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Use of ^{13}N as tracer for bacterial and algal uptake of ammonium from seawater

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ABSTRACT: The relative importance of phytoplankton and heterotrophic bacteria in the utilization of ammonium in a temperate coastal pelagic environment (Long Island Sound, New York, USA) was examined using the short-lived radioisotope, ^{13}N. Uptake of $^{13}NH_4^+$ into different size fractions under simulated *in situ* temperature and light conditions was compared to size fractionations of bacterial abundance, chlorophyll, and uptake of tritiated thymidine and ^{14}C-bicarbonate. In January and April, little ^{13}N entered the bacterial size fraction, but when bacteria were more metabolically active in May and July, about ⅓ of the total ^{13}N uptake appeared in the size fraction containing most of the bacteria but little of the chlorophyll (0.2 to 0.6 µm). Exposure to up to 8 mCi l^{-1} of ^{13}N radioactivity did not inhibit bacterial or phytoplankton activity, and killed controls showed virtually no uptake. Ammonium uptake rates measured by ^{13}N were comparable to those measured by the stable isotope ^{15}N in 2 of 3 experiments. The size fractionation results were consistent with estimates of bacterial and phytoplankton demand for nitrogen for growth. Turnover rates for the dissolved ammonium pool ranged from 0.6 % h^{-1} in April to 116 % h^{-1} in July. In separate experiments with mixed 'seawater cultures' of bacteria, the half-saturation constant (K_t) for ammonium uptake was <0.1 µ*M*, and ammonium uptake was reduced more by additions (100 to 1000 n*M*) of glutamine than by glutamate or other amino acids, suggesting that glutamine is directly involved in ammonium uptake in these bacteria.

INTRODUCTION

Nitrogen is probably the most important element limiting biological production on short time scales in the open ocean, and research to date suggests that dissolved ammonium is the most important source of nitrogen for phytoplankton (Dugdale & Goering 1967, McCarthy 1980). Thus, there is considerable interest in ammonium utilization in the sea. This property is most commonly measured with ammonium labelled with the stable isotope ^{15}N, and it is usually assumed that all of the uptake of this isotope is by phytoplankton. However, given new information on bacteria and their activities in the sea, this assumption may not be correct.

In recent years, it has become clear that heterotrophic bacterioplankton play a major role in carbon cycling in pelagic marine environments, consuming as much as 60 % of the total primary production (Hagström et al. 1979, Fuhrman & Azam 1980, 1982, Williams 1981). Less attention has been paid to the role of heterotrophic bacteria in nitrogen cycling, but some recent reports suggest that this group may indeed be responsible for a significant fraction of the total ammonium utilization. Horstmann & Hoppe (1981) showed that bacteria could successfully compete with phytoplankton from the Baltic Sea for uptake of the ammonium analog methylamine. Laws et al. (1985) invoked heterotrophic bacterial ammonium uptake to account for a large discrepancy between observed total ^{15}N ammonium uptake and an estimate of phytoplankton N demand from ^{14}C-bicarbonate incorporation into protein. In a study that used specific inhibitors and size fractionations in concert with uptake of ^{15}N labelled substrates, Wheeler & Kirchman (1986) concluded that heterotrophic bacteria may be responsible for more than half of the total ammonium utilization.

Most previous tracer studies of nitrogen utilization have used the stable isotope ^{15}N, which cannot always be added at true tracer levels (Harrison 1983, Cooper et al. 1985). Logistic difficulties associated with using the radioisotope ^{13}N, which has a 10-min half life and must therefore be produced, rapidly purified, and used immediately, has precluded its extensive use in studies of nitrogen cycling. However, ^{13}N has major advantages of sensitivity and detectability. It has an extremely high specific activity (1.89×10^{10} Ci $mole^{-1}$

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of pure ^{13}N), and therefore can be added and detected at true trace levels (e.g. picomolar) in experiments lasting only minutes. Detection of radioactive decay in filter-collected or liquid samples is accomplished in a gamma counter and is much simpler and quicker than for ^{15}N, which requires conversion to N_2 gas prior to mass or emission spectrometry. Furthermore, combustion to N_2 gas does not usually permit the use of organic membrane filters which are now in common use in studies of size fractionation. We have used ^{13}N to examine the roles of heterotrophic bacterioplankton in ammonium utilization by coastal marine organisms and have compared these estimates to those based on more conventional ^{15}N procedures.

