Estimation of ammonium regeneration efficiencies associated with bacterivory in pelagic food webs via a $^{15}$N tracer method

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**Abstract.** Phagotrophic protists are major components of pelagic food webs, both as consumers of bacterial and phytoplankton cells, and as regenerators of inorganic nutrients. In this study, we estimated the efficiency of ammonium regeneration by protists feeding on bacteria within natural planktonic assemblages, using a $^{15}$N tracer method, in which the excretion of $^{15}$N-labeled ammonium due to grazing on $^{15}$N pre-labeled bacteria was followed over time. We tested this approach in experiments based on the addition of heat-killed $^{15}$N-labeled bacteria to laboratory cultures and to samples of coastal seawater. During two experiments, variation in abundance of bacterivores and bacterioplankton resulted in non-constant grazing rates. Deterministic computer models that used abundance of bacteria and protists as variables were developed to estimate best-fit values of grazing mortality ($g$, h$^{-1}$) and of ammonium regeneration efficiency ($R_E$, fraction of the initial $^{15}$N label in added bacteria which is released as ammonium). Estimated ammonium $R_E$ were 0.30-0.35 for one trophic link systems with both a monospecific culture and a mixed species assemblage of bacterivorous flagellates. $R_E$ was higher for multi-trophic step food webs: 0.60 for 5 μm pre-screened coastal seawater and 0.90 for whole coastal seawater.

Introduction

Phagotrophic protists, including flagellates and ciliates, are recognized as having a central role in marine food webs. Protistan bacterivory is a major source of mortality for bacterioplankton (McManus and Fuhrman, 1988; Sanders *et al*., 1992) and for phytoplankton (reviewed in Sherr and Sherr, 1994). As a consequence of their grazing activity, protists are also responsible for most of the flux of regenerated nitrogen and phosphorus in marine systems (Caron and Goldman, 1990; Glibert *et al*., 1992). Understanding the details of protistan grazing within natural microbial assemblages is essential to understanding both the functioning of food webs and the cycling of bioactive elements in marine ecosystems.

To date, relatively few methodological approaches have been applied to the evaluation of the dynamics of specific pathways within natural microbial food webs. Most work on nutrient regeneration by protists has either been carried out using laboratory cultures of monospecific isolates of predator and prey (e.g. Andersson *et al*., 1985; Goldman and Caron, 1985; Caron *et al*., 1988; Goldman and Dennett, 1992) or has involved estimation of the overall rate of nutrient regeneration by planktonic assemblages (e.g. Glibert, 1982; Probyn, 1987; Wheeler *et al*., 1989; Glibert *et al*., 1992).

Here we describe a new method to study ammonium regeneration by intact communities in which we followed the release of $^{15}$N-labeled ammonium from $^{15}$N-labeled bacteria in order to estimate rates of ammonium regeneration due to protistan bacterivory on bacteria. In theory, the method also allows the estimation of overall ammonium regeneration efficiency by the food web, which can be used as an index of the complexity (i.e. number of trophic steps) within that food web.
Method

Theoretical considerations

In our assays, tracer amounts of $^{15}$N-labeled heat-killed bacteria were added to samples, and the accumulation of $[^{15}$N]ammonium in the dissolved fraction due to protistan ingestion and digestion of labeled bacteria was followed over time. This approach resembles the ammonium dilution assay (e.g. Glibert, 1982; Wheeler et al., 1989), in that the isotopic enrichment of ammonium is measured. However, while the ammonium dilution assay measures the change in nitrogen isotope abundance due to the dilution of the added $[^{15}$N]ammonium by $[^{14}$N]ammonium excreted by all heterotrophic organisms in the community, in our experiments we measured the quantitative increase of $[^{15}$N]ammonium solely as a result of excretion of $[^{15}$N]ammonium by bacterivores ingesting added pre-labeled bacteria. Previous studies have demonstrated that ammonium is the major nitrogen excretory product of heterotrophic protists (Andersson et al., 1985; Caron and Goldman, 1990).

Theoretically, the overall protist grazing rates will depend on the combined abundance of non-growing, added $^{15}$N-labeled bacterial cells + growing, non-labeled bacterial cells naturally present. However, the uptake of $^{15}$N-labeled bacteria and $[^{15}$N]ammonium excretion rates by protists will be a function of the continuously decreasing concentration of labeled bacteria. Consequently, the abundance of the added $^{15}$N-labeled bacteria should decline exponentially (Figure 1 A) and the concentration of $[^{15}$N]ammonium product should show an exponential rise to a maximum amount (Figure 1 B). Measuring the accumulation of $^{15}$N-labeled ammonium in the dissolved fraction is the most straightforward method of following the consumption of $^{15}$N-labeled bacteria, as the amount of label in the particulate fraction will be a continuously varying combination of label in ungrazed bacteria + label in protistan biomass.

