Letters in Applied Microbiology 2001, **33**, 455–460

Grazing rates of diverse morphotypes of bacterivorous ciliates feeding on four allochthonous bacteria

B. Ayo, E. Santamaría, A. Latatu, I. Artolozaga, I. Azúa and J. Iriberri

Departamento de Inmunología, Microbiología y Parasitología. Facultad de Ciencias. Universidad del Pais Vasco, Apartado 644, E-48080 Bilbao, Spain

2001/192: received 28 June 2001, revised 19 September 2001 and accepted 27 September 2001

B. AYO, E. SANTAMARÍA, A. LATATU, I. ARTOLOZAGA, I. AZÚA AND J. IRIBERRI. 2001.

Aims: The permanence in aquatic systems of allochthonous bacteria coming from sewage effluents is a risk for public health. This work aimed to analyse the elimination of the bacteria *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Escherichia coli* and *Klebsiella pneumoniae* by a riverine ciliate community.

Methods and Results: The ciliates were characterized and identified on the basis of morphological and behavioural features and grouped in nine morphotypes. Differential grazing by each morphotype on the four allochthonous bacteria was carried out by adding fluorescently labelled bacteria to the water samples, and measuring their uptake along time.

Conclusions: The nine morphotypes were present in all the samples but in different proportions, being the most abundant the small scuticociliates and hipotrichs. The smallest morphotypes showed grazing rates lower than 20 FLB ciliate^{-1} h^{-1}, with a preference towards *K. pneumoniae*. The larger morphotypes showed in general the highest grazing rates, but the selectivity was hardly attributable to bacterial size or type of cell wall.

Significance and Impact of the Study: The elimination of allochthonous bacteria in an aquatic system may be highly different depending on the diversity of the ciliated protistan community in the system and on the nature of the bacterial prey.

INTRODUCTION

The permanence of allochthonous bacteria in aquatic systems is relevant not only from an ecological point of view but also when public health is considered, since many of the bacterial species discharged in sewage effluents are potentially pathogens. Grazing by bacterivorous protists is one of the most relevant factors known to affect the disappearance of bacteria from freshwater systems. Several authors have reported significant grazing activities on autochthonous (Carlough and Meyer 1991; Vaqué *et al.* 1992) as well as on allochthonous bacteria (González *et al.* 1990; Iriberri *et al.* 1994; Barcina *et al.* 1997). Within the protistan community, the ciliates may become very active bacterial consumers, specially in summer (Sanders *et al.* 1989; Iriberri *et al.* 1993) when it has been observed that in many cases nearly all their carbon demand is satisfied by bacterivory (Vaqué *et al.* 1992; Simek *et al.* 1995).

Most studies carried out on bacterivory by ciliates in freshwater systems have been referred to the global community of these protists (Carlough and Meyer 1991 and references cited therein; Iriberri *et al.* 1993). However, when the studies have been focused on single genera or species, there have been noticeable differences in their grazing capacity (Sanders *et al.* 1989; Iriberri *et al.* 1995; Simek *et al.* 1995). In addition, not all bacterial prey types are grazed at the same rates, but some species seem to be more attractive than others to the grazer and, on the contrary, low preference or even rejection of some bacteria have also been described (Iriberri *et al.* 1994; Jürgens and Güde 1994).

On this basis, the aim of this study was to analyse the elimination by a riverine ciliate community of four allochthonous bacteria usually discharged in sewage effluents. First the diversity of the ciliate protistan community was studied, and then the analysis of differential grazing by each type of ciliate on the four allochthonous bacteria was carried out.

Correspondence to: J. Iriberri, Departamento de Inmunología, Microbiología y Parasitología, Facultad de Ciencias, Universidad del Pais Vasco, Apartado 644, E-48080 Bilbao, Spain (e-mail: oipirraj@lg.ehu.es).

MATERIALS AND METHODS

Study site and sampling

Four water samples were taken in Butrón River (42°22′N 2°51′W) from a depth of approximately 0·5 m in 10 L polypropylene bottles precleaned with diluted HCl, and processed in laboratory within two hours of sampling.

Bacteria studied and preparation of FLB

We used a collection of four bacterial strains allochthonous to aquatic systems from the C.E.C.T (Colección Española de Cultivos Tipo, Valencia, Spain): *Klebsiella pneumoniae* C.E.C.T. 517, *Escherichia coli* C.E.C.T. 11775, *Enterococcus faecalis* C.E.C.T. 19433, and *Staphylococcus epidermidis* C.E.C.T. 231.

