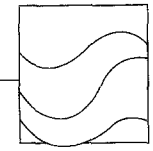

Release of dissolved amino acids by flagellates and ciliates grazing on bacteria

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(Received 25/01/97, revised 21/08/97, accepted 29/08/97)

Abstract – Release of amino acids was examined in the laboratory in the form of dissolved primary amine (DPA) by two marine planktonic protozoa (the oligotrichous ciliate, *Strombidium sulcatum* and the aplastidic flagellate *Pseudobodo* sp.) grazing on bacteria. DPA release rates were high (19–25 $\times 10^{-6}$ and 1.8–2.3 $\times 10^{-6}$ µmol DPA cell^{-1} h^{-1} for flagellates and ciliates, respectively) during the exponential phase, when the ingestion rates were maximum. Release rates were lower during the other growth phases. The release of DPA accounted for 10 % (flagellates) and 16 % (ciliates) of the total nitrogen ingested. Our data suggest that the release of DPA by protozoa could play an important role in supporting bacterial and consequently autotrophic pico- and nanoplankton growth, especially in oligotrophic waters, where the release of phytoplanktonic dissolved organic matter is low. © Elsevier, Paris

amino acids / protozoa / excretion rates / dissolved organic matter

Résumé – Excrétion d'acides aminés par des ciliés et des flagellés. Les taux d'excrétion d'acides aminés par deux protozoaires planctoniques marins (un cilié oligotriche, *Strombidium sulcatum,* et un flagellé hétérotrophe, *Pseudobodo* sp.), nourris avec des bactéries inactivées (par choc de température) ont été quantifiés expérimentalement. Les valeurs maximales sont mesurées en début de phase exponentielle et sont comprises entre 19 et 25 $\times 10^{-6}$ µmol·cellule^{-1}·h^{-1} pour les flagellés, entre 1,8 et 2,3 $\times 10^{-6}$ µmol·cellule^{-1}·h^{-1} pour les ciliés. Elles correspondent aux valeurs maximales des taux d'ingestion. Les flagellés et ciliés excrètent au maximum 10 et 16 % de l'azote ingéré sous forme d'acides aminés. Les acides aminés provenant des protozoaires pourraient jouer un rôle important dans la croissance des bactéries et du pico- et nanoplancton autotrophe, notamment dans les milieux oligotrophes où l'excrétion phytoplanctonique de matière organique dissoute est faible. © Elsevier, Paris

acides aminés / protozoaires / taux d'excrétion / matière organique dissoute

1. INTRODUCTION

It is now clear from recent studies that protozoa (both ciliates and phagotrophic flagellates) regenerate ammonium and phosphorus in significant quantities while grazing [1, 10, 12]. It has been suggested that they can also release dissolved organic matter (DOM) [16, 19, 25, 26, 35, 39]. However, data on release rates of DOM by protozoa are still scarce and conflicting. Some authors reported high release rates [2, 26, 34] whereas others estimated that this release is negligible [3, 9, 12]. Protozoa can also take up colloidal DOM, when it is available in the medium [13, 15, 36, 38].

The bulk of the DOM pool is composed of complex and refractory material, but a significant proportion includes

biologically active organic compounds such as dissolved free amino acids (DFAA). DFAA play an important role in oceanic waters since they are quickly taken up by heterotrophic bacteria [14, 32]. Hollibaugh et al. [17] showed a rapid turnover of the DFAA pool in enclosed water column, which suggested high DFAA release rates in sea water. Release of DFAA by phytoplankton and macrozooplankton has been well studied [4, 6, 22], but few studies investigated DFAA release rates by flagellates [3, 24, 25] and very little information is available on ciliates [16, 39].

The objective of the present study is to investigate the role of ciliates and flagellates as a source of dissolved free amino acids in the sea. The release of amino acids (in the dissolved primary amine form, DPA) by two protozoa (a flagellate and a ciliate) was therefore measured.