The amount of $^{15}$N-labeled ammonium in the dissolved pool at time $t$, $D(t)$, is:

$$D(t) = D_{\text{MAX}}(1 - e^{-gt})$$  \hspace{1cm} (1)

where $D_{\text{MAX}}$ is the maximum amount of $^{15}$N-labeled ammonium that can be produced from the amount of labeled bacteria added and $g$ is the protistan grazing rate (time$^{-1}$) on the labeled bacteria. Equation (1) can be solved for the best-fit values of $D_{\text{MAX}}$ and $g$, using the Marquard algorithm available in most statistics software packages. $D_{\text{MAX}}$ is the product of the regeneration efficiency ($R_e$) and the amount of labeled bacteria added at the start of the PRA experiment ($C_o$):

$$D_{\text{MAX}} = C_o R_e$$  \hspace{1cm} (2)

Thus, equation (2) can be used to estimate $R_e$ if $C_o$ is experimentally measured and $D_{\text{MAX}}$ is calculated from the time course of $[^{15}$NH$_4$]$^+$.

The grazing rate, $g$, can be expressed either as a function of rate of uptake of bacteria over time: $g = \left(\frac{db}{dt}/B\right)\times$ , where $db/dt$ is the number of bacteria ingested during time $t$ and $B$ is bacterial abundance, or as a function of protistan clearance rate: $g = CR\times P$, where $CR$ is the average per grazer clearance rate for the bacteria and $P$ is abundance of grazers. These relationships show that a change in either the total number of bacteria or grazer abundance during the course of an assay could...
Fig. 1. Hypothetical time course of (A) $^{15}$N remaining in the particulate fraction and (B) $^{15}$N appearing in the dissolved fraction, during the course of [$^{15}$N]ammonium release assays in which labeled prey are added in tracer amounts.

lead to a change in the grazing rate, and thus the associated rate of nutrient regeneration. One way to overcome the problem of varying grazing rates is to use deterministic models in which experimentally measured abundances of bacteria and bacterivores are input as variables in order to obtain best-fit values of $g$ and of $R_E$, as the shape of the [$^{15}$N]ammonium release curve is dependent on both these variables (Figure 2A and B).

The advantages of using $^{15}$N as a label include: (i) $^{15}$N is a stable isotope, thus avoiding the inconvenience of working with radioactive isotopes; and (ii) nitrogen is mainly present in cell proteins, thus the release of ammonium indicates both ingestion and digestion of labeled cells. Disadvantages of using ammonium as the waste product of interest include: (i) uptake of excreted ammonium can occur due to assimilation by both phytoplankton and bacteria; (ii) protocols for quantitative extraction of ammonium from seawater are tedious and require equipment difficult to use at sea; and (iii) measurement of nitrogen isotopic enrichment requires at least 5 μg of N in the sample for accurate analysis, which complicates the experimental design since the volume of water that is required to extract this much total nitrogen increases as the concentration of ammonium decreases.

We sought to minimize disadvantages (i) and (iii) by adding extra $^{14}$N to the incubation bottles. [$^{14}$N]ammonium was added to the incubation bottles to a final concentration of 1 μM (10 μM in Experiment 1) immediately after the addition of the $^{14}$N-labeled bacteria to curtail uptake of excreted [$^{15}$N]ammonium. The bottles...
we were also incubated in the dark to minimize phytoplankton uptake of $^{15}$N-ammonium. To ensure adequate amounts of ammonium-N in samples prepared for isotopic analysis, a known amount of unlabeled carrier nitrogen was added to those samples immediately prior to analysis. This step decreased the final $^{15}$N enrichment of the sample and thus the sensitivity of the method. This means that addition of labeled bacteria must be at levels sufficiently low to avoid saturated feeding, but sufficiently high to result in measurable $^{15}$N enrichment of extracted ammonium. While this requirement can be satisfied when working with coastal seawater, it is a drawback for use of the method in oligotrophic open-ocean water where microbial abundances are low. To overcome the problems involved with extraction of ammonium at sea, a procedure in which samples for ammonium extraction were frozen and stored for later analysis on shore was developed as part of this study.

Fig. 2. Simulation of the time course of appearance of excreted label in the dissolved fraction during a $[^{15}$N]ammonium release assay in which (A) regeneration efficiency ($R_e$) and initial concentration of label in added prey ($C_o$) are constant, while grazing rate ($g$) varies and (B) $C_o$ and $g$ are constant, while $R_e$ varies.
Preparation of labeled bacteria

A rod-shaped bacterial strain that formed yellow colonies on marine agar was isolated from Oregon coastal waters. The bacteria were labeled with $^{15}$N by growing them on a minimal medium with $[^{15}N]$ammonium as the sole nitrogen source. After 48 h of growth, the bacteria were heat killed at 60°C and harvested by centrifugation for 20 min at 10 000 g. The bacteria were brought up in a small volume of sodium pyrophosphate buffer to prevent clumping, and stored frozen until used in the experiments, at which time aliquots of the $^{15}$N-labeled bacteria were sonicated (twice for 5 s at 100 W) to disperse the cells and added in known quantities to experimental bottles.