Fluorescently-labelled bacteria (FLB) were obtained as described by Sherr *et al*. (1987). We cultured the four bacterial strains in nutrient broth to obtain a dense suspension of cells at the end of their exponential growth phase. The bacteria were collected as a pellet after high speed centrifugation (19 149 RCF*g*, 15 min), resuspended and stained with 5-(4,6-dichlorotriazin-2-yl) aminofluorescein (DTAF) 200 mg l^{-1} final concentration for 2 h at 60°C. The FLB suspensions were centrifuged and resuspended four times to remove the excess fluorochrome. After a brief sonication, FLB abundance in the suspensions was determined, and one ml aliquots were placed in cryotubes and stored in the dark at –20°C.

Microbial counts and characterizations

Bacterial abundance in formalin fixed samples (2% final concentration) was measured by acridine orange epifluorescence direct counting (AODC) (Hobbie *et al*. 1977). Bacteria or FLB present in at least 30 randomly selected fields were counted.

For protistan enumeration, samples were preserved with alkaline Lugol (0·5% final concentration) – formalin (3% final concentration) (Sherr *et al*. 1988) and stained with diamidino-phenylindol (DAPI) for epifluorescence microscopy (Porter and Feig 1980). Ciliate counts were carried out at a magnification of ×250, and the entire filter surface was examined.

Protistan live observations in the samples and after enrichment were made directly on Sedgewick-Rafter chambers (Graticules Ltd, Tonbridge, Kent, UK). The material was observed by light microscopy at a magnification of ×200 and ×1000 (Nikon Diaphot-TMD and Optiphot, both equipped with Nomarski DIC system) (Nikon Corporation, Tokyo, Japan). Protists were characterized and identified on the basis of their morphological and behavioural features as described in Small and Lynn (1985); Patterson and Hedley (1992) and Foissner and Berger (1996).

Ciliates were grouped on the basis of their morphological characteristics in nine morphotypes. The sizes of FLB and ciliates were determined from fixed and stained samples. Cell sizes were calibrated with a stage micrometer at a magnification of ×1250 on a Nikon epifluorescence microscope equipped with a video camera (Hamamatsu 2400, Hamamatsu Photonics, Hamamatsu City, Japan) and a semiautomatic image analysis system (VIDS IV). We measured the maximum length and width of at least 200 randomly selected FLB and 20 ciliates of each morphotype. FLB cell volumes were calculated by the equation $V = [4/3[\pi(W/2)^3] + [\pi(W/2)^2 \times (L\text{-}W)]$, where W is cell width and L is cell length.

Grazing estimations

A volume of suspension of FLB was added to the water samples until achieving a 10 ± 1% of the natural bacterial density ($2{\cdot}9 \pm 1{\cdot}4 \times 10^6$ bacteria ml^{-1}) in each experiment. The samples were incubated for one hour at *in situ* temperature. During the first 20 min, 50-ml subsamples were taken at 2-min intervals, and at 5–10-min intervals during the next 40 min. Subsamples were preserved with alkaline Lugol-formalin, and stored for at most one week at 2°C in the dark until microscopical processing.

The preserved subsamples were stained with DAPI (Porter and Feig 1980) and filtered onto 3·0 μm pore size polycarbonate filters. The filters were first observed under u.v. light at a magnification of ×200, and when a ciliate was located, the incident light was changed to blue light, which allowed the counting of FLB inside it. At least 20 ciliates of each morphotype were inspected for FLB ingestion in each subsample. FLB ingestion rates by ciliate were obtained from the slopes determined via regression analysis, of the linear portions of the plots of the numbers of FLB ingested per ciliate vs time.