2. MATERIAL AND METHODS

Experiments were performed with the oligotrichous ciliate, *Strombidium sulcatum*, and the heterotrophic nanoflagellate, *Pseudobodo* sp., fed with either live or heat-killed bacteria. Protozoa were both isolated from the North-West Mediterranean Sea and grown on a mixed bacterial assemblage as prey [28]. Cultures were incubated in darkness at 12 °C. Heat-killed bacteria were prepared as described by Sherr et al. [31]: a mixed bacterial assemblage was harvested in the stationary growth phase and heated for 4 h at 60 °C until no enzyme activity (aminopeptidase and glucosidase, measured by spectrofluorometry) could be detected in the incubation medium. The heat-killed bacteria were filtered through a 4 µm Nuclepore membrane to remove bacterial aggregates and stirred for 10 min. These suspensions were then used as a food source at a final concentration of ca. 2×10^7 ·cell·mL^{-1}. Two sets of bacterial suspensions (heat-killed and live bacteria) were used in flagellate experiments. Only heat-killed bacteria were used in ciliate experiments. Ciliates and flagellates were inoculated into the bacterial media at a final concentration of ca. 1 and 10^3 cell·mL^{-1}, respectively. Both flagellates and ciliates were previously grown on heat-killed bacteria, in order to avoid the carry-over of live bacteria. According to Landry et al. [21], flagellates selectively graze live bacteria compared to heat-killed ones. Therefore, after sub-culturing protozoa in heat-killed bacteria for several generations, contamination by live bacteria was assumed to be minimal. Bacteria (heat-killed and live) were also incubated during control experiments to measure the bacterial release of dissolved primary amine (DPA).

All cultures were simultaneously run in triplicate, with samples taken once or twice a day from each culture to determine protozoan and bacterial abundances as well as DPA concentrations. Ciliates were fixed using a Lugol's solution (2 % vol/vol final concentration) and counted with an inverted Zeiss microscope. Heterotrophic bacteria and flagellates were preserved with borax-buffered formaldehyde (0.3 % vol/vol final concentration), stained with DAPI [27], and counted with a Zeiss Axiophot epifluorescence microscope. DPA bulk concentrations were determined using a Spex spectrofluorimeter and a fluorogenic reagent (o-phthalaldehyde) according to Strickland and Parsons [33]. Fluorescence produced by the OPA-ammonium complex has been subtracted from total fluorescence. In order to avoid an increase in DPA concentrations due to cell disruption, samples were prefiltered: 1) through 0.1 µm Millipore filters at ≤ 70 mm·Hg (0.1 bar) vacuum in the flagellate experiment; greater pore size filters may have induced high linear water velocities and high shear [11]; moreover, gentle vacuum filtration (<70 mm·Hg) has a minor effect on DFAA concentrations [24]; 2) through syringe filtration using Acrodisc (Gelman) in the ciliate experiment, according to Nagata and Kirchman [24]. Disc filters are indeed more adapted to ciliates than classical membrane filtration units because ciliates are structurally less rigid and more susceptible to breakage during filtration than flagellates. We observed no significant filtration damage due to cell rupture: this would have increased DPA concentration with increasing protozoan number.

Protozoan growth rate (μ, h^{-1}) was obtained as follows:

$$\mu = (\ln C_1 - \ln C_0)/(t_1 - t_0) \quad (1)$$

where C_0 and C_1 are the ciliate or flagellate concentrations (cell mL^{-1}) at the beginning (t_0) and the end (t_1) of the incubation time (h).

DPA release rates by protozoa (R_R, µmol protozoa^{-1} h^{-1}) were estimated for bottles containing heat-killed or live bacteria as follows :

$$R_R = (DPA_1 - DPA_0) / [C \cdot (t_1 - t_0)] \quad (2)$$

where DPA_0 and DPA_1 are the dissolved primary amine concentrations (µM) at the beginning (t_0) and the end (t_1) of incubation (h). C (cell mL^{-1}) is the average concentra-

tion of protozoa during incubation which was defined assuming exponential growth :

$$C = (C_1 - C_0) / (\ln C_1 - \ln C_0) \quad (3)$$

In the 'live bacteria' vessels, release rates (R_{RL}) represented 'net' rates (i.e. the difference between protozoan excretion and bacterial uptake). In the vessels containing heat-killed bacteria, these rates (R_{RHK}) should represent actual protozoan release rates.

Protozoan specific nitrogen ingestion rates were calculated as follows :

$$I_N = Q_N \cdot (B_1 - B_0) / [C \cdot (t_1 - t_0)] \quad (4)$$

where: I_N is the particulate organic nitrogen ingestion rate (µmol N protozoa^{-1} h^{-1}); Q_N is the bacterial nitrogen cell quota (µmol N cell^{-1}); B_0 and B_1 are the bacterial concentrations (cell mL^{-1}) at the beginning (t_0) and at the end (t_1) of the incubation (h). The N content of heat-killed and live bacteria ranges between 1.24 and 1.47 $\times$ 10^{-9} µmol N cell^{-1} for live and heat-killed bacteria, respectively.

3. RESULTS

Heat-killed and live bacterial cultures: Changes in the concentration of heat-killed bacteria were measured daily in the control bottles to check for the absence of cell division. No growth could be observed and concentrations actually decreased from 8.50 to 5.64 $\times$10^7 cell mL^{-1} in one week, perhaps due to cell disintegration. Moreover, there was no significant DPA release in bacterial cultures without protozoa. In live bacterial cultures, the amount of DPA quickly decreased (from ca. 3.6 to 1.8 µM) whereas it remained stable (ca. 4.3–3.8 µM) in the heat-killed suspensions (*table I*).

