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Use of ^{14}C-protein-labelled bacteria for estimating clearance rates by heterotrophic and mixotrophic flagellates

Kari Nygaard, Dag O. Hessen

Norwegian Institute for Water Research (NIVA), PO Box 69 Korsvoll, N-0808 Oslo 8, Norway

ABSTRACT: Three cultured species and mixed seawater communities of heterotrophic or mixotrophic flagellates were fed with non-cultivated aquatic bacteria labelled with ^{14}C-protein hydrolysate, i.e. the method of radioactive labelled bacteria (RLB). Seawater bacteria incorporated protein-hydrolysate more rapidly and far more efficiently than ^{14}C-glucose. Loss of label was less in the protein-labelled bacteria compared with those labelled with glucose. All tested flagellate species were found to ingest bacteria; individual clearance rates ranged from 0.014 µl ind.$^{-1}$ h^{-1} (36 bacteria ind.$^{-1}$ h^{-1}; *Ochromonas minima*) to 0.156 µl ind.$^{-1}$ h^{-1} (156 bacteria ind.$^{-1}$ h^{-1}; *Paraphysomonas* sp.). Uptake of bacteria by *O. minima* and *Bodo* sp. was linear for ca 10 min, and levelled off between 10 to 40 min. *Paraphysomonas* sp. had a linear uptake for 20 min. Grazing rates obtained with *O. minima* fed labelled bacteria and grazing rates calculated from chemostat culture were not significantly different. RLB proved rapid and efficient for estimating clearance by small heterotrophs and mixotrophs. Grazing rates in *Bodo* sp. and *Paraphysomonas* sp. from a 'direct-count' method using DTAF-stained bacteria were 10 % of those obtained with RLB. RLB is shown to work well even with small size differences between predator and prey. This is obtained by use of pre-starved bacteria, which are reduced to a size where they may be separated from predators using a 1.0 µm filter. Field experiments with mixed seawater communities gave 10 to 30 times higher grazing rates using RLB compared to stained bacteria. The method accounts for food vacuoles containing bacteria, egested upon preservation of the bacterivory flagellates.

INTRODUCTION

Development of epi-fluorescent techniques in the late 1970's and in the 1980's (Hobbie et al. 1977, Porter & Feig 1980, Caron 1983 and references therein) have shown that bacteria, as well as pico- and nano-plankton, may constitute an important part of the planktonic biomass. Autotrophic pico- and nano-plankton are now regarded important producers in the pelagic system (60 to 90 % of total production) (Sieburth et al. 1978, Bienfang & Takahashi 1983).

Azam et al. (1983) formulated the hypothesis of a microbial loop with high relevance to the pelagic flux of energy. This entailed a need for methods to measure mass transport within the loop, and its relevance for higher trophic levels. A comprehensive search for methods to quantify grazing rates on pico- and nano-plankton has resulted in an abundance of new methods. The most frequently used of these methods are: fluorescent particles (Børsheim 1984, MacManus & Fuhrman 1988, Nygaard et al. 1988), fluorescent-stained bacteria (Albright et al. 1987, Sherr et al. 1987, Nygaard et al. 1988), dilution methods (Landry & Hassett 1982), growth inhibitors (Campbell & Carpenter 1986, Sherr et al. 1986, Taylor & Pace 1987, Tremaine & Mills 1987), grazing rates measured by bacterial growth after fractional filtering (Wright & Coffin 1984, Andersen & Fenchel 1985, Rivier et al. 1985), and labelling with radioactive isotopes (Hollibaugh et al. 1980, Rieman 1985).