MATERIALS AND METHODS

Water samples were collected in an acid-washed bucket from a pebble beach at Crane Neck, New York (USA), on Long Island Sound (Lat. 40° 55.3' N Long. 73° 09.3' W). These were transferred into clean plastic bottles and taken within 1 h to either the Marine Sciences Research Center (for everything but the ^{13}N work) or the Cyclotron Laboratory of Brookhaven National Laboratory (for ^{13}N) where they were stored under simulated *in situ* temperature and light conditions for at most 2 h before experiments.

^{13}N-ammonium uptake by various size fractions was measured in January, April, May, and July 1985. Other properties that were measured in various size-fractions included chlorophyll content measured fluorometrically (Parsons et al. 1984), direct bacterial counts by acridine-orange epifluorescence microscopy (Hobbie et al. 1977), and uptake of 5 n*M* [methyl-^{3}H] thymidine and ^{14}C-bicarbonate (Fuhrman et al. 1986). The size fractionation protocol followed that of Fuhrman et al. (1986) and essentially consisted of parallel filtrations through 25 mm diameter Nuclepore filters with pore sizes ranging from 0.2 to 8.0 µm. Volumes filtered were 10 ml except for chlorophyll, which required 50 ml. ^{14}C-bicarbonate size fractionations did not include all filter pore sizes (see 'Results').

^{13}N-ammonium was produced by the $^{16}O(p,a)^{13}N$ reaction (Cooper et al. 1985) bombarding 1 ml of ^{16}O water with 17 MeV protons using the BNL Division of Chemistry Japan Steel Works 41 inch BC1710 cyclotron (Vaalberg et al. 1975). The irradiated water contains predominantly $^{13}NO_3^-$ with some $^{13}NO_2^-$, with yields of up to 100 mCi ml^{-1}. $^{13}NO_2^-$ and $^{13}NO_3^-$ were converted to $^{13}NH_4^+$ with de Varda's alloy (Gelbard et al. 1975, Vaalberg et al. 1975). Irradiated water was transferred from the target by remote application of pressure into a vessel containing 0.5 g of de Varda's alloy and 0.5 g of NaOH. $^{13}NH_3$ in the effluent gas from the vessel was purged into 1 ml of water, which traps the activity as $^{13}NH_4^+$, and this was passed through 1 ml of an anion exchange resin (AG 1 × 8 Cl^-) to remove traces of $^{13}NO_3^-$ and $^{13}NO_2^-$. The resin was rinsed with 1 ml of water HPLC analysis (Chasko & Thayer 1981) of the resulting water solution found only $^{13}NH_4^+$ with no traces of $^{13}NO_3^-$ or $^{13}NO_2^-$. Typical realized yields of $^{13}NH_4^+$ are 1 to 10 mCi ml^{-1} (not decay corrected). Because of contaminant ammonium in the reducing agents (Cooper et al. 1985), the final NH_4^+ concentration in the trapping solution was about 100 µ*M*, resulting in a specific activity of 10^5 to 10^6 Ci mol^{-1}.

Uptake of ^{13}N-ammonium was measured by closely-spaced time course analysis of (a) radioactivity accumulated onto the different pore-size filters and (b) disappearance from the filtrates. Owing to the short half-life, measurements could only be made over a period of ca 1.5 to 2 h. Additions of the ^{13}N stock solutions were typically 1 to 6 mCi l^{-1} of sample. Radioactivity was measured immediately in a Packard gamma counter and all counts were decay-corrected to a common time, usually the start of the experiment. Ammonium concentrations in filtered water (0.2 µm or Whatman GF/F) were determined by the phenol-hypochlorite method (Solorzano 1969) as adapted for an autoanalyzer (Whitledge et al. 1981).