3.1. Ciliate experiment

Ciliates exhibited a typical growth curve, with a lag, an exponential and a stationary phase of 2, 4 and 4 days respectively. Bacterial abundance decreased with the increase of ciliate concentration (*figure 1a*). Ciliate growth rates reached maximum values at the end of the exponential phase (0.08 h^{-1}), remained high during ca. 48 h (0.06 to 0.08 h^{-1}) and decreased thereafter (*figure 1b*). Particulate nitrogen ingestion rates were maximal during the exponential phase (2 to 4 $\times$ 10^{-5} µmol N cell^{-1} h^{-1}, *figure 1b*) when the bacterial food was still abundant. DPA concentrations varied from 0 to 2.5 µM from the beginning to the end of the incubation (*figure 1a*). Maximum DPA release rates occured during the lag and early exponential phase and varied from 1.80 to 2.30 $\times$ 10^{-6} µmol N cell^{-1} h^{-1} (*figure 2*). These rates were lower at the end of the exponential growth phase (0.02 to 0.1 10^{-6} µmol N cell^{-1} h^{-1}) and dropped during the stationary phase (2 $\times$ 10^{-8} to 0.9 $\times$ 10^{-9} µmol cell^{-1} h^{-1}).

3.2. Flagellate experiment

Flagellates exhibited growth patterns similar to those obtained for ciliates (*figure 3, 4a*). When fed heat-killed bacteria, flagellates exhibited a lag but no stationary phase and their concentrations sharply decreased after the peak (*figure 3a*). However, the highest concentrations were similar in live and heat-killed bacterial cultures (ca. 5 – 8 $\times$ 10^4 cell mL^{-1}). During the exponential phase, flagellates grew at rates equal to 0.05 to 0.25 h^{-1} and 0.05 to 0.15 h^{-1} on heat-killed and live bacteria, respectively. Growth rates decreased from the beginning to the end of the incubation time in the live bacterial media, whereas they presented several peaks in the heat-killed one (*figures 3, 4b*). As for ciliates, maximum values of particulate nitrogen ingestion rates (*figure 3, 4b*) were obtained during the lag and early exponential phases (4 or 5 $\times$ 10^{-5} µmol N cell^{-1} h^{-1} for flagellates fed live and heat-killed bacteria, respectively). The amount of DPA increased regularly from 0 to 3.3 µM and from 0.2 to 2.4 µM in the heat-killed and live bacterial media respectively (*figures 3–4a*). Maximum DPA release rates (*figure 2*) also occured during the lag and early exponential phases (0–48 h) and varied from 19 to 24.6 $\times$ 10^{-6} and from 2.1 to 3.5 $\times$ 10^{-9} µmol DPA flagellate^{-1} h^{-1} for flagellates fed heat-killed and live bacteria respectively. These rates were lower at the end of the exponential phase (1 to 7 $\times$ 10^{-9} and 0.1 to 0.3 $\times$ 10^{-9} µmol cell^{-1} h^{-1} for flagellates fed heat-killed and live bacteria, respectively), and decreased during the stationary phase (0.1 to 3 $\times$ 10^{-9} and 0 to 0.1 $\times$ 10^{-9} µmol cell^{-1} h^{-1} for flagellates fed heat-killed and live bacteria, respectively). DPA release rates in the ciliate and flagellate experiments (log transformed) were significantly ($p < 0.05$) correlated with ingestion rates (R^2 equal to 0.91, 0.90 and 0.96 for ciliates and flagellates fed heat-killed bacteria and flagellates fed live bacteria respectively *figure 5*).