Most of these methods are laborious, and they often include a serious system perturbation during incubation, and/or require use of artifical food tracers. A prerequisite for approaching 'true' grazing rates is to minimize such unnatural conditions. This can often be overcome by labelling assemblages of natural food and incubating natural communities. When studying flagellate grazing on bacteria, a serious problem arises due to the small size difference between food particles and the grazer, which results in the inability to separate these 2 groups by filtration. We present here a tracer method which circumvents these shortcomings by using

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radiolabelled non-cultivated bacteria as food tracers added to samples incubated in situ to minimise system perturbation. Additonal separation problems inherent in using filter-techniques may be overcome by pre-starving the bacteria to a size where almost all cells pass through a 1.0 µm filter. The data presented here demonstrate that problems concerning discrimination towards 'unnatural' particles can be avoided. Some preliminary results and comparisons with other methods are presented.

MATERIAL AND METHODS

Isotope uptake in bacteria and flagellates. To determine the best compound for radiolabelling bacteria (RLB), and quantifying bacterivory, an initial test was conducted comparing ^{14}C-labelled amino acids and glucose. This experiment was performed on both bacteria from the Oslo fjord, southern Norway, and a mixed culture of *Vibrio* spp.

A final concentration of 1.25 µCi ^{14}C-protein-hydrolysate (Amersham CFB 25) was added to 1 l Pyrex bottles with prefiltered (1 µm) seawater or culture. Bottles to which 1.3 µCi final concentration of ^{14}C-glucose (Amersham CFB 96) was added were run as parallels. Samples for analysis of particulate activity were taken at times 0, 5, 10, 15, 30, 60 and 120 min by filtering subsamples of 500 µl on 0.2 and 1.0 µm polycarbonate membrane filters (3.0 µm filters were used in this particular experiment, because bacteria were too large to pass a filter with smaller pore-size, otherwise 1.0 µm filters were used). Particulate activity was determined after 1 to 5 wk of freezing (− 20 °C), to test for cellular leakage of isotope, and to determine whether frozen, labelled bacteria could be used in grazing experiments upon thawing.

To determine grazing rates and efficiency of isotope retention, mixed communities of sea bacteria were used. To obtain small bacteria, thereby avoiding high background on 1 µm filters, we used bacteria from 10 l beakers of seawater stored at 15 °C for 1 to 2 mo. Epifluorescent microscopic examination revealed dominance of small bacteria, with a mean volume of 0.021 µm^3 on the 0.2 µm filters, and only negligible retention of bacteria was obtained on the 1.0 µm filters. The seawater was filtered through a 1.6 µm cellulose acetate filter, in order to remove any large particles. The bacteria were labelled with 0.5 kBq (30 µCi final concentration) protein-hydrolysate for 24 to 30 h (15 °C). Total activity, total particulate activity (0.2 µm) and 'background' activity on the 1.0 µm filters were determined from triplicate samples.

Three flagellate species were used in experiments: the mixotroph *Ochromonas minima* and the heterotrophs *Bodo* sp. and *Paraphysomonas* sp. The flagellates were cultured in a continuous culture system as described in Nygaard et al. (1988), or in batch cultures.

The grazing experiment, in which laboratory cultures of flagellates were fed with labelled bacteria, was performed in 500 ml bottles. Samples (100 ml) of the flagellate culture were gently added to 400 ml aliquots of the bacterial suspension. Triplicate samples were taken at 0, 5, 10, 20 and 40 min. At each time-interval, ingestion was stopped by preserving 10 ml samples with $HgCl_2$, final concentration 11 mM. Activity in the flagellates was determined by filtering 1 ml subsamples on 1.0 µm polycarbonate filters at low vacuum (< 50 mm Hg). To obtain net activity in the flagellates, the 'background' values caused by bacteria retained on the 1.0 µm filter were subtracted from these values. In general the background values were negligible compared to activity on the 0.2 µm filters and low (< 10 %) compared to total activity on the 1.0 µm filters, i.e. few bacteria were retained on the 1.0 µm filters. This was confirmed by microscopic examination of the filters.