General protocol for the experiments

Replicate 4 l polycarbonate bottles covered with black tape to ensure darkness were used for incubations. Two of the bottles contained both bacteria and bacterivorous protists, and one bottle contained bacteria-only control samples prepared by filtering water three times through 0.8 μm polycarbonate membrane filters. The experiments were started with the addition of $^{15}$N-labeled bacteria and unlabeled ammonium chloride to each treatment, and the bottles were incubated at 15°C. At each time point sampled, 20 ml samples were taken from each replicate bottle, preserved, stained and filtered onto 0.2 or 0.8 μm Nuclepore filters for enumeration of bacteria via acridine orange direct counts (Kirchman, et al., 1982) and of heterotrophic flagellates via DAPI staining (Sherr et al., 1993).

At each sampling time, 250–300 ml samples from each bottle were filtered through 0.47 mm cellulose acetate filters. A manual punch was used to punch 5 mm discs from the sample area of the filter. Four of the discs from each filter were placed into a tin capsule for mass spectrometry. A 70 μl volume of a 20 mM unlabeled ammonium chloride solution was pipetted onto the discs inside the capsule to ensure that there was sufficient nitrogen in the sample for accurate nitrogen isotopic analysis. The tin capsules were dried at 80°C, folded and stored in a desiccator until analysis.

Aliquots of the filtrate were immediately taken for analysis of ammonium concentration in the bottles (Koroleff, 1983). Unlabeled ammonium was added to the remaining filtrate to 10 μM and the ammonium was then converted to indophenol blue (Dudek et al., 1986). After 24 h of reaction, the indophenol blue samples were frozen for later analysis. The indophenol was extracted for isotopic analysis from thawed samples via solid-phase extraction after the protocol of Wheeler et al. (1989). The indophenol red was eluted in acetone and spotted onto 5 mm glass fiber discs that were placed in mass spectrometry tin capsules and dried at 80°C. The folded capsules were stored in a desiccator prior to analysis. The $^{15}$N enrichment and total nitrogen content of the samples were determined via mass spectrometry with a Europa Scientific mass spectrometer. From these values, the total amount of $^{15}$N in each sample was calculated.

To estimate the best-fit (lowest SE) values of the parameters $D_{\text{MAX}}$ and $g$ of equation (1), the time series data were tabulated in a spreadsheet and analyzed by the
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Marquard non-linear regression procedure using the statistical software Statgraphics (Statistics Graphics Co.).

A series of experiments was carried out using a monospecific culture of a marine flagellate, a mixed flagellate assemblage enriched from coastal seawater, natural microbial assemblages in whole and 5 μm filtered coastal seawater, and microbial assemblages in open-ocean water. The results of three experiments are reported here; no usable data were obtained from the open-ocean experiments due to the low rates of bacterivory and thus of ammonium regeneration by bacterivores that were obtained in those samples. In the three experiments described below, no measurable accumulation of $^{15}$N-ammonium was observed in the 0.8 μm filtered control bottles, thus production of labeled ammonium was ascribable to excretion by bacterivores ingesting and digesting the added labeled bacteria, and by protists feeding on bacterivores in multiple-step food webs.

**Experiment 1: one trophic step, flagellate culture**

A culture of the marine bacterivorous flagellate, *Cafeteria* sp., previously isolated from Oregon coastal seawater, was diluted with autoclaved artificial seawater (AW; Harrison et al., 1980) to yield 5 l of sample with a final abundance of $10^5$ flagellates ml$^{-1}$. Then 2.5 l of sample were poured into two 4 l bottles. Unlabeled, heat-killed marine isolate was added to the bottles for a final concentration of $10^7$ bacteria ml$^{-1}$. The bottles were incubated in the dark at 15°C until flagellate abundance reached $10^4$ ml$^{-1}$, at which time the experiment was started by adding $5 \times 10^6$ $^{15}$N-labeled bacteria ml$^{-1}$ to the samples. Labeled bacteria were also added to a control bottle containing 1 l of artificial seawater. Unlabeled ammonium was added to each bottle to a 10 μM final concentration, to curtail uptake of excreted $^{15}$N-ammonium by any live bacteria in the samples, and the bottles were sampled for cell counts and amount of $^{15}$N in particulate and dissolved fractions at 0, 7, 11, 15, 23, 35 and 48 h.