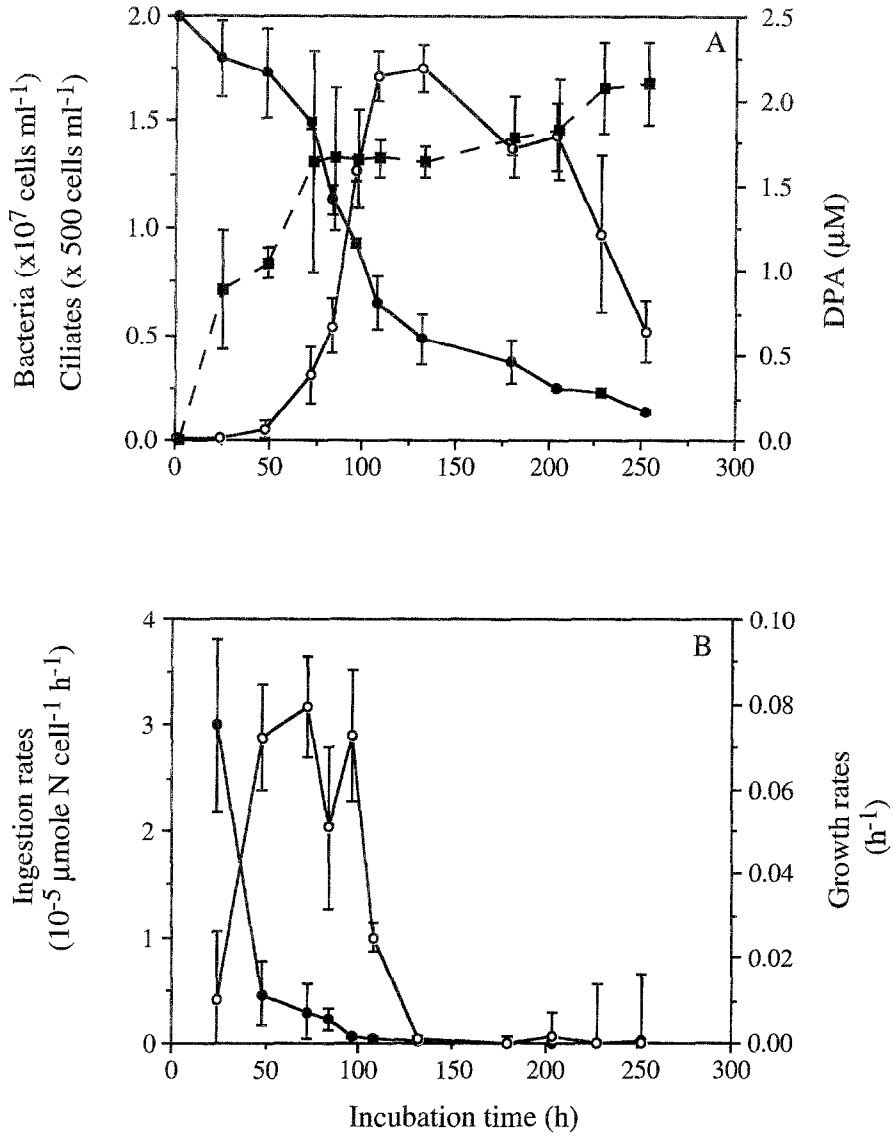

Figure 1. Ciliates fed heat-killed bacteria. A) concentrations of bacteria (black circles), ciliates (white circles) and dissolved primary amine (dashed line) during the incubation time. B) Ciliate ingestion rates (black circles) and growth rates (white circles). Data are given as mean and standard deviation of three replicate experiments.

4. DISCUSSION

Our data suggest that protozoa release dissolved organic matter derived from the ingested bacteria. Since cultures containing bacteria alone did not show any increase in DPA concentrations with time, bacterial protein ingested by protozoa was hydrolyzed in food vacuoles to amino acids, and then released. The ecological importance of the release of DOM by protozoa has only recently been recognized [26] and the organic nitrogen pool has been shown to be an important nutrient reservoir. Any attempt to budget the pathways of total nitrogen in the euphotic zone must therefore account for DON fluxes.

DPA release rates measured in the flagellate experiments are in agreement with the results on DFAA obtained by Nagata and Kirchman [25]. This suggests that the method used in this experiment (with heat-killed bacteria as a food source) is satisfactory for this type of measurement. As far as ciliates are concerned, we are not aware of any study measuring amino acid release rates during their