Preservation followed by filtration within 10 min was performed for all flagellate species except for *Ochromonas minima*. Previous testing showed breakdown of the cell wall and an almost complete leakage of cell content of the other, more fragile, species when not subjected to fixation. For the somewhat hardier *O. minima*, gentle filtration of living cells could be done without loss of material.

Total number of flagellates per volume was determined by microscopic counts on 1.0 µm filters. Activity per cell (DPM bacterium^{-1}) was determined from counts of total number of DAPI-stained bacteria.

Number of ingested bacteria per flagellate per unit time was calculated from

$$I = \frac{Af}{Cf \times T(t_0 - t_1)} \left(\frac{Ab}{Cb}\right)^{-1} \quad (1)$$

where I = number of bacteria ingested flagellate^{-1} min^{-1}; Af = activity in flagellates (DPM ml^{-1}); Cf = number of flagellates per ml; Ab = activity in bacteria (DPM ml^{-1}); Cb = number of bacteria per ml; T = minutes of elapsed grazing ($t_0 - t_1$). For all the present experiments t_1 = 10 min. Subsequently, this was converted to grazing rate (G = filtered volume of water flagellate^{-1} unit time^{-1}) as I = G × (cells ml^{-1}).

To account for unincorporated label in solution during the grazing experiment, we tested for direct uptake of free amino acids. Grazing experiments were performed in the bacterial cultures with added label. Over the duration of the grazing experiments 90 % of added amino acid was incorporated into the bacteria. In general, the uptake of free amino acids was expected to be negligible in the flagellates within the short incubation time of the grazing experiments (40

min). A test on direct uptake of labelled amino acids was carried out with a culture of *Ochromonas minima* pretreated with additions of 0.075 g l^{-1} streptomycin. This reduced bacterial numbers to almost 10 % of initial numbers ($< 5 \times 10^4$ cells ml^{-1}), while numbers (microscopic examination) of *O. minima* were not affected. Protein-hydrolysate, 0.5 kBq, was added to this pretreated culture, and sampling and procedures were as described for the grazing experiment above. Uptake of label in the > 1 µm fraction from the test on isotope uptake in the seawater samples also served as an indication of direct uptake of label by the flagellates, as this sample contained 10^4 to 10^5 flagellates ml^{-1}.

All samples were counted in Opti-Fluor cocktail (Packard) for aqueous samples for a 10 min period in a Minaxi Tri-Carb liquid scintillation counter (Packard) pre-programmed for direct calculation of DPM by use of internal standards at the ^{14}C window settings.

Field experiments. Field experiments were executed to determine whether RLB could be used to measure grazing rates in natural systems.

During a cruise in the inner part of the Oslo fjord, water was sampled from 5 m depth at 2 different sites (Stns 1 and 2). Water samples received additions of equal amounts of isotope-labelled bacteria, volume 1:1, and incubated at in situ temperature. Procedures for incubation and filtration were as described above. Direct uptake of amino-acids in bacteria and eucaryotes was tested for each sample, by adding radiolabelled amino acids direct to seawater samples, using the same procedure for incubation and filtration as described above.

Method comparisons. Results obtained by use of RLB were compared with an independent method. Grazing experiments with the 3 selected flagellates were performed with fluorescently labelled bacteria (FLB) as described below.

A culture of *Vibrio* sp. was stained with 5-(4,6-dichlorotriazin-2-yl) aminofluorescein (DTAF). We used the standard procedure of Sherr et al. (1987). For measurements of grazing rates, the different flagellate cultures were incubated with FLB using the same incubation periods and number of bacteria as used for the RLB method described above. Uptake of FLB in flagellate vacuoles was counted using epifluorescent microscopy, as described by Nygaard et al. (1988).

Microscopic enumeration. Samples were preserved with $HgCl_2$, final concentration 11 mM, and filtered onto Nuclepore polycarbonate filters, pore-size 0.2 µm (bacteria) or 1.0 µm (eucaryotes). All samples were stained with DAPI (diamidimophenylindole) (Porter & Feig 1980) and Primuline (Caron 1983) for enumeration of bacteria and flagellates respectively.

