to evaluate the utilization of amino acids by the growing bacterial population and compare this to the composition of THAA in seawater, as well as to the amino acids generally found in bacterial protein. A listing of the average molar composition of THAA in the 4 water samples, the average of those consumed, and the molar composition of amino acids in bacterial protein (Reeck 1983, Simon & Azam 1989) is given in Table 4. The values of Simon & Azam (1989) are from proteins extracted from a natural bacterial assemblage in seawater. The values of Reeck (1983) are the average of known bacterial proteins from different cellular components. These 2 do not differ much from each other.

Little variation among the 4 experiments was observed in the molar composition of the ambient total amino acids and of those that were consumed. Glutamic acid, glycine (our HPLC system clearly separates glycine from threonine), and serine were the

Table 4. Average molar composition (mole %) of total hydrolyzable amino acids in the media, of those consumed, and of bacterial protein amino acids (BPAA). (–) below detection limit of 1 nM

Amino acid	Media	Consumed	BPAA 1[a]	BPAA 2[b]
Aspartic acid	11.3	8.5	13.3	11.3
Glutamic acid	19.3	20.3	12.4	10.5
Serine	16.8	17.5	6.4	5.5
Histidine	–	–	2.7	2.3
Threonine + glycine	31.1	35.9	16.1	13.7
Alanine + arginine	11.7	9.1	16.6	14.1
Tyrosine	0.5	0.5	3.6	3.1
Valine	–	–	8.7	7.4
Phenylalanine	–	–	4.1	3.5
Isoleucine	–	–	6.4	5.5
Leucine	5.2	4.7	9.9	8.5
Lysine	4.0	3.5	–	6.0
Methionine	–	–	–	2.2
Tryptophane	–	–	–	3.1
Cysteine	–	–	–	1.1

[a] From Simon & Azam (1989)
[b] From Reeck (1983)

dominant amino acids in the medium as well as of those which were consumed. It was observed that several of the amino acids found in bacterial protein could not be obtained from the seawater, which implies the necessity for cellular synthesis (see 'Discussion').

DISCUSSION

The classical function of heterotrophic marine bacteria has been the remineralization of nitrogen (Fig. 5, Path 4). This view has been modified recently by studies showing that microbial growth rates reflect a high nitrogen demand which cannot be totally supported by the utilizable portion of DON in seawater, i.e. amino acids (Wheeler & Kirchman 1986). Ammonium was found to supplement total bacterial nitrogen demands (Path 2a); this has been shown to occur commonly in different marine waters (Wheeler & Kirchman 1986, Goldman et al. 1987, Kirchman et al. 1989).

The net uptake and regeneration of ammonium by bacteria can be described as a function of the carbon and nitrogen content of the bacteria and of bacterial substrates (Goldman et al. 1987, Newell et al. 1988). However, this stoichiometric relationship, referred to as the C : N ratio model, has little physiological basis and is limited to predicting only net effects of nitrogen metabolism.

Interpretations from this model are based on the generally accepted idea that cells metabolize their substrates for maximum economy of growth. The idea that ammonium is utilized as a supplemental nitrogen source teleologically implies that ammonium regeneration will be minimal or not take place at all because of the high demand for nitrogen (Goldman et al. 1987). Conversely, because of the preferential utilization of amino acids over ammonium, the mineralization of such compounds is held to imply sufficiency of nitrogen; thus, ammonium is not considered necessary for bacterial growth (Kirchman et al. 1989).

Our work, however, demonstrates that bacterial populations actively assimilate ammonium with the simultaneous mineralization of DON. The time-course of ^{15}N changes is evidence that these 2 processes take place simultaneously (Fig. 2). The dual function observed here appears to be regulated not by substrate specifications but rather by metabolic constraints (Hollibaugh 1978, Zehr et al. 1985). The cumulative amounts of ammonium uptake and DON mineralization with time (Figs. 3 & 4) indicate that these processes roughly follow the patterns of PON accumulation and DON consumption. Specific rates are difficult to determine, however, because they appear to vary during the incubation, even within the same growth phase. The net result of these processes would be addition or consumption of ammonium in the water. Regardless of the net result, however, this study identifies the function and importance of heterotrophic bacterial populations in consumption and regeneration of ammonium (Fig. 5, Paths 2a and 4), processes which occur concomitantly, and in certain cases equally.