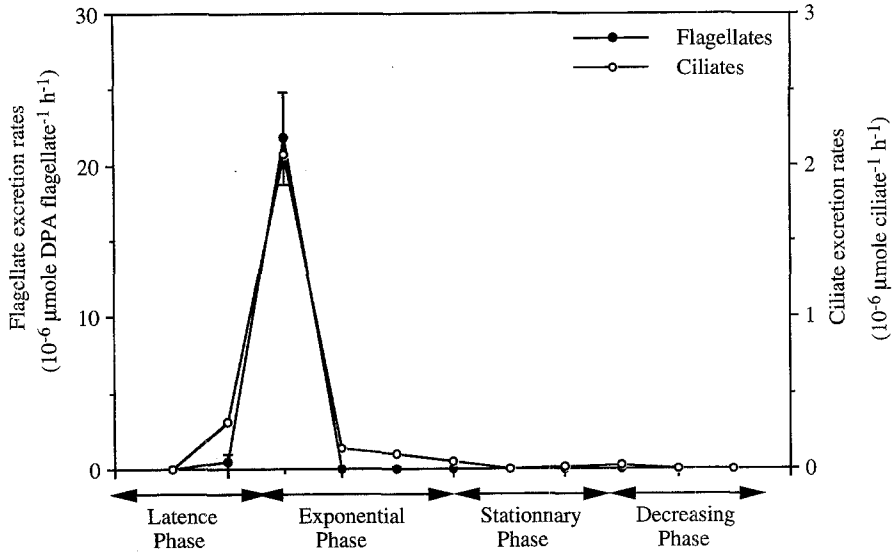

Figure 2. DPA release rates (µmol cell^{-1} h^{-1}) for the flagellates and ciliates fed heat-killed bacteria.

growth. Taylor et al. [34] found, for natural microzooplankton populations, that dissolved organic carbon release accounts for 3 to 88 % of the ingested carbon biomass in an eutrophic environment. Joseph and Nair [18] as well as Tranvik [35] demonstrated that flagellates excrete dissolved and colloidal organic carbon from ingested bacteria. However, no information is available on dissolved organic nitrogen. Hoch et al. [16] recently demonstrated that not only flagellates but also ciliates release equal proportions of ammonium and DFAA during exponential growth, but excretion rates were not quantified. In the present experiment, we demonstrate that ciliates can release large amounts of DPA when food is sufficient.

Planktonic oligotrichous ciliates belonging to the genus *Strombidium* represent more than 50 % of the total concentration of ciliates inhabiting Mediterranean waters [30]; therefore, the excretion of organic nitrogen by these protozoa is likely to play an important role in nutrient cycling. Maximum DPA release rates were obtained during the lag and early exponential phase. This could be due to high metabolic rates of ciliates due to the high bacterial concentrations. During the exponential growth phase, release rates varied between 1.8 and 2×10^{-6} µmol cell^{-1} h^{-1}. These rates were of the same order of magnitude (although lower) as those measured during the same growth phase for the phagotrophic flagellates (19–24 $\times$ 10^{-6} µmol cell^{-1} h^{-1}). DPA excretion rates by flagellates were in agreement with DFAA release rates found previously by Nagata and Kirchman [25] for the same culture phase. These authors measured maximum release rates of 0.15 to 30×10^{-6} µmol flagellate^{-1} h^{-1} (depending on the prey type) for the phagotrophic flagellate *Paraphysomonas imperforata*. However, decrease in release rates during the stationary phase was higher in this experiment (10^{-9} to 10^{-10} µmol flagellate^{-1} h^{-1} than in previous observations [25]. Excretion could be greatly underestimated if DPA uptake by heterotrophic bacteria was not taken into account. In this experiment, maximum release rates for flagellates fed live bacteria (0.20 to 9×10^{-9} µmol cell^{-1} h^{-1}) were well below those measured for flagellates fed heat-killed ones. DPA release was underestimated by factors of 100 to 1000 in cultures containing live bacteria. High amino acid uptake rates by bacteria were already reported in previous studies [5, 20, 29, 37].

Expressed in terms of dry weight, DPA release rates for ciliates (0.41 to 0.46 µg mg DW^{-1} h^{-1}) were lower than those obtained for flagellates (8.70 to 11.10 µg mg DW^{-1} h^{-1}). This was previously observed in ammonium studies [7, 10]. When compared with ammonium excretion rates measured during the exponential growth phase for the same ciliates and flagellates fed with the same bacterial prey ([10]; 8.72×10^{-6} and 7.72×10^{-9} µmol NH_4^+ cell^{-1} h^{-1} respectively), DPA release rates were in the same range. During the stationary phase, decrease in ammonium excretion rates was lower than the decrease in DPA release rates. Moreover, positive relations were obtained between ammonium or DPA release rates and nitrogen