To assess the possible toxicity from the gamma decay of the added ^{13}N or from any contaminants within the solutions, the water remaining in the containers used for the ^{13}N experiments (after the experiments were over and most of the ^{13}N had decayed) were taken to the Marine Sciences Research Center for measurements of thymidine incorporation and ^{14}C-bicarbonate uptake (0.2 µm pore size filters only). These measurements occurred 4 to 6 h after similar measurements had been made with freshly-collected samples.

Uptake of the ^{15}N ammonium was assessed with simulated *in situ* incubations. ^{15}N-ammonium (97 % enriched) was added to 1 l (April) or 500 ml (May and July) samples in 1 l Nalgene PMP bottles, at levels of about 10 % of the ambient ammonium concentrations. Incubations were terminated by filtration onto precombusted Whatman GF/F glass fiber filters. Filters were dried at 60 °C overnight and were stored in combusted glass vials until analysis. A subsample of each filtrate was reserved for the t_{final} nutrient analysis, and the remainder was frozen for later distillation and analysis. Incubations lasted 1 h. In May and July, a second time point was also run (at 6.5 h and 4 h respectively), but the ammonium turnover time was too rapid (see 'Results') for the data from the second time point to be useful.

For the ^{15}N work, the ammonium pool was recovered by distillation using the method of Glibert et al. (1982). Carrier ammonium was added to the 300 ml sample to

supply sufficient nitrogen for the requirements of the mass spectrometer. The distillate was spotted onto a Whatman GF/F glass fiber filter immediately before analysis. Mass spectrographic analysis of both the particulate pools and the distilled ammonium pools was done on an AEI MS-10 mass spectrometer (McCarthy et al. 1977). Data were analyzed using the iterative modeling program of Garside & Glibert (1984) to estimate the uptake rate corrected for isotopic dilution of the substrate (ammonium) pool and the turnover time (1/k; Glibert et al. 1982).

For an additional estimate of nitrogen demand by phytoplankton in May and July, we followed the procedure of DiTullio & Laws (1983). This procedure, which is essentially a ^{14}C-bicarbonate uptake experiment followed by extraction with trichloroacetic acid, estimates the phytoplankton protein synthesis rate. We used the conversion factors (N per unit protein) presented by DiTullio & Laws (1983) to calculate phytoplankton N demand.

'Seawater cultures' of bacteria (Ammerman et al. 1984) were grown by inoculation of 0.6 μm polycarbonate Nuclepore filtered seawater into seawater filtered through a 0.22 μm mixed-ester Millipore filter, with inoculum : total ratios of 0.05 to 0.1. Cultures were grown to late log phase (1 to 2 d). Cultures grown for the May 20 experiments included an unenriched control and ones enriched with ammonium (70 n*M*) and amino acids (asp, glu, ser, his, gly, thr, arg, ala, tyr, met, val, phe, ile, leu, lys, pro, cystine, each at 70 n*M*), 100 μg l^{-1} bovine serum albumin (BSA), glucose and ammonium (1 μ*M* each), or 0.3 to 10 μ*M* ammonium chloride at the time of inoculation. The culture used for analysis of uptake kinetics on 3 June was enriched at the time of inoculation with 5 μ*M* D-glucose to cause bacterial depletion of dissolved nitrogen, and aliquots were further enriched with different levels of unlabelled ammonium chloride (0.3 to 10 μ*M*) 15 min before addition of the ^{13}N tracer. Cultures (originally unenriched) used for determining the effects of amino acids on ammonium uptake were dispensed and enriched 20 min before addition of tracer with 1 μ*M* glutamic acid, 1 μ*M* glutamine, 100 n*M* glutamine, or a mixture consisting of 50 n*M* each of the same 17 amino acids listed above.