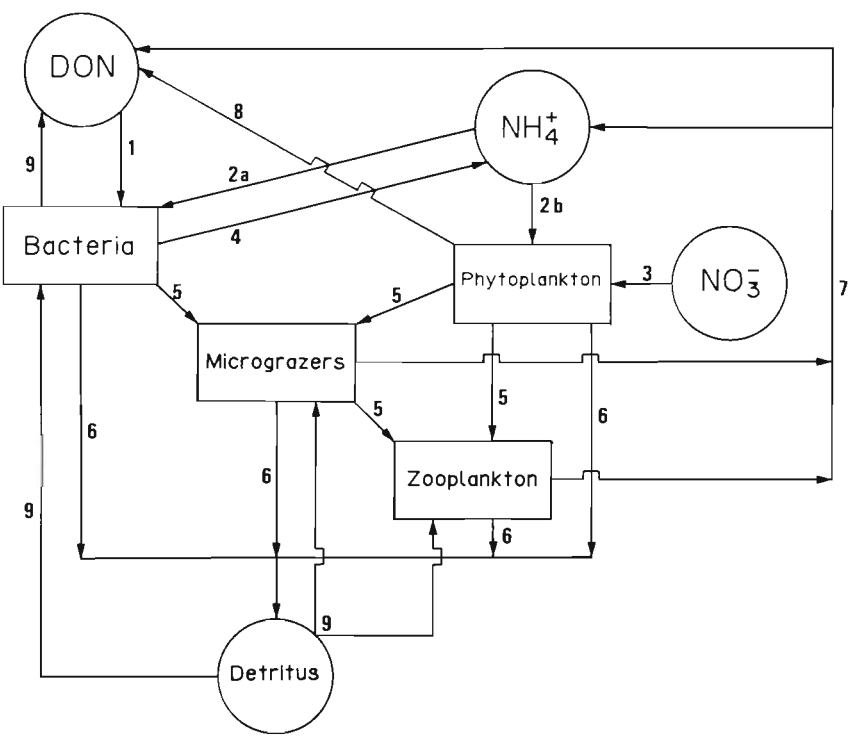

Fig. 5. Nitrogen cycling through the microbial food web. Pathways involved are (1) dissolved organic nitrogen (DON) assimilation, (2) ammonium assimilation, (3) nitrate assimilation, (4) DON mineralization (ammonium regeneration), (5) grazing, (6) detrital formation, (7) excretion/sloppy feeding, (8) excretion/release, and (9) detrital assimilation/degradation

Even if we consider literature values of ammonium uptake by the chl *a*-containing population in the cultures (Pennock 1987), this amount would be <10 % of the total amount of estimated ammonium uptake. In addition, chl *a* concentration did not change during the experiment (Table 2), and all incubations were done in the dark. The effect of autotrophic ammonium assimilation under these conditions, therefore, would be insignificant if not negligible.

The presence of bacterial grazers in seawater would contribute to the production of ammonium; however, for the experiments conducted here, their presence and activity were greatly suppressed if not totally eliminated. Fractionation of seawater resulted in near elimination of the potential grazer population (Table 1); the small number in the media did not change during incubation (Table 2), because the duration of incubation was short and did not allow for development of the grazer population. Grazing activity, if any, would therefore have contributed an insignificant amount of ammonium to the total regenerated nitrogen.

Estimates of bacterial nitrogen demand can be derived from the increase in PON. Assuming a bacterial C : N ratio (atom : atom) of 5 (Nagata 1986), and a gross growth efficiency of 30 % (Bjørnsen 1986), bacterial carbon requirements would range from 24 to 47 μg-at. C l^{-1}. Since the integrated C : N ratio for amino acids in the seawater media was ca 4 (Table 4), carbon contribution from the amino acids consumed from the media would only be ca 10 to 20 % of total carbon demand. This implies that major carbon demands are met by non-nitrogen-containing carbon compounds. Although the concentration of total dissolved amino acids was greatly reduced at the end of 1 growth phase of the population, there were still large amounts of ammonium remaining, implying that the bacterial population may have been limited by carbon rather than nitrogen. This condition of carbon limitation has been shown to occur in natural waters (Kirchman 1990, Kirchman et al. 1990).