RESULTS AND DISCUSSION

The morphological features used to distinguish the morphotypes of the ciliates found in the four samples taken in Butrón River are shown in Table 1. The correspondence between these morphotypes, obtained from epifluorescence microscopy on DAPI-stained samples, and concrete taxons detected after observation of live samples by optical microscopy was possible only in some cases. On the basis of the cell sizes and morphological features, the most common scuticociliates found in this aquatic system, such as *Uronema* sp. and *Cyclidium* sp., would be inside the morphotype C1, while the morphotypes C2 and C3 represented other scuticociliates

Table 1 Morphological features of DAPI stained ciliates

Ciliate morphotype	Cell morphology	Width (μm) (mean ± S.E.)	Length (μm) (mean ± S.E.)	Macronuclei (number/shape/location)	Micronuclei (number/shape/location)
C1	Oval-shaped	8·3 ± 0·24	18·2 ± 0·5	1/oval/terminal	1/round/terminal
C2	Oval-shaped	8·3 ± 0·3	17·8 ± 0·6	2/round/terminal	1/round/terminal
C3	Oval-shaped	9·6 ± 0·2	20·8 ± 0·5	3/round/central	N.S.*
C4	Oval-shaped	10·3 ± 0·6	21·9 ± 1·5	1/oval/central	1/round/central
C5	Elongated	33·1 ± 1·1	90·7 ± 4·5	1/oval/central	1/round/central
C6	Oval-shaped	32·3 ± 0·8	39·3 ± 3·5	1/C-shaped/cell contour	1/round/terminal
C7	Elongated	23·4 ± 2·2	42·5 ± 4·5	1/oval/central	N.S.
C8	Spherical	10·0 ± 2·8	10·2 ± 0·9	1/oval/central	1/round/central
C9	Pyryform	21·1 ± 1·8	50·2 ± 4·5	2/round/central	N.S.
Others	Various	N.D. †	N.D.	N.D.	N.D.

*N.S. not seen

†N.D., not determined because this group includes untyped and scarce specimens.

(*Colpidium* sp., *Glaucoma* sp.). The morphotype C6 was also easily recognized as small and medium hipotrichs, possibly *Aspidisca* sp. and *Euplotes* sp. It was not possible to assign taxons to the other morphotypes due to the deformation observed after fixation of the samples. These taxons found in Butrón River were typical of freshwater systems in which bacterial prey was abundant, and they have been detected in some other freshwater ecosystems (Sanders *et al*. 1989; Simek *et al*. 1995; Fernández-Leborans and Novillo 1996).

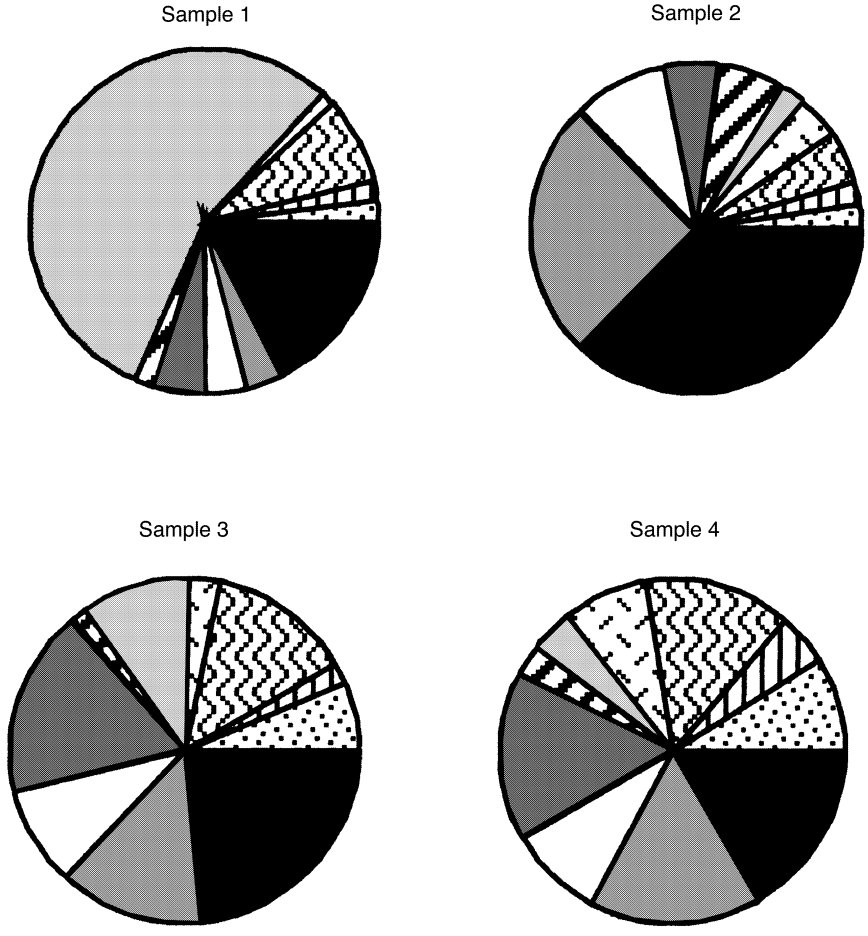

Fig. 1 Distribution of the morphotypes of cilliates in the analysed samples. (■), C1; (▩), C2; (□), C3; (▦), C4; (▨), C5; (▤), C6; (▧), C7; (▩), C8; (▥), C9; (▩), others