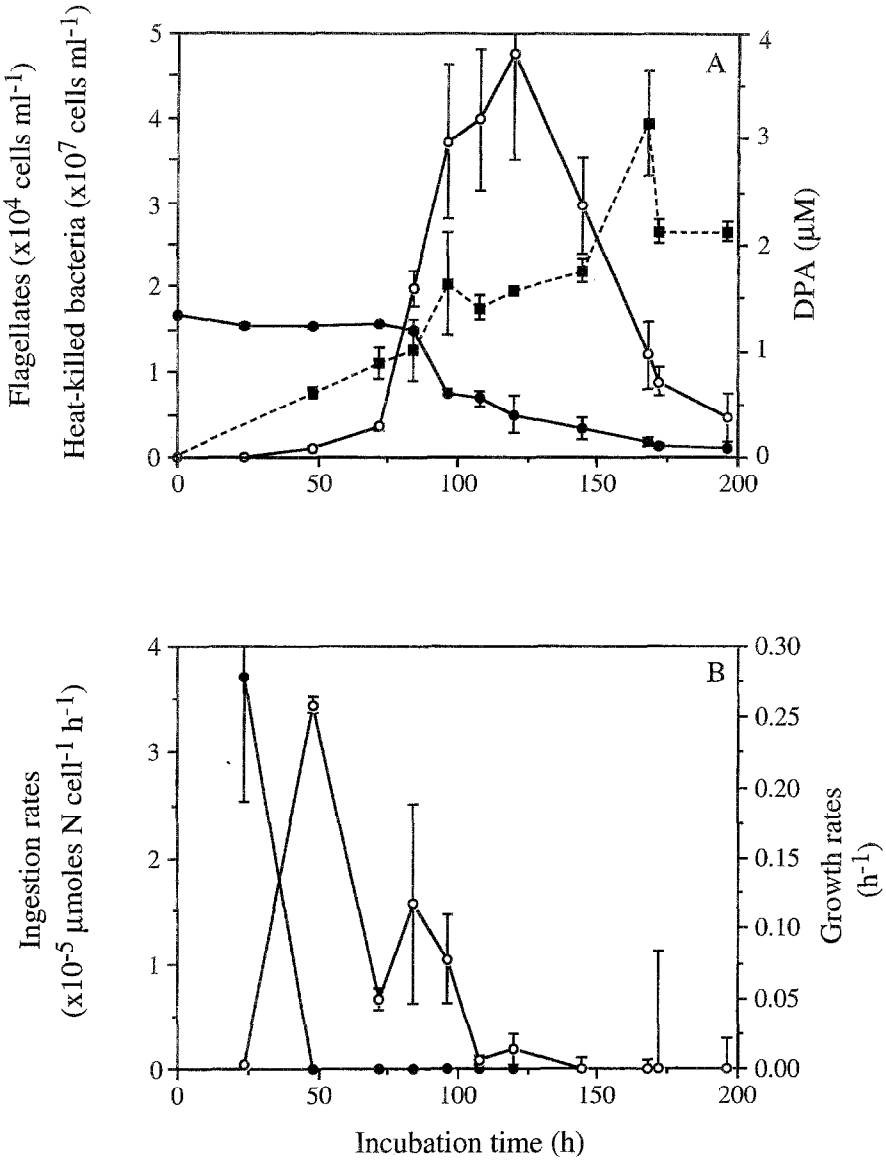

Figure 3. Flagellates fed heat-killed bacteria. A) concentrations of bacteria (black circles), flagellates (white circles) and dissolved primary amine (dashed line) during the incubation time. B) Flagellate ingestion rates (black circles) and growth rates (white circles). Data are given as mean and standard deviation of three replicate experiments.

ingestion rates and confirmed results from previous studies [2, 25].

To determine whether the "protozoa-bacteria" system may be a significant source of nitrogen in the sea, the percentage of bacterial nitrogen ingested by protozoa and released as amino acids was calculated. In order to estimate this percentage, an average of 12.5 % N in amino acids was assumed [3]. In our experiments, 10 % and 16.5 % of the ingested nitrogen was released as amino acids during the exponential growth phase of heterotrophic flagellates and ciliates, respectively. In contrast, ammonium release during the same growth phase (both for ciliates and flagellates) accounted for ca. 30 % of the ingested nitrogen [10]).

While some authors found no evidence for protozoan amino acid release [3, 12], our results are in agreement