In this study, THAA were the major form of DON utilized. The bacteria consumed about 70 % of the total amino acids in the media, implying that at least this portion of amino-acid nitrogen was available for the bacteria. Despite this, only half of amino-acid nitrogen taken up was used as bacterial biomass, while external ammonium provided the major portion of nitrogen demand. We speculate that metabolic requirements govern the assimilation of carbon and nitrogen compounds, and that amino acids are assimilated largely for energy and carbon skeletons, with nitrogen being released outside the cell to be assimilated later through ammonium metabolic pathways.

It has been argued that the use of FAA is energetically more advantageous than processing combined forms or ammonium and carbon skeletons (Kirchman & Hodson 1986). In the marine environment, however, the concentration of DFAA is only a small fraction of total dissolved amino acids, which are largely in the form of combined amino acids. Dissolved combined amino acids (DCAA), such as simple peptides or proteins, are considered to be a more important source of nitrogen for marine bacteria than are FAA (Azam & Cho 1987, Coffin 1989).

The major portion of amino acids utilized from the seawater in our experiments were in the form of DCAA, since the concentration of total DFAA in Ohtsuchi and Aburatsubo Bay waters is very low (ca 0.2 μM). Bacteria are capable of transporting a peptide with as much as 6 amino-acid residues but cannot select all the amino acids in compliance with their requirements (Payne 1976). It has been suggested that these excess amino acids are catabolized and excreted (Coffin 1989), since bacteria would not store amino acids non-essential for protein synthesis (Kirchman & Hodson 1986). Transport mechanisms that function to supply amino substrates as carbon sources rather than for protein synthesis have been identified in bacteria (Alper & Ames 1978, Payne 1980).

Amino acids were consumed in roughly the same proportion as that of their molar composition in seawater (Table 4). This suggests that there may be little selection for certain amino acids. Amino acid composition of bacterial proteins (Reeck 1983, Simon & Azam 1989) shows that histidine, valine, phenylalanine, and isoleucine compose about 22 % of bacterial protein. These amino acids, however, are not present in seawater media and therefore must be synthesized. Amino acids such as glutamate, glycine, and serine, which are consumed in ratios much higher than those in cells, may provide the material for such synthesis. Construction of new amino acids may also occur through anabolic processes using ammonium and carbon skeletons, which appear to be considerable in these experiments. Utilization of ammonium for amino acid synthesis would be more energy-consuming but yield a more versatile means of providing the necessary amino acids for bacterial proteins.

The results presented here are from a mixed assemblage of natural bacterial populations. Specific metabolic mechanisms of individual species cannot be ascertained. The assemblage would be composed of different populations assimilating ammonium or DON, exclusively or mutually. Most bacteria, however, possess transport mechanisms for both organic and inorganic nitrogen (Payne 1980). It would be reasonable to assume then that a single cell would be capable of mutually assimilating amino acids and ammonium, while simultaneously mineralizing organic nitrogen.

Examination of the metabolic mechanisms of axenic cultures would give definite information on this.

Microorganisms < 1 μm in size have been reported to be responsible for up to 60 % of total ammonium assimilation in coastal marine waters (Wheeler & Kirchman 1986, Harrison & Wood 1988), and the major portion of these organisms were prokaryotes. The identity of major contributors to ammonium regeneration, on the other hand, is only speculative at present since organisms at many trophic levels are capable of such a function. It has been argued on the basis of discounting DON mineralization by marine bacteria that heterotrophic microflagellates and microzooplankton are mainly responsible for ammonium regeneration. Our experiments suggest, however, that bacterial regeneration of ammonium occurs to a large extent, even when C : N models predict no net production of ammonium.