RESULTS AND DISCUSSION

^{13}N uptake into different size fractions could be followed easily on all dates and, as expected, the smaller pore size filters accumulated radioactivity generally faster than did the larger ones (Fig. 1). Formalin-killed controls did not accumulate substantial radioactivity into any size fraction (Fig. 1C). The rates of uptake into cells collected with the GF/F glass fiber filters were as high or higher than those collected with the smallest pore-size Nuclepore filters, although the data from the GF/F filters were 'noisier' on 15 May. The 'noise' may be related to the larger liquid hold-up volumes of these filters, which makes effective rinsing more difficult.

Comparisons of the different parameters in each size fraction indicate which properties were associated with which organisms. On all dates, thymidine incorporation partitioned into size-fractions very similarly to bacteria (mostly <0.6 μm), confirming that bacteria are responsible for this property (Fig. 2). Uptake of ^{13}N partitioned into size-fractions similarly to chlorophyll (large size fractions) in January and April, but diverged from chlorophyll in May and July, with a larger amount associated with smaller size fractions. Of particular note was the 25 to 30 % of the ^{13}N uptake in May and July that passed through the 0.6 μm filters, and the 40 % that passed 1 μm filters in July, despite the lack of chlorophyll in the smaller size fractions (Fig. 2). These data, in combination with the size fractionations of bacterial numbers and thymidine incorporation, strongly suggest that bacteria can be directly responsible for ⅓ or more of the total ammonium uptake in spring and summer.

The experiments comparing the activities of bacteria and phytoplankton with and without prior exposure to ^{13}N showed no significant differences (*t*-test, $p > 0.05$, data not shown). This indicates little or no toxicity either from the radiation itself or from any contaminants in the isotope solutions.

Estimates of ammonium uptake from ^{13}N and ^{15}N were similar in April and July, but differed markedly in May (Table 1). We do not know the reason for the discrepancy in May, but it appears that there was either inhibition (or competition) in the ^{13}N sample or stimulation in the ^{15}N sample. In any case, there is general agreement in 2 out of 3 comparisons.

Comparisons of estimated N demand by phytoplankton and bacteria demonstrated that the demand by each of these 2 groups of organisms was similar. While the phytoplankton production (carbon fixation) was about 3 to 4 times higher than the bacterial secondary production estimated from thymidine incorporation (Table 1), the different C : N ratios of the 2 groups (6 to 10 for phytoplankton, ~ 4 for bacteria) (Lee & Fuhrman 1987), account for the comparable demands.

Note that the N demand and estimated ammonium uptake rates shown in Table 1 do not always match. When comparing estimates of N demand to ammonium uptake, it is important to keep in mind that ammonium is not the only N source. Bacteria undoubtedly use organic N compounds (see Paul 1983), and phytoplankton use nitrate, nitrite, urea, and possibly organic N as