**Experiment 2: one trophic step, mixed flagellate enrichment**

Surface seawater was collected in February 1992 at the mouth of Coos Bay, Oregon (43°21'N 124°20'W). The seawater was gently screened through a 10 μm Nitex mesh net and left in a 20 l carboy at room temperature for 2 days in order to increase the biomass of bacterivorous protists. When a mixed species assemblage of bacterivorous flagellates had grown up to an abundance of $10^3$ ml$^{-1}$, the experiment was started. $^{15}$N-labeled bacteria were added to $5 \times 10^6$ ml$^{-1}$ in duplicate bottles containing 2.5 l of the flagellate assemblage. Based on the results of Experiment 1, we decided to decrease the final concentration of $^{14}$NH$_4^+$, as we suspected that high ammonium concentrations might have been deleterious to the protists (see Results), and so unlabeled ammonium was added to a 1 μM final concentration. A control bottle consisted of the same concentration of labeled bacteria and unlabeled ammonium added to 1 l of 0.8 μm filtered seawater from the carboy. The bottles were incubated as above and sampled at 1, 6, 9, 12, 24, 36 and 48 h.
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**Experiment 3: multiple trophic steps, natural microbial assemblage**

Surface seawater was collected in March 1992 on an incoming tide at the mouth of Coos Bay, Oregon. Two 4 l bottles were filled with either 2.5 l of unscreened seawater or 2.5 l of seawater filtered through 5 μm pore size, 47 mm diameter Gelman acrodiscs. A third bottle filled with 1 l of 0.8 μm filtered seawater served as a non-grazing control. $^{15}$N-labeled bacteria and unlabeled ammonium were added to final concentrations of $5 \times 10^5$ ml$^{-1}$ and 1.0 μM in each bottle. The bottles were incubated in the dark at 15°C, and sampled at 1, 4, 7, 12, 24, 35 and 48 h.

**Simulation models to account for variation in grazing during the experiments**

As explained in the Results, an initial analysis of flagellate and bacterial abundance during the experiments indicated that the assumption of a constant grazing rate by the bacterivore community during the incubations was likely to have been violated. Therefore, two differential equation models based on $^{15}$N concentrations were developed using the software package Stella (High Performance Systems Inc.) to simulate the time course of accumulation of excreted labeled ammonium. In the models, the abundances of bacterivorous flagellates and of total bacteria were input as a time series based on individual samplings during the course of the experiment. Also, it was assumed that the ammonium regeneration efficiency, $R_E$, was constant even though flagellate grazing rate parameters might vary.

In both models, the amount of $^{15}$N in the labeled bacteria ($^{15}$N$_{BAC}$) decreases solely as function of protistan grazing $G$, as:

$$^{15}\text{N}_{BAC}(t) = ^{15}\text{N}_{BAC}(t - dt) - G \cdot dt$$  \hspace{1cm} (3)

The total protist grazing is the product of the protistan grazing rate and the amount of $^{15}$N in the labeled bacteria.

Conversely, the amount of $^{15}$N-labeled ammonium is solely the function of the protistan ammonium excretion, $E$:

$$^{15}\text{NH}_4^+(t) = ^{15}\text{NH}_4^+(t - dt) + E \cdot dt$$  \hspace{1cm} (4)

Since $R_E$ is assumed to be constant, the protistan ammonium excretion is described as:

$$E = R_E \cdot G$$  \hspace{1cm} (5)

**Model I: cell-specific clearance rates are assumed to be constant.** In Model I (Figure 3A), it was assumed that the bacterivores had a constant cell-specific clearance rate ($CR; \text{nl cell}^{-1} \text{ h}^{-1}$) during the experiment. The grazing rate $g$ was a product of the cell-specific clearance rate $CR$ and the protist abundance (estimated by direct counts). Therefore, grazing on labeled bacteria ($G$) was directly dependent on the abundances of bacterivores and the amount of $^{15}$N in the labeled bacteria. The constants $CR$ and $R_E$ were estimated by sensitivity analyses as described below.

**Model II: ingestion rates are assumed to be constant.** In Model II (Figure 3B), it was assumed that bacterivores modulated their cell-specific clearance rates as the bacterial abundance varied, thus keeping their ingestion rates on bacteria ($IR; \text{bacteria flagellate}^{-1} \text{ h}^{-1}$) constant. This implied that the flagellates changed their feeding behavior in order to adapt themselves to conditions of limiting food concentrations, and so the clearance rates were a function of the total number of labeled + unlabeled bacteria (estimated by direct counts):

$$CR = IR/\text{bacterial numbers}$$  \hspace{1cm} (6)
In this model, the grazing rate $g$ was again the product of the cell-specific clearance rate $CR$ and the protist abundance (estimated by direct counts). Thus, grazing on labeled bacteria ($G$) was directly dependent on the abundances of the total numbers of bacteria as well as bacterivores, and the amount of $^{15}$N in the labeled bacteria. The constants $IR$ and $R_g$ were estimated by sensitivity analyses as described below.