We speculate that in the natural environment, the flow of inorganic nitrogen through the bacterial component of the planktonic food web occurs in 2 directions (Fig. 5), from inorganic nutrients to bio-particles, i.e. the bacteria (Path 2a), and through DON mineralization (Path 4). Due to their ability to absorb low concentrations of nutrients from their environment (Paths 1 and 2a), marine bacteria are effective utilizers and competitors for limiting nutrients, such as ammonium. At the same time, their ability to regenerate ammonium from DON allows for the steady production of ammonium through bacterial metabolism (Path 4).

This work is consistent with the traditional view that bacteria are efficient mineralizers of nitrogen in the marine environment. At the same time it also supports the idea that marine bacteria subsist on ammonium for growth, in some cases to a large extent. These 2 previously conflicting ideas are reconciled in the light of these new findings. Uptake and regeneration of ammonium appear to be basic characteristics of nitrogen metabolism by bacterial assemblages. Amino acids in seawater are apparently inadequate for providing the necessary kind of building blocks for bacterial proteins; thus, quality not quantity of substrates may be significant in controlling bacterial growth and production.

LITERATURE CITED

Alper, M. D., Ames, B. N. (1978). Transport of antibiotics and metabolite analogues by systems under cyclic AMP control: positive selection of *Salmonella typhimurium cya* and *crp* mutants. J. Bacteriol. 138: 149–157

Azam, F., Cho, B. C. (1987). Bacterial utilization of organic matter in the sea. In: Fletcher, M., Gray, T R. (eds.) Cambridge, Univ. Press. p. 261–281

Azam, F. Fenchel, T., Field, J. G., Gray, J. S., Meyer-Reil, L. A., Thingstad, F. (1983). The ecological role of water column microbes in the sea. Mar. Ecol. Prog. Ser. 10: 257–263

Bjørnsen, P. K. (1986). Bacterioplankton growth yield in continuous seawater cultures. Mar. Ecol. Prog. Ser 30: 191–196

Blackburn, T. H. (1979). Method for measuring rates of NH_4^+ turnover in anoxic marine sediments, using a ^{15}N-NH_4^+ dilution technique. Appl. environ. Microbiol. 37: 760–765

Coffin, R. B. (1989). Bacterial uptake of dissolved free and combined amino acids in estuarine waters. Limnol. Oceanogr. 34: 531–542

Dugdale, R. C., Wilkerson, F. P. (1986). The use of ^{15}N to measure nitrogen uptake in eutrophic oceans; experimental considerations. Limnol. Oceanogr. 31. 673–689

Fuhrman, J. A. (1987). Close coupling between release and uptake of dissolved free amino acids in seawater by an isotope dilution method. Mar. Ecol. Prog. Ser. 37: 45–52

Gardner, W. S., Chandler, J. F., Laird, G. A., Carrick, H. J. (1987). Sources and fate of dissolved free amino acids in epilimnetic Lake Michigan water. Limnol. Oceanogr. 32: 1353–1362

Glibert, P. M., Lipschultz, F., McCarthy, J. J., Altabet, M. A. (1982). Isotope dilution models of uptake and mineralization of ammonium by marine plankton. Limnol. Oceanogr. 27: 639–650

Goldman, J. C., Caron, D. A., Dennet, R. M. (1987). Regulation of gross growth efficiency in bacteria by substrate C : N ratio. Limnol. Oceanogr. 32: 1239–1252

Harrison, W. G., Wood, L. J. E. (1988). Inorganic nitrogen uptake by marine picoplankton: evidence for size partitioning. Limnol. Oceanogr 33: 468–475

Hollibaugh, J. T (1978). Nitrogen regeneration during the degradation of several amino acids by plankton communities collected near Halifax, Nova Scotia, Canada. Mar. Biol. 45: 191–201

Keeney, D. R., Nelson, D. W. (1982). Nitrogen-inorganic forms. In: Black et al. (eds.) Methods of soil analysis, Part 2, 2nd edn. Am. Soc. Agron. p. 643–693

Kirchman, D. L. (1990). Limitation of bacterial growth by dissolved organic matter in the subarctic Pacific. Mar. Ecol. Prog. Ser. 62: 47–54