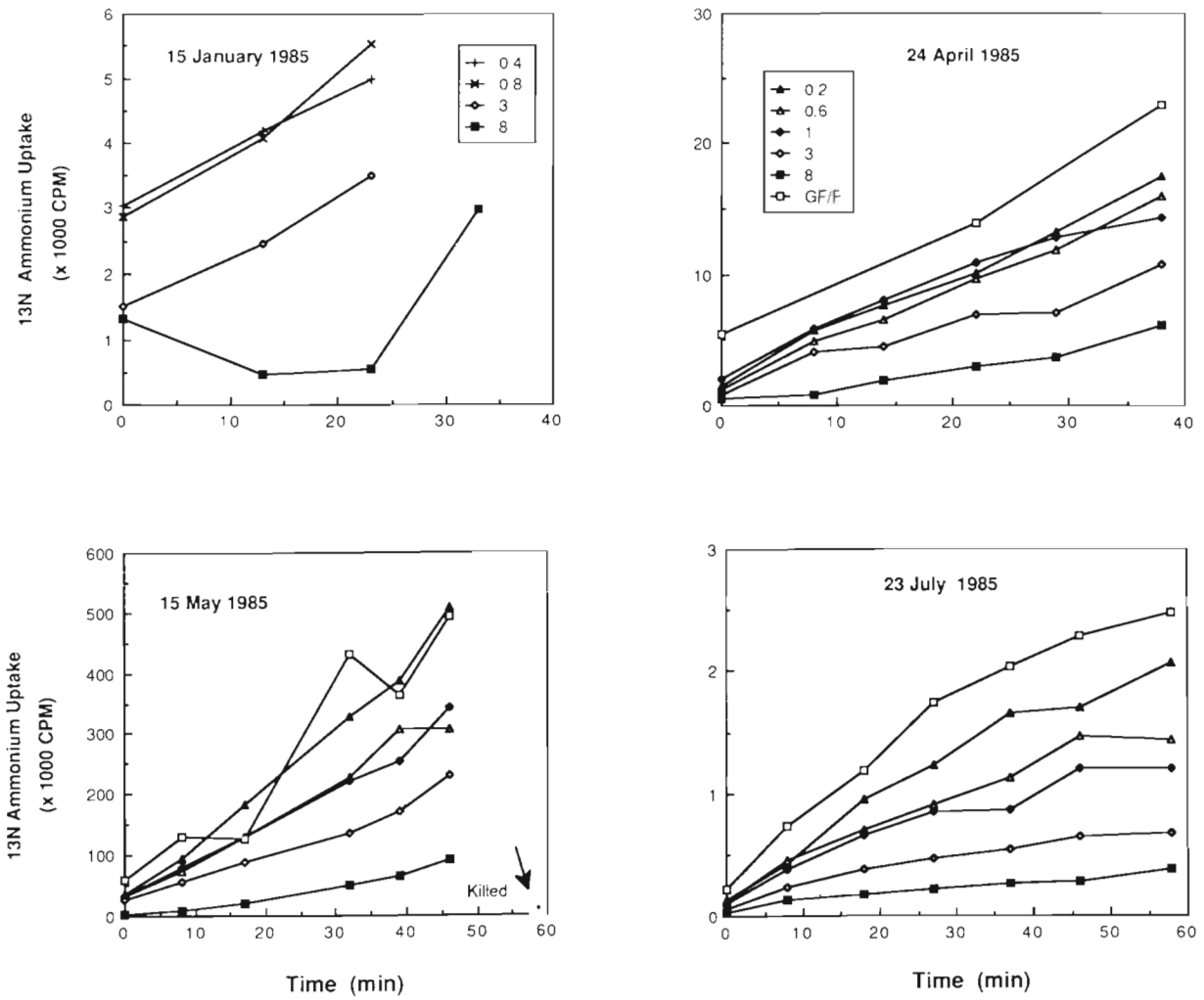

Fig. 1 Time courses of $^{13}NH_4^+$ uptake onto different pore size filters. Symbols as noted, with pore sizes of polycarbonate filters indicated in μm; GF/F: Whatman type GF/F glass fiber filters. All dates in 1985

well. Therefore, ammonium uptake less than the estimated N demand is not unreasonable. However, if the calculations indicate that ammonium uptake significantly exceeds the N demand, as is the case for the July data and the ^{15}N May data, then either there is 'luxury uptake' of ammonium or there is something wrong with the data or calculations. Ammonium analyses for all dates were performed by Autoanalyzer While ammonium concentrations during April and May were within the range of the analyzer, they were at the limit of detectability in July. If ammonium concentrations were overestimated, either through contamination or inaccuracy, then N utilization as determined by both isotopes would also have been overestimated, explaining the high apparent N demand compared to C.