Biovolume determinations. Samples were prepared as described for counting of bacteria and flagellates, except that acridine orange was used for staining (Hobbie et al. 1977), giving a better size-estimate (Bratbak 1985). The size of the stained bacteria was measured using a calibrated eyepiece graticule (New Porton G12; Graticules, Ltd, England) (Bratbak 1985). This graticule has an array of 11 globes and circles with different diameters. The diameter increase in a progression of N2 at magnification × 1000, the smallest graticule circle had a diameter equivalent to 0.2 µm, and the largest had a diameter equivalent to 7.6 µm. The sizes of 50 bacteria were measured in each sample. Cell volumes were calculated as $(\pi/4)W^2(L - W/3)$, where L = cell length, W = cell width. This formula applies for both rods and cocci (L = W).

RESULTS

Isotope uptake in bacteria

Bacterial uptake of the ^{14}C-labelled amino-acid mixture gave higher DPM values than that of the ^{14}C-labelled glucose in the non-cultured bacteria (Fig. 1a, b). Although the 2 substrates were added in similar amounts (1.3 µCi l^{-1}), the final activity of glucose-labelled bacteria was less than 25 % of that in the amino acid labelled bacteria. Moreover, uptake of glucose showed a time-delayed response (lag-phase). As bacterial biomass was identical in these tests, these differences would be the same if reported as specific activity. For seawater with added isotopes, uptake in the > 1 µm fraction was negligible, indicating that bacteria were responsible for the major uptake of added substratum. A cultured *Vibrio* sp. showed the reverse: efficient uptake of glucose, whereas uptake rates of amino acids were 10 to 15 % of that for glucose (Fig. 1c).

Loss of activity on deep-freezing was slightly higher in the glucose-labelled bacteria. Loss of label was less than 20 % of initial activity in the amino acid labelled bacteria, and became stable after 1 wk of freezing (Table 1). Hence, both glucose and amino acid labelled bacteria may be used for grazing experiments even after freezing.

Uptake of dissolved amino acids in flagellates

From Fig. 1a, it is evident that uptake of label in particles > 1 µm was negligible, i.e. the flagellates retained on the 1 µm filter were unable to utilize the free amino acids to any detectable extent. The test with *Ochromonas minima* pretreated with streptomycin was performed at high activity (4 kBq l^{-1}), but uptake was nonetheless only slightly above background values.