Equations developed for the above models were also used to write FORTRAN routines that simulated the time course of the excreted labeled ammonium,
compared the output of the model with the observed time series, and calculated the residual mean of squares (RMS) between the model and the data over a range of values of either clearance or ingestion rates, and ammonium regeneration efficiencies. The clearance rate (Model I) or ingestion rate (Model II) and regeneration efficiencies that produced the lowest RMS were taken to be the best-fit values of these parameters for a particular data set.

Freezing and storage of the indophenol blue–ammonium complex for later nitrogen isotopic analysis

Six liters of coastal seawater were filtered through Gelman GFF filters. The natural ammonium content was measured and a known amount of 5% 15N-enriched ammonium chloride was added to yield a final ammonium concentration of 4 μM with a 4% 15N enrichment. The water was split into 12 × 500 ml subsamples, and the ammonium in each subsample was complexed with indophenol using the procedure of Dudek et al. (1986). After a 24 h development of the indophenol blue, the indophenol complex in three of the subsamples was extracted following the protocol of Wheeler et al. (1989). The remaining samples were stored frozen at -20°C. Triplicate subsamples were thawed and extracted after 1, 7 and 14 days of storage. The extracted indophenol complex from each sample was spotted onto a glass fiber filter which was then dried, ground to a powder, combusted in evacuated glass tubes, and the 15N enrichment of the resulting nitrogen gas was measured using a JASCO emission spectrometer (Wheeler and Kirchman, 1986). The effect of storage time was evaluated by one-way analysis of variance (ANOVA) of the data using Statgraphics software.

Results

Freezing and storage of the indophenol blue complex

The 15N enrichments of the ammonium extracted from replicate seawater samples did not appear to be affected by freezing and storage of the indophenol blue complex at -20°C for periods of up to 2 weeks (Table I). The results of the ANOVA showed no significant difference among treatments. Thus, short-term freezer storage of ammonium samples complexed with indophenol blue should not affect subsequent nitrogen isotope analysis. This makes the use of 15N isotopes somewhat easier for sampling programs, e.g. short cruises on ships with limited laboratory space, in which immediate extraction of the indophenol blue complex in samples is inconvenient.

Experiment 1: one trophic step, flagellate culture

Bacterial abundance steadily decreased by two orders of magnitude during the experiment (Figure 4A). The initial abundance of Cafeteria sp. declined sharply at the beginning of the experiment, to ~10^3 cells ml^-1, perhaps due to an inhibitory effect of the 10 μM unlabeled ammonium addition. The initial decline resulted in growth of flagellates during the course of the experiment (Figure 4B). Owing to changes in abundance of both bacterivorous protists and bacteria, the data could not be analyzed via simple non-linear regression using the function of equation (3),
Table I. Percent $^{15}$N enrichment of 12 replicate samples of indophenol blue developed from ammonium for 24 h. Initial triplicates were extracted immediately after the development of indophenol. The remaining triplicates were extracted after freezing and storage for 24 h, 7 days and 14 days.

<table>
<thead>
<tr>
<th>Storage time</th>
<th>Initial</th>
<th>24 h</th>
<th>7 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>a (%)</td>
<td>3.367</td>
<td>3.818</td>
<td>3.785</td>
<td>3.758</td>
</tr>
<tr>
<td>b (%)</td>
<td>3.841</td>
<td>3.870</td>
<td>3.809</td>
<td>3.758</td>
</tr>
<tr>
<td>c (%)</td>
<td>3.830</td>
<td>3.709</td>
<td>3.729</td>
<td>3.773</td>
</tr>
<tr>
<td>Average (%)</td>
<td>3.679</td>
<td>3.799</td>
<td>3.774</td>
<td>3.781</td>
</tr>
</tbody>
</table>

which assumes constant grazing during the experiment. Instead, the simulation models were used to analyze the data set. Model I, which assumes that the flagellate cell-specific clearance rate is constant during the experiment, yielded a better fit (lower RMS) than did Model II. The lowest RMS of 0.033–0.042 for Model I were obtained for values of clearance rate of 7–9 nl cell$^{-1}$ h$^{-1}$ and $R_E$ of 0.30–0.35 (Figure 5). Values for grazing mortality, $g$, in this experiment, based on the model results, varied from 0.005 to 0.108 h$^{-1}$, and protistan ingestion rates were calculated to be on the order of 60–70 bacteria flagellate$^{-1}$ h$^{-1}$.