Kirchman, D. L., Hodson, R. (1986). Metabolic regulation of amino acid uptake in marine waters. Limnol. Oceanogr. 31: 339–350

Kirchman, D. L., Keil, R. G., Wheeler, P. A. (1989). The effect of amino acids on ammonium utilization and regeneration by heterotrophic bacteria in the subarctic Pacific. Deep Sea Res. 36: 1763–1776

Kirchman, D. L., Keil, R. G., Wheeler, P. A. (1990). Carbon limitation of ammonium uptake by heterotrophic bacteria in the subarctic Pacific. Limnol. Oceanogr. 35: 1258–1266

Kogure, K., Koike, I. (1987). Particle counter determination of bacterial biomass in seawater. Appl. environ. Microbiol. 53: 274–277

Lindroth, P., Mopper, K. (1979). High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatizations with o-phthaldialdehyde. Anal. Chem. 51: 1667–1674

Nagata, T. (1986). Carbon and nitrogen content of natural planktonic bacteria. Appl. environ. Microbiol. 52: 28–32

Newell, R. C., Moloney, C. L., Field, J. G., Lucas, M. I., Probyn, T. A. (1988). Nitrogen models at the community level: plant-animal-microbe interactions. In: Blackburn, T. H., Sørensen, J. (eds.) Nitrogen cycling in coastal marine environments. Wiley, SCOPE, p. 379–414

Ohtsuki, A., Ino, Y., Fujii, T. (1983). Simultaneous measurement and determination of stable carbon and nitrogen isotope ratios, and organic carbon and nitrogen contents in biological samples by coupling a small quadropole mass

spectrometer and a modified carbon-nitrogen elemental analyzer. Int. J. Mass Spectrom. Ion Phys. 48: 343–346
Payne, J. W. (1976). Peptides and microorganisms. Adv. Microbiol. Physiol. 13: 55–113
Payne, J. W (1980). Regulation of peptide transport in bacteria. In: Payne, J. W (ed.) Microorganisms and nitrogen sources. Wiley, New York, p. 335–358
Pennock, J. R. (1987). Temporal and spatial variability in phytoplankton ammonium and nitrate uptake in the Delaware estuary. Estuar. coast. Shelf Sci. 24: 841–857
Porter, K. G., Feig, Y. S. (1980). The use of DAPI for identifying and counting aquatic micro-flora. Limnol. Oceanogr. 25: 943–948
Reeck, G. R. (1983) Amino acid composition of selected proteins. In: Laskin, A. I., Lechevalier, H. A. (eds.) Handbook of microbiology, Vol. 2: Microbial composition. CRC Press, Boca Raton, p. 15–25
Robertson, K. J., Williams, P. M., Bada, J. L. (1987). Acid hydrolysis of dissolved combined amino acids in seawater: a precautionary note. Limnol. Oceanogr. 32: 996–997

This article was submitted to the editor

Simon, M., Azam, F. (1989). Protein content and protein synthesis rates of planktonic marine bacteria. Mar. Ecol. Prog. Ser. 51: 201–213
Strickland, J. D. H., Parsons, T R. (1972). A practical handbook of seawater analysis, 2nd edn. Bull. Fish Res. Bd Can. 167
Suzuki, R., Ishimaru, T. (1990). An improved method for the determination of phytoplankton chlorophyll using N,N-dimethylformamide. J. Oceanographical Soc. Japan 46: 190–194.
Tupas, L., Koike, I. (1990). Amino acid and ammonium utilization by heterotrophic marine bacteria grown in enriched seawater. Limnol. Oceanogr. 35: 1145–1155
Wheeler, P. A., Kirchman, D. L. (1986). Utilization of organic and inorganic nitrogen by bacteria in marine systems. Limnol. Oceanogr. 31: 998–1009
Zehr, J. P., Axler, R. P., Goldman, C. R. (1985). Heterotrophic mineralization of amino acid nitrogen in subalpine Castle Lake. Mar. Chem. 16: 343–350

Manuscript first received: August 28, 1990
Revised version accepted: December 14, 1990