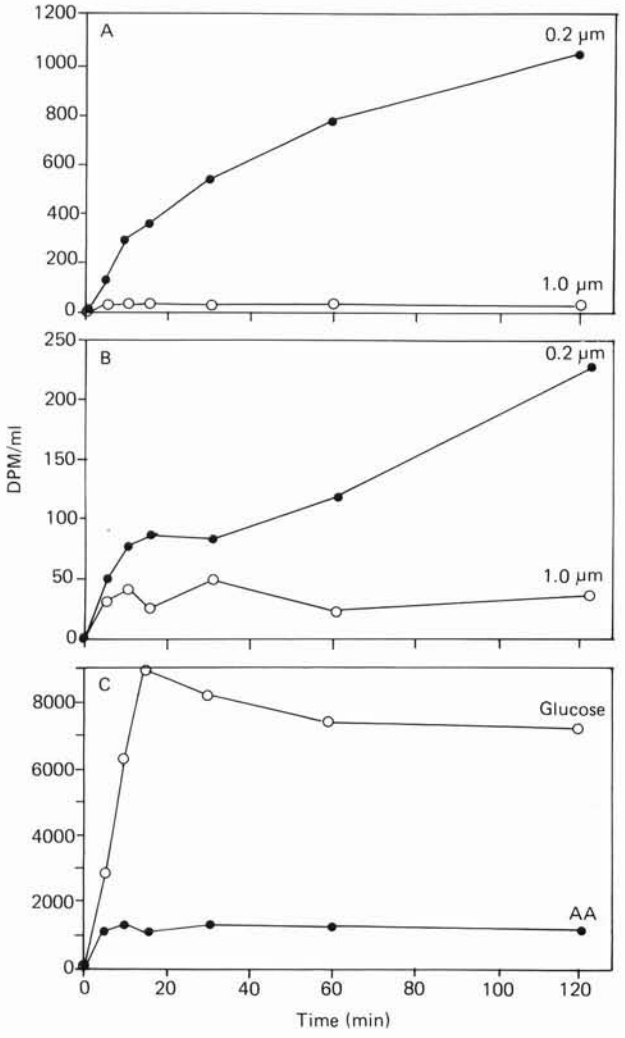

Fig. 1. (A) Incorporation of ^{14}C-protein-hydrolysate in natural seawater seston 0.2 µm (mainly bacteria) and 1.0 µm (mainly algae). (B) Incorporation of ^{14}C-glucose in natural seawater seston. (C) Incorporation of glucose and amino-acids (AA) in a cultured strain of *Vibrio* sp.

The somewhat higher activity at 40 min was probably due to grazing on the few remaining bacteria (10^4 ml^{-1}). This increase in activity would still amount to less than 10 % of the increase in activity in the grazing experiments.

Grazing experiments

Isotope-labelled bacteria

The laboratory experiments showed an efficient uptake of bacteria for the 3 flagellate species tested. *Ochromonas minima* had an almost linear increase in activity up to 10 min, and a decreased uptake from 10 to 40 min, indicating a 'turnover-time' (vacuole-filling)

Table 1. Decrease in radioactivity (DPM ml^{-1}) in bacteria after 1, 2 and 5 wk of freezing. 1 ml filtered on 0.2 µm filter. Mean and SD of 3 replicates

Time (wk)	Amino acids	(SD)	Glucose	(SD)
t_0	1040		233	
1	843	(125)	120	(37)
2	730	(66)	173	(48)
5	842	(89)	170	(59)

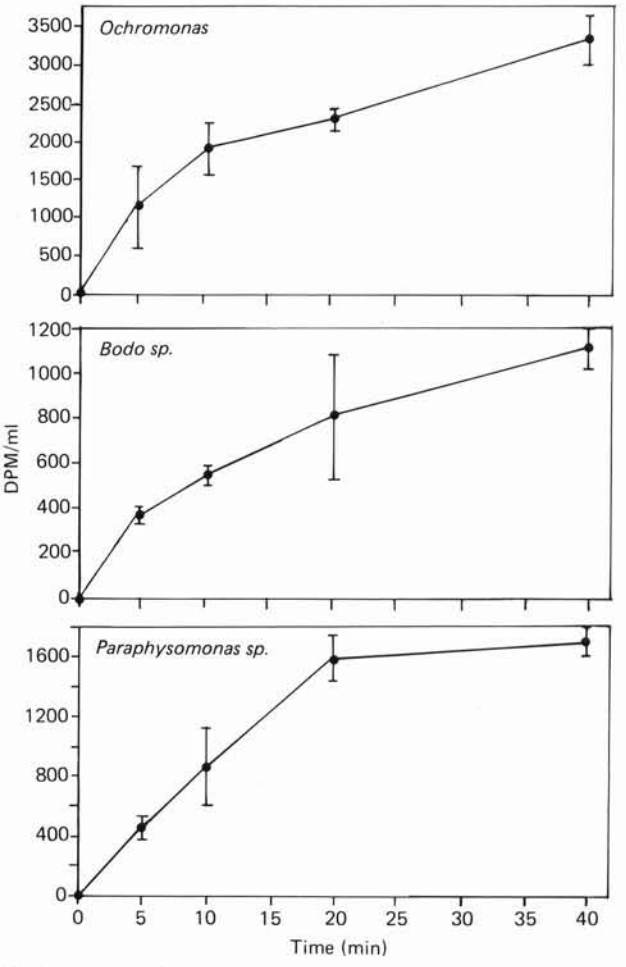

Fig. 2. Mean grazing rates measured as incorporated activity (DPM) per filtered (1 µm) volume (ml) of water in cultures of *Ochromonas minima*, *Bodo* sp., and *Paraphysomonas* sp. SD's for 3 replicate samples given as vertical bars. Increase in activity for the initial 10 min grazing period is used for calculating the specific grazing rates given in Table 1