The ammonium pool turnover rates that we measured ranged from 0.6 % h^{-1} in April to 116 % h^{-1} in July. Such rapid summertime turnover indicates the importance of ammonium regeneration in this system. Apparently, the ammonium is taken up within a very short time of its release into the water. In such a system, the processes that regenerate the ammonium are probably major agents in the control of primary productivity, and any organisms, such as the bacteria, that compete for ammonium uptake, exert further control. Until very recently, it was usually believed that the bacteria were mostly nitrogen regenerators. For example, Harrison (1978), Caperon et al. (1979), Glibert (1982), and Harrison et al. (1983) showed that most of regeneration of ammonium was carried out by organisms that passed 35 μm filters, and often much of that passed even 1 μm filters. It should be noted that (1) there were big differences in the size fractionation patterns between authors and locations; (2) several of the ^{15}N studies (e.g. Harrison et al. 1983) also found significant uptake by the <1 μm fraction, but bacterial uptake was not usually discussed. Our observed bacterial competition for ammonium uptake may seem to contradict that view,

Table 1. Comparison of ammonium uptake and estimated N demand

	Uptake N by N-13 (ng-at N l^{-1} h^{-1})	Uptake N by N-15 (ng-at N l^{-1} h^{-1})	Calc. demand Bact. N* (ng-at N l^{-1} h^{-1})	Calc. demand Phyto. N** (ng-at N l^{-1} h^{-1})	Calc. demand Phyto. N*** (ng-at N l^{-1} h^{-1})	% N-13 uptake < 1 µm	Primary production C (µg C l^{-1} h^{-1})	Production ratio bact/phyto
April	12.1	11.4	20.7	30.0	–	20	3.4	0.29
May	47.1	750	142	178	100	34	20	0.35
July	1228	1721	150	257	193	42	29	0.25

* From thymidine incorporation; assumes 2×10^{18} cells produced per mole incorporated and 5.7 fg N $cell^{-1}$
** From primary production, assumes C/N = 8
*** From estimate of protein synthesis (method of DiTullio & Laws 1983)

but it must be realized that the 'bacteria' may consist of many types of prokaryotic organisms (including a variety of heterotrophs and autotrophs), and some may be regenerating ammonium while others are assimilating it.

'Seawater cultures' of naturally derived marine bacteria (0.6 µm filtered seawater inoculated into filter-sterilized seawater and grown in the dark) were used to determine ammonium uptake properties of the bacteria under controlled conditions. The experiment on 20 May 1986 showed no significant difference between ammonium uptake rates (^{13}N-measured turnover rate times ammonium concentration, $p > 0.05$) in unenriched cultures and those enriched at time of inocula-