**Experiment 2: one trophic step, mixed flagellate assemblage**

The abundances of flagellates and of bacteria were relatively constant during the experiment, thus a constant grazing rate was assumed. Non-linear regression of the time course of accumulation of $^{15}$N ammonium in the dissolved fraction, using equation (3), produced curves for both replicate bottles that fit the data with high correlation coefficients using a low number of iterations (Figure 6). Grazing mortality values were $0.031 \pm 0.017$ and $0.034 \pm 0.011$ h$^{-1}$ for duplicate bottles; the average $R_E$ was calculated to be 0.30. Using grazing mortality and abundance data, the cell-specific clearance rate of the flagellates was estimated to be $\sim$4.8–8.9 nl cell$^{-1}$ h$^{-1}$, and the ingestion rate to be on the order of 4–9 bacteria flagellate$^{-1}$ h$^{-1}$.

**Experiment 3: multiple trophic steps, natural microbial assemblage**

The initial abundance of non-pigmented flagellates was $1.5 \times 10^3$ ml$^{-1}$ in the unscreened water sample and $\sim 0.5 \times 10^3$ ml$^{-1}$ in the 5 $\mu$m filtered sample (Figure 7B). Flagellates grew up to a maximum concentration of $\sim 2.5 \times 10^3$ ml$^{-1}$ in both unscreened and in 5 $\mu$m filtered water during the experiment. Bacterial abundance decreased with time in the whole water sample after 6 h and in the 5 $\mu$m filtered treatment after 36 h of incubation (Figure 7A). Growth of flagellates and decrease in bacterial abundance resulted in variable grazing impact during the course of the experiment. Both simulation models were applied to the two data sets, as in Experiment 1. In this case, Model II, which assumes a constant per cell ingestion rate, fit the time course of labeled ammonium with lower RMS than Model I. The best fit (RMS = 0.0017–0.0048) was attained using values for ingestion rate of 8 bacteria flagellate$^{-1}$ h$^{-1}$ and $R_E = 0.60$ for the 5 $\mu$m pre-filtered treatment (Figure 8B) or $R_E = 0.90$ for the whole water (Figure 8A). Based on the model results, $g$ varied
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**Fig. 4.** Time course of (A) bacterial abundance and (B) flagellate abundance in replicate bottles during Experiment 1: one trophic step, culture study with *Cafeteria* sp. Open squares, bottle a; solid circles, bottle b.

from 0.001 to 0.011 h$^{-1}$ in the 5 $\mu$m filtered treatment, and from 0.003 to 0.017 h$^{-1}$ in the whole water sample, and clearance rates were calculated to be on the order of 2–10 nl flagellate$^{-1}$ h$^{-1}$ in both treatments.

Weight-specific ammonium excretion rates for bacterivorous flagellates were calculated from the experimental data: changes in flagellate abundance and in total ammonium concentration over time, during the first 24 h of each experiment (Table II). An average flagellate cell size of 6 $\mu$m diameter and a biovolume:dry weight (DW) ratio of 0.35 pg DW $\mu$m$^{-3}$ of biovolume of preserved cells (based on 0.14 pg C $\mu$m$^{-3}$ and a carbon:dry weight ratio of 2.5) were used for these calculations. Excretion rates varied from 6.1 to 148 $\mu$g N mg$^{-1}$ DW h$^{-1}$. Highest excretion rates were found during the initial 7–15 h of each experiment; lower rates were obtained during the following 8–12 h (Table II).

**Discussion**

The method described here differs from other methods designed to study the dynamics of protist grazing in that instead of measuring rates of ingestion or disappearance of bacteria, accumulation of $^{15}$N-labeled ammonium due to grazing on $^{15}$N-pre-labeled bacteria is assessed. The approach was attractive in that the $R_{e}$ of the nitrogen in a specific type of prey, in this case bacteria, could be estimated from...
Fig. 5. Best fit results of Stella Model I to experimentally determined excretion of $^{15}$NH$_4^+$ from added labeled prey during Experiment 1 (culture study), for replicate bottle a. $C_0$ was 3.12 $\mu$mol l$^{-1}$ $^{15}$N, flagellate clearance rate was 9 $nl$ cell$^{-1}$ h$^{-1}$, $R_E$ was estimated to be 0.30. For replicate bottle b, the estimated clearance rate was 8 $nl$ cell$^{-1}$ h$^{-1}$ and $R_E$ was 0.35.

Fig. 6. Best fit of non-linear regression model to experimentally determined excretion of $^{15}$NH$_4^+$ from added labeled prey during Experiment 2 (one trophic step, mixed flagellate study) for replicate bottle a. Best-fit value for $D_{max}$ was 0.125 $\mu$M $^{15}$NH$_4^+$ and for $g$ was 0.034, $r^2$ of the regression was 0.963. For replicate bottle b, best fit of the non-linear regression model yielded estimates of 0.145 $\mu$M $^{15}$NH$_4^+$ for $D_{max}$ and of 0.031 for $g$, $r^2$ of the regression was 0.911. $R_E$ calculated from these results was 0.30.

the data. No other approach has been suggested which allows the calculation of $R_E$ from specific prey within intact microbial food webs. Community-level $R_E$ should be positively related to complexity of the food web beginning with the added labeled prey. Based on nitrogen regeneration efficiencies estimated from experiments in laboratory cultures (Caron and Goldman, 1992), $R_E$ values of 0.30–0.50 would indicate one trophic step. Values of $R_E$ higher than these should thus indicate multiple trophic steps in the food web, beginning with the prey of interest.