of 10 min or less (Fig. 2). *Bodo* sp. showed an almost identical curve; however the turnover-time in this species is apparently less than 10 min. *Paraphysomonas* sp. had a linear uptake up to 20 min, with no increase in activity from 20 to 40 min, indicating a negligible assimilation of ingested bacteria (Fig. 2).

Table 2. Mean grazing rate per organism obtained with labelled and DTAF-stained bacteria

	Labelled (RLB)		Stained (FLB)	
	µl ind.$^{-1}$ h^{-1}	Bact. ind.$^{-1}$ h^{-1}	µl ind.$^{-1}$ h^{-1}	Bact. ind.$^{-1}$ h^{-1}
Ochromonas minima	0.014	36	0.001	29
Bodo sp.	0.132[a]	132	0.012	12
Paraphysomonas sp.	0.156[a]	156	0.024	24
Mixed seaw. comm. Stn 1	0.188[a]	188	0.006	6
Mixed seaw. comm. Stn 2	0.130[a]	130	0.006	6

[a] Fed with bacteria from a batch of seawater containing 1×10^6 labelled bacteria

Ingestion rates ranged from 0.6 bacteria flagellate^{-1} min^{-1} (*Ochromonas minima*) to 2.6 bacteria flagellate^{-1} min^{-1} (*Paraphysomonas* sp.) (Table 2). Corresponding grazing rates ranged from 0.012 µl flagellate^{-1} h^{-1} to 0.156 µl flagellate^{-1} h^{-1}.

DTAF-stained bacteria (FLB)

To compare methods, the same 3 species of flagellates were fed with FLB.

Ochromonas minima was grown in the second step of a 2-step continuous culture system, fed with bacteria from the first step. We could therefore use the grazing rates in the continous culture as a 'grazing' rate standard for *O. minima*. Our results (Fig. 3a) show that grazing rates were identical for the first 20 min for labelled bacteria compared with FLB. For this species, grazing rates measured with the 'grazing rate standard' was slightly lower than for the 2 other methods.

The 2 heterotrophic flagellates, *Bodo* sp. and *Paraphysomonas* sp., were fed with FLB. Both of them had a grazing rate on FLB that was about 10 % of the grazing rate measured with labelled bacteria (Fig. 3b, Table 2).