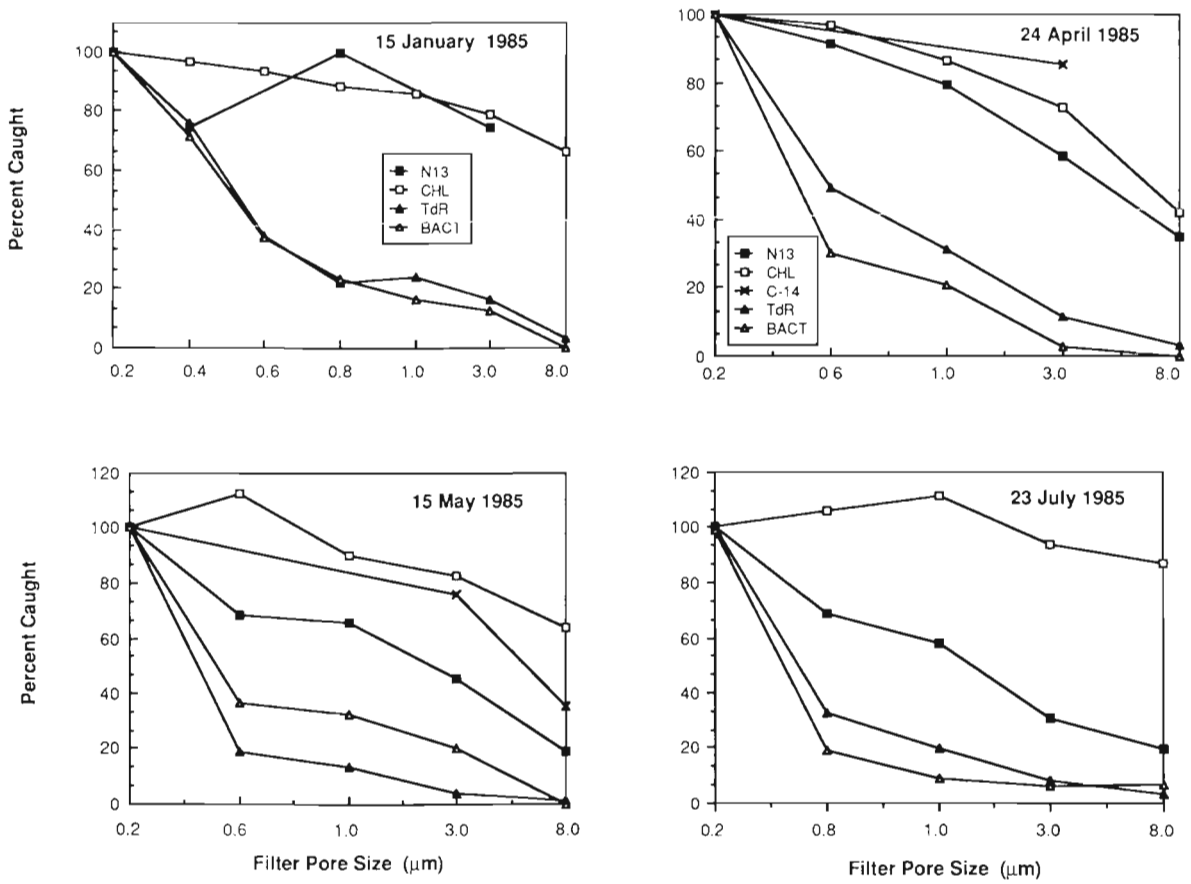

Fig. 2. Size fractionation of measured parameters plotted as percent caught on a filter vs pore size of the filter. Symbols as noted, with abbreviations: N13, $^{13}NH_4^+$ uptake; CHL, chlorophyll *a*; C-14, uptake of [^{14}C]bicarbonate; TdR, uptake of tritiated thymidine; BACT, direct counts of bacteria. All dates in 1985

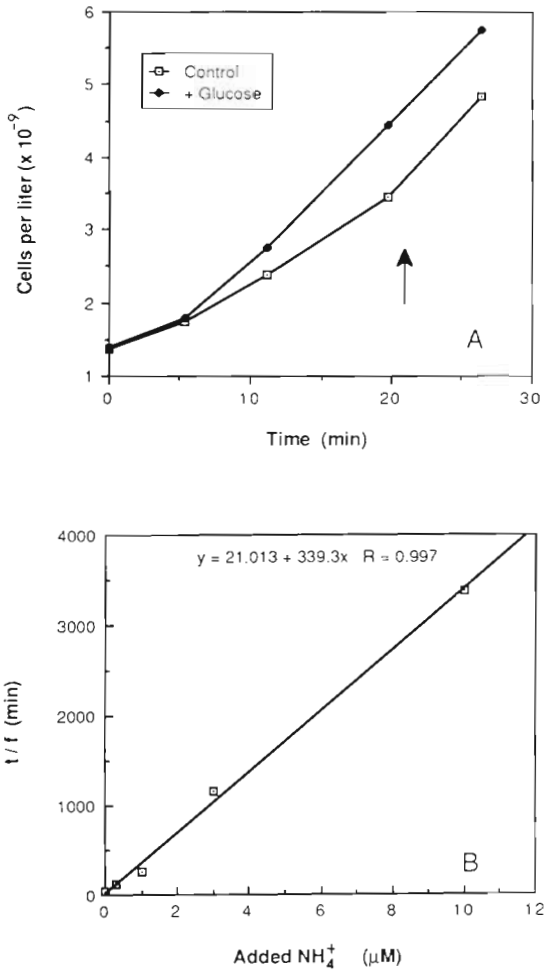

Fig. 3. (A) Growth of 'seawater cultures' used for measurement of uptake kinetics and effects of added amino acids (3 June 1986); arrow: time of experiments. (B) 'Wright-Hobbie' plot of ammonium uptake kinetics in a seawater culture; x-intercept: negative sum of half-saturation constant for uptake (K_t) plus ambient substrate concentration (S_n); slope indicates the reciprocal of the maximum uptake rate (V_{max}) (Wright & Hobbie 1966)