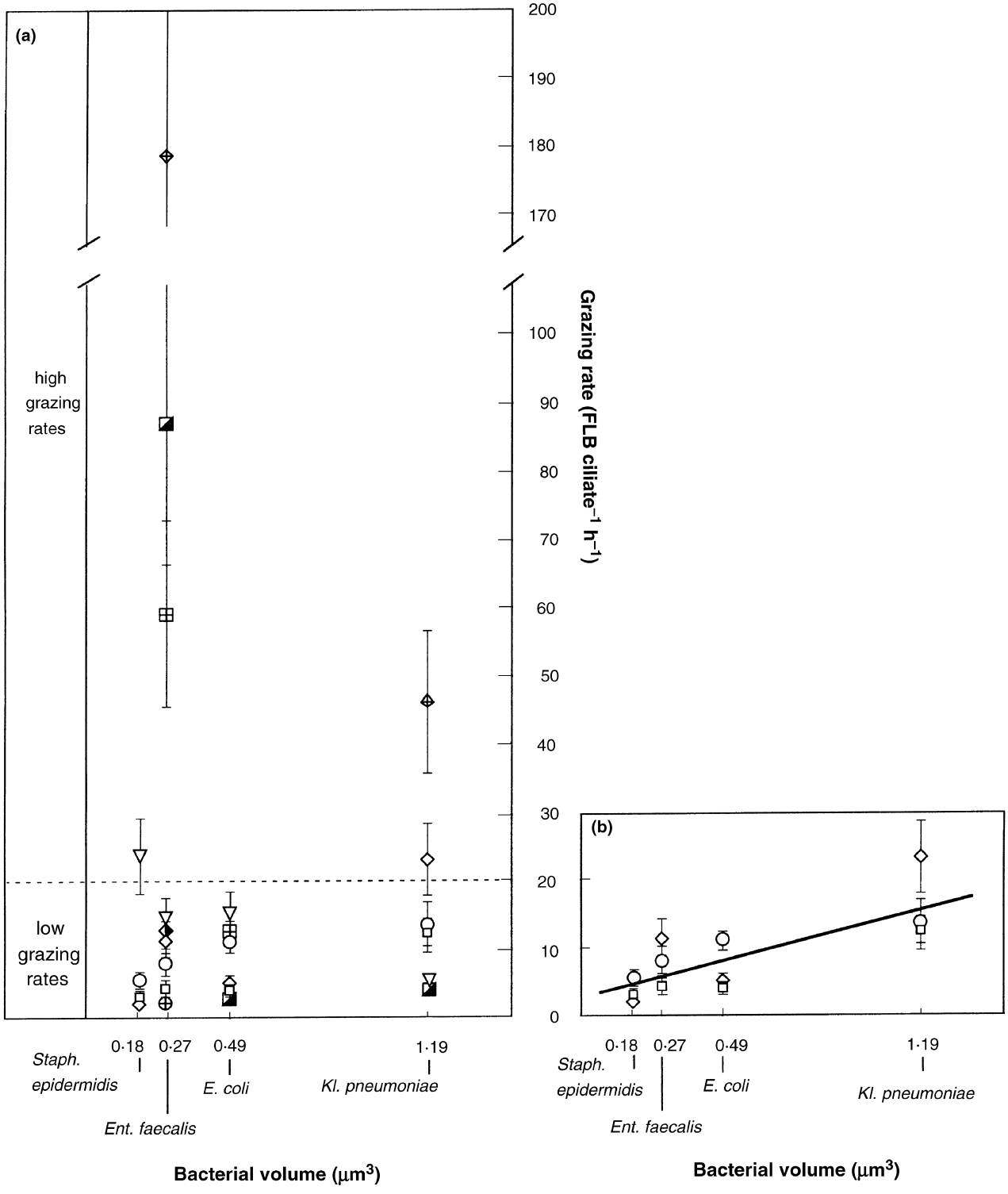

Fig. 2 (a) Grazing rates observed for the nine morphotypes of ciliates on the four allochthonous bacteria. (b) Regression between the grazing rates of scuticociliates (C1 + C2 + C3) and bacterial volume (slope = 11·31, $r^2 = 0{\cdot}601$, $P < 0{\cdot}01$) (□), C1; (◇), C2; (○), C3; (△), C4; (⊞), C5; (⊕), C6; (⊕), C7; (▽), C8; (◪), C9; (◆), others