A drawback to using $^{15}$N to label prey cells is that a fairly substantial amount of nitrogen is required for nitrogen isotopic analysis (a minimum of 10 $\mu$g is required by our mass spectrometer). We had to add extra unlabeled nitrogen to our
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Fig. 7. Experiment 3 (multiple trophic steps, natural microbial assemblage). (A) Time course of bacterial abundance in whole water (solid circles) and in <5 μm screened water (open squares). (B) Time course of flagellate abundance in whole water (solid circles) and in <5 μm screened water (open squares).

ammonium samples prior to extraction in order to obtain a sufficient quantity of nitrogen for isotopic analysis. This addition diluted the isotopic enrichment resulting from $^{15}$N ammonium excreted by protists grazing on labeled bacteria during the experiments. For samples with quantitatively low rates of bacterivory, e.g. in oligotrophic ocean water, $^{15}$N enrichment after addition of unlabeled carrier ammonium was so low that $g$ and $R_e$ values could not be calculated from the data set. However, in both mesotrophic coastal seawater and in laboratory cultures, higher grazing rates on added $^{15}$N-labeled bacteria produced a final $^{15}$N enrichment of ~0.5% in the experiments.

Other problems encountered in carrying out the experiments were the potential for re-uptake of excreted $[^{15}$N]ammonium by phytoplankton and by bacteria, and violation of the assumption of constant protistan grazing rates during the course of the assay. The first problem was addressed by adding 1 μM (10 μM in Experiment 1) final concentration of unlabeled ammonium to samples at the start of the experiments. This amount of nitrogen was ~10 times the maximum amount that would be expected to be released as ammonium via grazing from added $^{15}$N-labeled bacteria. For example, if $^{15}$N-labeled bacteria were added at a concentration equivalent to 10⁹ cells l⁻¹, this would represent a carbon biomass of 20 μg C l⁻¹ and a nitrogen biomass of 4 μg N l⁻¹ or 0.3 μM N. If half of the bacteria were grazed during the experiment, then the maximum amount of labeled ammonium released would be only 0.15 μM ammonium-N.
The problem of variable grazing rate due to changes in abundance of protists and total bacteria during the course of the experiments was addressed by formulating deterministic computer models in which measured abundances of bacterivores and bacteria at sampling time points were input as data in order to simulate change in grazing rate during the assays. Two simulation models were used: one in which the per cell clearance rate by the grazing protists was assumed to be constant and one in which protists varied cell-specific clearance rates as bacterial abundance varied, in order to maintain a constant rate of ingestion of bacteria. In two of the experiments described in this study (Experiments 1 and 3), the simulation models yielded better fits (lower RMS) to the time course of labeled ammonium than did the regression analysis to equation (1) (RMS values presented in Suzuki, 1994).

As a check on the results obtained with the assays, our estimated rates for flagellate clearance rates and bacterial ingestion rates can be compared to literature values from other studies of bacterivorous flagellates using a variety of techniques (Table III). Values for both of these parameters obtained using our new approach...
Table II. Calculated ammonium excretion rates, μg N mg⁻¹ DW h⁻¹, for bacterivorous flagellates in the three experiments during periods of time over the initial 24 h of the experiments

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<thead>
<tr>
<th>Experiment</th>
<th>Elapsed time (h)</th>
<th>Ammonium excretion rate (μg N mg⁻¹ DW h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture study, <em>Cafeteria</em> sp.</td>
<td>(A) 11-15</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>11-23</td>
<td>25</td>
</tr>
<tr>
<td>One trophic step, mixed species</td>
<td>(A) 9-12</td>
<td>61.6</td>
</tr>
<tr>
<td></td>
<td>12-24</td>
<td>7.0</td>
</tr>
<tr>
<td>Multiple trophic steps, &lt; 5μm fraction</td>
<td>4-7</td>
<td>53.6</td>
</tr>
<tr>
<td>mixed species</td>
<td>4-12</td>
<td>16.0</td>
</tr>
</tbody>
</table>

Table III. Comparison of values of bacterivore clearance rate (nl cell⁻¹ h⁻¹) and ingestion rate (bacteria cell⁻¹ h⁻¹) obtained in various studies, including the present one