tion with 1.2 µ*M* mixed amino acids, 100 µg l^{-1} protein, and 1 µ*M* each ammonium and glucose. The same culture also showed no changes in uptake rates upon addition of 0.3 to 10 µ*M* ammonium, probably indicating that uptake was already saturated in the unenriched culture. It is significant that the ammonium concentrations in all the experiments on that date were at least 4.1 µ*M*, suggesting that the cultures were not nitrogen limited; this may help explain the similar uptake rates under the various conditions.

Table 2. Effects of amino acids on bacterial ammonium turnover rate from a seawater culture*

Amino acid added	% Reduction
1 µ*M* glutamic acid	38 %
1 µ*M* glutamine	74 %
100 n*M* glutamine	68 %
50 n*M* each of 17 amino acids (no gln)	53 %

* Ammonium turnover rate in the control culture was 0.184 h^{-1}, or ca 130 nmol l^{-1} h^{-1}

To measure uptake kinetics, it was necessary to lower the ammonium concentration in the seawater cultures, so for the experiment on 3 June 1986, one seawater culture was grown in the presence of 5 µ*M* added glucose to induce nitrogen starvation, in an attempt to cause the bacteria to remove almost all of the ammonium. The culture with glucose grew 50 % faster than the control (μ = 0.06 vs 0.04 h^{-1}; Fig. 3A). Analysis of ammonium uptake kinetics from the culture with glucose indicated that the sum of the half-saturation constant for uptake plus the ambient concentration ($K_t + S_n$) was 0.05 µ*M*, and the calculated V_{max} was 2.9 nmol l^{-1} min^{-1} at a cell density of 4.5×10^9 cells l^{-1} (Fig. 3B). Thus, the glucose apparently did cause the bacteria to deplete the ambient ammonium. The very low K_t (<0.05 µ*M*) indicated a high bacterial affinity for ammonium, consistent with the observed bacterial competition with algae for ammonium.

The control culture on 3 June was used to determine the immediate effects of added amino acids on ammonium uptake. There was significant reduction of the rate by all the additions tested, but glutamine had the largest effect, even at 100 n*M* (Table 2). It is significant that glutamine at 100 n*M* had a greater effect on uptake than did a mixture of 50 n*M* each of 17 other amino acids, including 2 other basic amino acids, arginine and lysine. We view this special effect of glutamine as an indication that the uptake mechanism for ammonium by the natural bacteria directly involves glutamine (e.g. glutamine synthetase and/or glutamate synthase), as has been shown for most microorganisms studied to date (Brown et al. 1972, Magasanik 1982, Falkowski 1983).

As our view of the role of bacterioplankton in carbon and energy flow in planktonic systems has recently changed, it appears that we may also need to modify our perception of the role of bacteria in the pelagic nitrogen cycle. Several studies, including the present one, have indicated that bacteria can at times dominate N demand. The generality of this observation needs to be examined in a wider range of environments, especially warm oligotrophic waters that make up most of the ocean's surface. For instance, Probyn (1985) found that picoplankton (<1 µm) accounted for 27 % of the ammonium uptake in oligotrophic offshore waters, compared to 10 % nearshore. The rapid turnover and bacterial uptake of ammonium observed in this study requires that we examine more closely which organ-

isms are doing the regeneration. It is possible that either some bacteria are taking ammonium up while others are regenerating or other organisms (e.g. protozoa) are the dominant regenerators (Harrison 1978, Goldman et al. 1985).

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