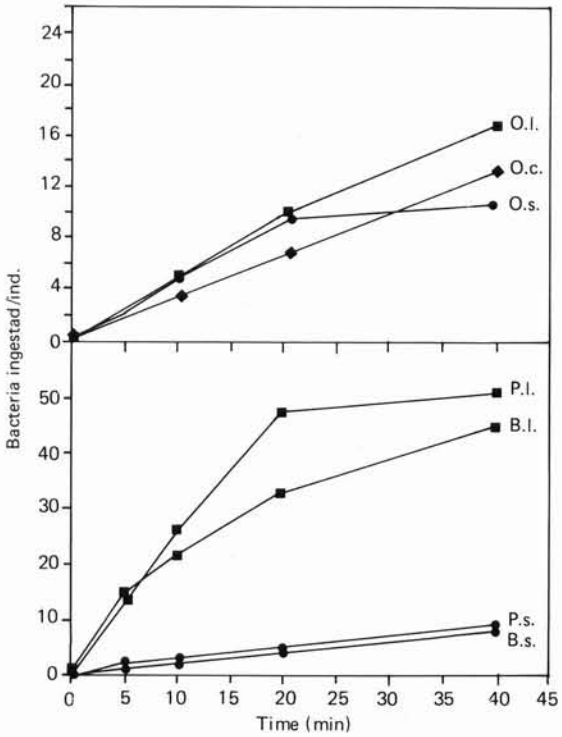

Fig. 3. Grazing rates measured for 3 species of flagellates, using radiolabelled bacteria, stained bacteria and chemostat (*Ochromonas minima* only). O.: *O. minima*; P.: *Paraphysomonas* sp.; B.: *Bodo* sp. Small letter indicates which method is used to measure grazing rates: l.: radio-labelled bacteria; s.: stained bacteria; c.: chemostat measurements

Field experiments

The methods were tested on a cruise in the Oslo fjord. Grazing rates were measured with both RLB and FLB. The results show grazing rates obtained by RLB 10 to 30 times those measured by FLB (Table 2).

Possible direct uptake of isotope in the flagellates during incubation was measured, and a control of uptake of isotope by the natural bacterial flora was made, by adding isotope direct to seawater samples. A negligible uptake of isotope, not significantly different to background values, was recorded both in the 0.2 µm and the 1.0 µm fraction, indicating low uptake of isotope even in the bacterial fraction at the prevailing low temperature (4.0 °C).

DISCUSSION

The use of radioactive tracers for measuring grazing rates in flagellates and ciliates has the obvious advantage that feeding on natural assemblages of algae and bacteria can be followed in situ with minimum disturbance of test organisms. Moreover, the method is rapid, simple, and allows a large number of replicates with low effort. Two problems may arise, however. First, when the size difference between prey (bacteria) and predator (flagellate) is small, a separation procedure based on filtering may not be sufficient. In the present laboratory experiments, we used 'starved' bacteria of

very small sizes (mean volume: 0.021 μm^3). In the cruise experiments, the bacteria in natural assemblages also were small, with sizes comparable to those in the laboratory experiment. Hence, the retention of bacteria on 1 µm filters was low or negligible (as also confirmed by epifluorescent microscopic examination). In general, however, this method will not work well with natural assemblages of bacteria, due to the often large proportion of cells retained on 1.0 µm filters. Preferably, prestarved non-cultivated bacteria should also be used for cruise experiments. Protozoan bacterivores appear to selectively graze larger bacterial cells in the bacterioplankton assemblage (Andersson et al. 1986, Tobiessen 1990). This could be a problem using pre-starved, small bacteria, as grazing rates would be underestimated, but has not been observed, either in laboratory experiments or during summer and autumn cruises in 1989 (unpubl.).

During all experiments, corrections were made for the 'background activity' of non-ingested bacteria on the 1 µm filters. Correspondingly, flagellates may pass the 1 µm filters, giving an underestimation of true grazing rates. The passage of whole cells is negligible; Fuhrman & McManus (1984) reported that about 1 % of naturally occurring flagellates will pass when using 1 µm filters, but certainly broken cells may not be retained. Even at low vacuum, soft-bodied genera like *Bodo* sp. and *Paraphysomonas* sp. may be completely broken when unfixed. The use of mercuric chloride as pre-filtering fixative apparently solved this problem. For the more robust *Ochromonas minima* a fixing procedure was superfluous.