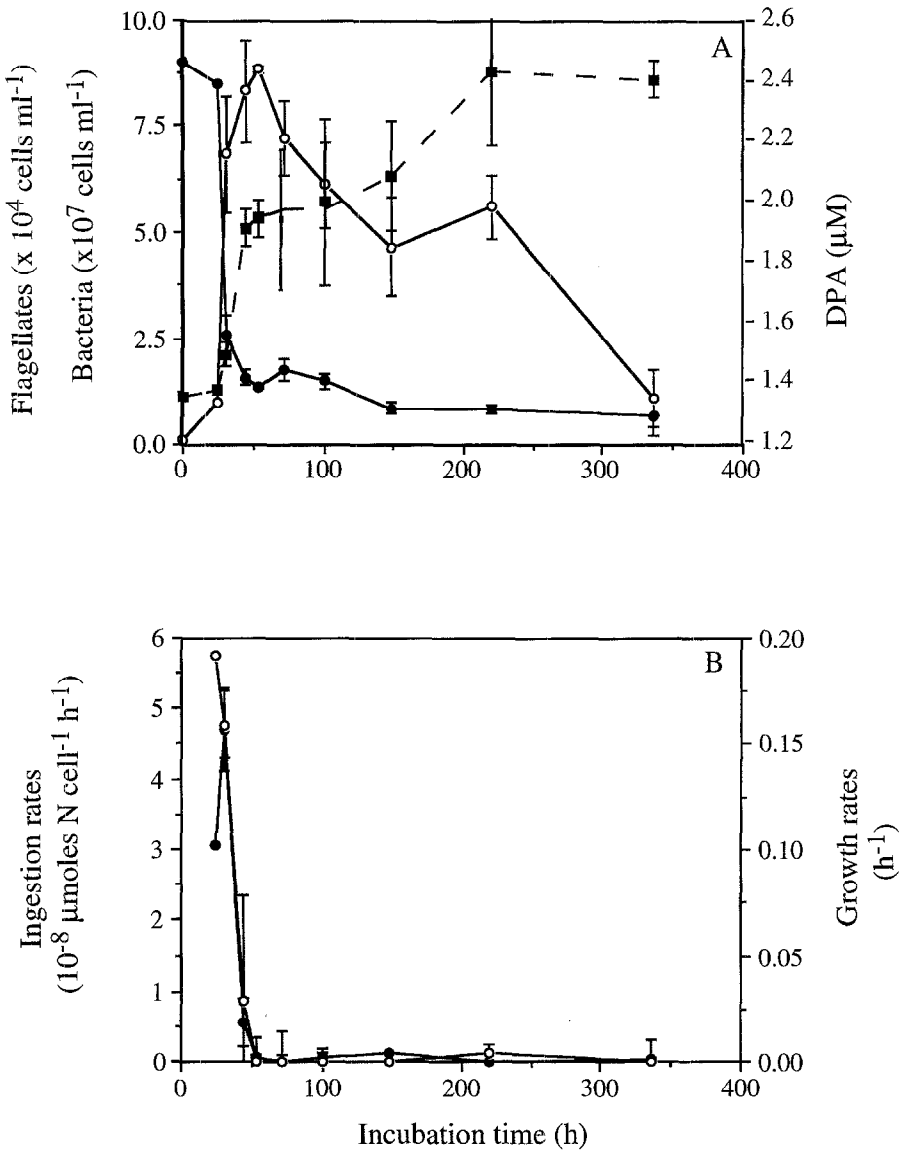

Figure 4. Flagellates fed live bacteria. A) concentrations of bacteria (black circles), flagellates (white circles) and dissolved primary amine (dashed line) during the incubation time. B) Flagellate ingestion rates (black circles) and growth rates (white circles). Data are given as mean and standard deviation of three replicate experiments.

with other estimates of dissolved organic matter release rates in flagellates. Andersson et al. [3] estimated that release of DFAA by the flagellate *Ochromonas* sp. was 7 % of the ingested nitrogen biomass. Nagata and Kirchman [25] calculated for another flagellate (*Paraphysomonas imperforata*) that DFAA release varied between 4 to 22 % of the ingested nitrogen depending on type of prey. Finally, Van Wambeke [39] estimated that the dissolved organic nitrogen excreted by the ciliates in a culture of *Phaeodactylum tricornutum* constituted more than 50 % of the identified nitrogen forms (dissolved and particulate).

High DPA release rates obtained for both ciliates and flagellates suggest that they are an important source of available dissolved organic matter. Since DPA release