<table>
<thead>
<tr>
<th>Method</th>
<th>Bacterivore</th>
<th>Clearance rate (nl cell⁻¹ h⁻¹)</th>
<th>Ingestion rate (bacteria cell⁻¹ h⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uptake of fluorescent microspheres</td>
<td>Nanoplankton, Chesapeake Bay</td>
<td>0.62-2.30</td>
<td>1.8-25</td>
<td>McManus and Fuhrman, 1986</td>
</tr>
<tr>
<td>Uptake of fluorescently labeled bacteria</td>
<td>Flagellates, Czech reservoir</td>
<td>2.0-17.4</td>
<td>4-44</td>
<td>Simek and Straskrabova, 1992</td>
</tr>
<tr>
<td>Size fractionation</td>
<td>Flagellates, Gulf of Finland</td>
<td>0.8-6.1</td>
<td>2.6-21.7</td>
<td>Kuupu-Leinikki and Kuosa, 1990</td>
</tr>
<tr>
<td>Size fractionation</td>
<td>Flagellates, Central North Atlantic</td>
<td>3-30</td>
<td>9-36</td>
<td>Weisse and Scheffel Moser, 1991</td>
</tr>
<tr>
<td>Selective metabolic inhibitors</td>
<td>Nanoplankton, Southeastern US estuary</td>
<td>2-10</td>
<td>29-80</td>
<td>Sherr <em>et al.</em>, 1986</td>
</tr>
<tr>
<td>Selective metabolic inhibitors</td>
<td>Nanoplankton, Gulf of Aden (Red Sea)</td>
<td>39-94</td>
<td>21-58</td>
<td>Weisse, 1989</td>
</tr>
<tr>
<td>Range of dilutions of whole water</td>
<td>Flagellates, Lake Constance S. FRG</td>
<td>0.2-123</td>
<td>10-100</td>
<td>Weisse, 1990</td>
</tr>
<tr>
<td>Ammonium release</td>
<td><em>Cafeteria</em> sp. culture</td>
<td>8</td>
<td>60-70</td>
<td>This study</td>
</tr>
<tr>
<td>Ammonium release</td>
<td>Marine flagellate enrichment</td>
<td>4.8-8.9</td>
<td>4-9</td>
<td>This study</td>
</tr>
<tr>
<td>Ammonium release</td>
<td>Unscreened and 5 μm screened water, Oregon coast</td>
<td>2.3-10.4</td>
<td>8</td>
<td>This study</td>
</tr>
</tbody>
</table>
were within the range of published values (Table III). In addition, the calculated rates of ammonium excretion by bacterivorous flagellates in this study (6.1–148 μg N mg⁻¹ DW h⁻¹; Table II) were comparable to ammonium excretion rates (3–140 μg N mg⁻¹ DW h⁻¹) previously reported for heterotrophic flagellates (Caron and Goldman, 1990).

The ammonium regeneration efficiencies determined here support both theoretical and empirical conclusions about the regeneration of microbial nitrogen in planktonic food webs. In laboratory studies, protists have been found to have a high gross growth efficiency for nitrogen, in the range of 0.70 for flagellates in log phase growth, to 0.30–0.50 for protists in stationary phase growth (Caron and Goldman, 1990). Thus, single-step food webs beginning with bacteria would be expected to have ammonium regeneration efficiencies of 0.30–0.50. The $R_E$ values calculated from Experiments 1 and 2, in which there were only bacteria and bacterivorous flagellates, were both low, averaging 0.30.

Previous direct measurements of ammonium regeneration rates via $^{15}$N dilution experiments have suggested high ammonium regeneration efficiencies in natural marine planktonic assemblages, with most of the regeneration being carried out by organisms that pass a 35 μm mesh screen (Glibert, 1982; Probyn, 1987; Glibert et al., 1992). The observed rapid growth rates of phytoplankton in oligotrophic seawater also require high ammonium regeneration efficiencies in the food web (Goldman, 1984). For a sample of coastal seawater, we estimated $R_E$ values of 0.60 in 5 μm screened water and of 0.90 in unscreened water. If we assume that single trophic step food webs have a $R_E$ of 0.30, then these higher $R_E$ values would imply food webs beginning with bacteria of 2–3 steps in the 5 μm screened water and of 4–5 steps in unscreened water. Such complexity in the heterotrophic ‘microbial loop’ supports the notions of multistep food webs in <20 μm size fractions (Wikner and Hagstrom, 1988) and of bacterivory being primarily a regenerative process (Ducklow et al., 1986).

The method described in this study may not be the optimum one for the simultaneous study of protistan grazing and nutrient regeneration by the measurement of rates of excretion of label. Labeling prey cells with radioisotopes, e.g. $^{32}$P, and following the accumulation of radiolabel in the dissolved fraction to study phosphorus regeneration, could be an alternative to the use of $^{15}$N-labeled prey. Determination of in situ efficiencies of growth and regeneration within microbial food webs is important for understanding the cycling of bioactive elements in aquatic ecosystems; the general approach described here may allow estimation of these parameters for specific trophic pathways.

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**References**

Estimation of ammonium regeneration via a $^{15}$N tracer method


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