When using dissolved organic compounds as tracers, a second error may be introduced if direct uptake of tracer by the eucaryotes occurs. As shown by Güde (1988), this may be the case with some freshwater algae when using amino acids as tracers. We cannot reject this possibility, but the error introduced in short-term experiments (< 1 h) is probably negligible. Hessen et al. (1989) found a rapid uptake of ^{14}C-amino acids in freshwater bacteria, while only 3 % of activity was found in the > 1 µm fraction during a 3 h incubation. Part of this 3 % was likely caused by large bacteria. The same pattern of particulate activity was found when using ^{3}H-thymidine. During these experiments (Hessen et al. 1989), flagellates (*Ochromonas minima, Monochrysis* sp., *Chromulina* sp.) were present in high numbers. In the present study, the initial tests (Fig. 1) similarly indicated a negligible uptake in the > 1 µm fractions over a 1 h interval. This is also supported by uptake rates in the streptomycin-treated culture of *O. minima*. A further indication of negligible uptake of isotope in the algae is the slopes of the grazing curves. A decrease in uptake after only 20 min would not be expected for the algae. This is best illustrated by Fig. 2c, showing no net increase in radioactivity in *Paraphysomonas* sp. after a grazing period of 20 min.

Tests from the cruise experiments showed low uptake in the bacterial fraction (< 1 µm), and a negligible uptake of isotope in the eucaryotic fraction (> 1 µm). This may in part be accredited to the low ambient temperature, but shows that eucaryotic isotope uptake of isotope in the grazing experiments with pre-labelled bacteria must be due to grazing activities, and not a direct uptake of isotope. For this type of experiments, with surplus of dissolved tracers available in the grazing medium, such 'direct-uptake assays' should always be performed as controls.

High conformity was found between the chemostat 'grazing rate standard' and isotope experiments. Lower uptake rates of stained bacteria compared to uptake rates of radio-labelled bacteria may be due to use of cultured bacteria for the stained bacteria method. These bacteria are much larger (mean volume: 0.283 μm^3, this study) than the starved, non-cultivated bacteria (mean volume: 0.021 μm^3) used in the isotope experiments; small flagellates like *Bodo* sp. might find these laboratory bacteria too large.

Flagellates are able to differentiate particles, as shown by Nygaard et al. (1988). The method used for staining bacteria (FLB) includes heat killing, which could make them 'unattractive' as food particles (Landry et al. 1987). Sherr et al. (1989) did not find this kind of selectivity, although number of flagellates containing FLB in mixed community (max. 74 %) was somewhat lower than uptake measured in pure culture (max. 97 %). *Ochromonas minima* do not seem to select between FLB and RLB. This may be an effect of the culturing conditions by this flagellate strain, which has made it more adapted to laboratory conditions. The other flagellates used for these experiments, *Bodo* sp. and *Paraphysomonas* sp., were both isolated from seawater prior to the experiments.

Methods that work well in laboratory experiments have often been shown to give low grazing rates when applied in field studies. In general, there is a gap between measured total grazing losses of bacteria, and 'true' losses which can be calculated from bacterial growth rates and biomasses. Methods like FLB (Sherr et al. 1987) have been showed to give grazing estimates close to bacterial production rates, though the rates varied from 10 to 125 % of bacterial production (Sherr et al. 1989). Bloem et al. (1989) found that bacterivory protozoan grazed about 25 % of bacteria produced per day, using the FLB method. The gap between bacterial production and measured grazing rates has been explained as problems due to fixation which leads to egestion of food vacuoles (Sieracki et al. 1987). The method presented here can account for this problem, as egested food vacuoles should not pass the

1.0 µm filter. Bacterial mortality due to viruses might also be an important regulating factor for bacterial biomass (Servais et al. 1985, Bergh et al. 1989). Low calculated grazing rates may be a result of the use of methods which disturb the grazers, or present them with a food particle that is too different from natural food, entailing selectivity towards natural food particles in the sample. A serious problem is that the degree of selectivity probably changes with species composition (Nygaard et al. 1988).

Proper estimates of true grazing losses are important, not only to evaluate the role of bacteria in the overall food-web, but also to evaluate 'top-down' or 'bottom-up' regulation of the bacterial biomass. Grazing rates as measured on 2 different stations in the Oslo fjord (Table 2) gave rates equal to a turnover of the bacterial population of 1.7 and 2.25 d^{-1}. This imply a potential for 'top-down' control of bacterial biomass by heterotrophic flagellates.

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