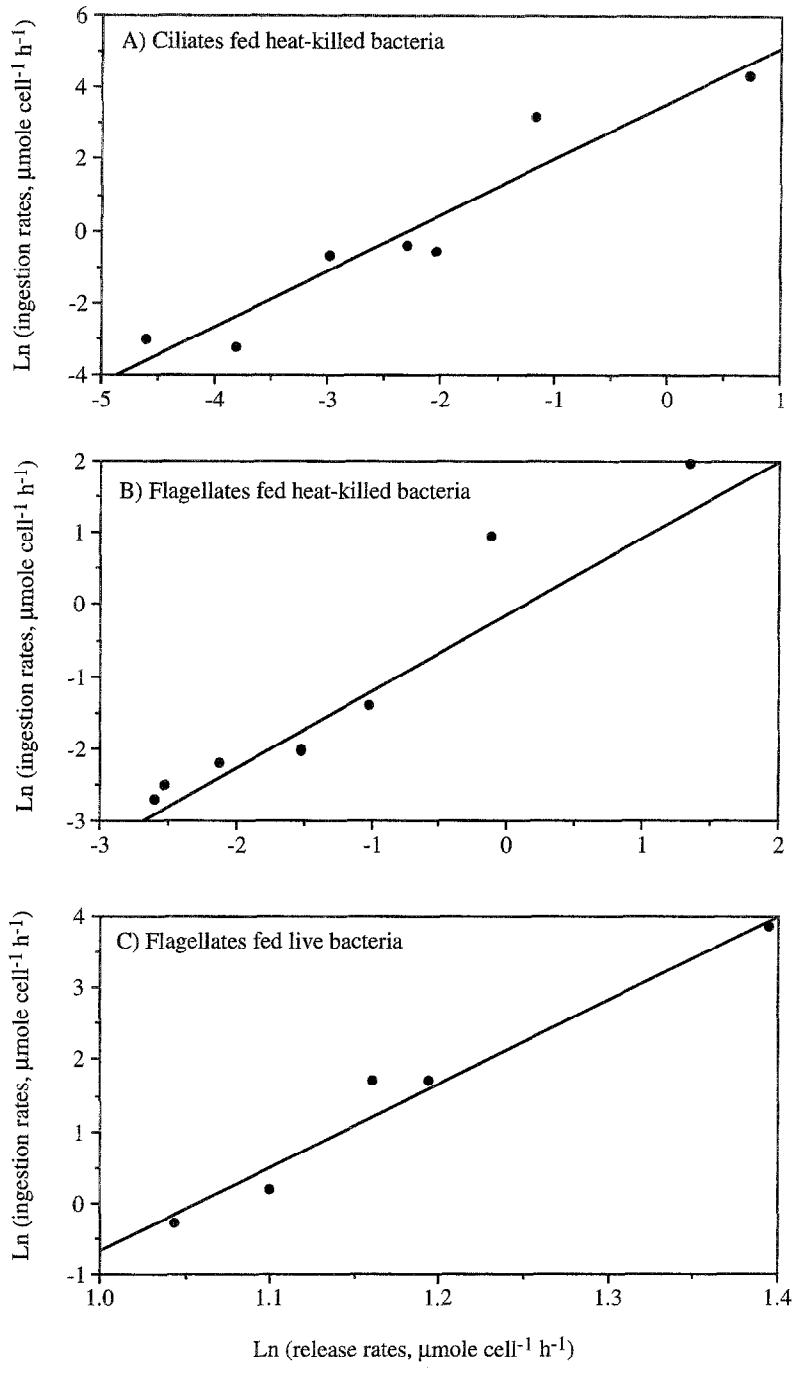

Figure 5. Correlation between nitrogen ingestion rates and DPA release rates. Data represent mean values of three replicate experiments

Table I. Changes in bacterial and DPA concentrations in the live and heat-killed bacterial cultures. Data are given as mean and standard deviation of three replicate experiments.

Incubation time (days)	Live bacteria (10^7 cell mL^{-1})	Heat-killedbacteria (10^7 cell mL^{-1})	DPA concentrations in the live bacterial culture (µM)	DPA concentrations in the heat-killed bacterial culture (µM)
1	11.94 ± 1.93	8.50 ± 0.35	3.60 ± 0.51	4.22 ± 1.13
2	10.85 ± 1.51	8.17 ± 0.31	3.69 ± 1.45	4.30 ± 0.23
3	10.27 ± 1.86	6.87 ± 1.50	2.77 ± 0.21	4.37 ± 0.65
4	10.74 ± 0.06	5.95 ± 1.34	1.82 ± 0.51	3.39 ± 0.09
5	10.90 ± 0.52	6.06 ± 1.80	1.77 ± 0.32	3.55 ± 0.39
6	10.66 ± 1.11	5.95 ± 1.19	1.34 ± 0.43	3.86 ± 0.15
7	11.90 ± 1.06	5.64 ± 1.02	1.71 ± 0.26	3.80 ± 0.31

rates can be as high as ammonium excretion rates, protozoa could play a key role in nutrient remineralization, mainly in oligotrophic environments where microphytoplanktonic biomass is generally low [23], and the release of phytoplanktonic DOM is also likely to be low. Thus, even small amounts of amino acids provided by protozoa could be important for bacterial growth (net nutrient production) and after recycling, for autotrophic pico- and nanoplankton growth. Moreover, nutrient turnover during grazing is relatively fast [8]. Even though bulk amino acid concentrations might be low, rapid cycling could make them highly significant agents for carbon and nitrogen cycling and major contributers to overall food web processes.

Acknowledgements

The authors would like to thank J. Fuhrman, T. Nagata, D. Kirchman, J. Dolan and J.-P. Gattuso for comments. This research is a contribution of the "Microzooplankton team" and was supported by CNRS (URA 716) and the EEC. MAST II programme targeted on the Mediterranean (sub-project Mast 2-CT93-0063 MEDIPELAGOS) grants.

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