The frequency distribution of the nine morphotypes of ciliates in the four analysed samples is shown in Fig. 1. The high variation of the proportions of the morphotypes among samples was noticeable: the three morphotypes formed by scuticociliates (C1, C2 and C3) represented from 24·8% to 71·6% of the total community. All the other morphotypes were always present in the samples, but they accounted for a small fraction of the community of ciliates. The only exception was morphotype C6 in sample 1, where it was the dominant morphotype.

The ciliate grazing rates on the four allochthonous bacteria are given in Fig. 2. The analysis of the grazing capacity of the nine morphotypes indicated that they might be grouped into two different clusters. The smallest ciliates included in morphotypes C1, C2, C3 and C8, had relatively low grazing rates, lower than 20 FLB ciliate^{-1} h^{-1}. The small morphotype C7 and the mixed subcommunity grouped in 'Others' were also inside this cluster, in view of their low grazing rate detected on *Ent. faecalis*. The larger morphotypes, such as C5, C6, and C9 should be put together in the second cluster, considering that they generally showed higher grazing rates which reached values of as much as 178·5 FLB ciliate^{-1} h^{-1}. Regarding the morphotype C4, we did not detect any grazing activity on the four allochthonous bacteria.

The preference by these morphotypes towards their prey was analysed. The four allochthonous bacteria were the Gram negative rods *Kl. pneumoniae* (1·19 ± 0·05 μm^3) and *E. coli* (0·49 ± 0·024 μm^3) and the Gram-positive cocci *Ent. faecalis* (0·27 ± 0·015 μm^3) and *Staph. epidermidis* (0·18 ± 0·008 μm^3). From the analysis of the grazing rates of the group of scuticociliates (C1 + C2 + C3), a significant regression (see Fig. 2b) was observed between the prey volume and the observed grazing rates. Thus, *Kl. pneumoniae* was the largest bacteria analysed and the most quickly consumed one, and the lowest grazing rate values were detected for the smallest bacteria, *Staph. epidermidis.* However, the significance of this regression comes from the relatively very high grazing rates observed for the three morphotypes on *Kl. pneumoniae*. It may be possible that not only the size but also some other features of this bacterial species, such as the chemical composition of the cell periphery, may be implied on this preference.

The remaining morphotypes showed clear preferences for species of bacteria, but they could not be attributable to bacterial size, morphology or even cell wall type. The morphotype C8 showed the highest grazing rates on *Staph. epidermidis* and *E. coli* (23·6 and 14·9 FLB ciliate^{-1} h^{-1}, respectively), while the morphotypes C5 and C9 showed very high grazing rates on *Ent. faecalis* (59·0 and 87·6 FLB ciliate^{-1} h^{-1}, respectively) but very low grazing activity on *E. coli* (12·3 and 3·5 FLB ciliate^{-1} h^{-1}, respectively). Moreover, the morphotype C6 showed the highest grazing rates on *Kl. pneumoniae* (46·1 FLB ciliate^{-1} h^{-1}) and *Ent. faecalis* (178·5 FLB ciliate^{-1} h^{-1}), two bacteria which are very different in size and other cell characteristics. Some other authors (Sherr *et al*. 1988; Epstein and Shiaris 1992) have also found that different protistan taxons from natural samples had preferences for bacteria of specific size ranges. However, in these studies the prey offered to the protists was bacterioplankton and not single bacterial species, which could have provided more information about the basis of these selective grazing activities.

These results indicated that the elimination of allochthonous bacteria in an aquatic system may be highly different in function of the diversity of the ciliated protistan community inhabiting that system and of the nature of the bacterial prey. In ecosystems which are dominated by small scuticociliates (morphotypes C1, C2 and C3), the allochthonous bacteria will be grazed at relatively low rates, and a longer permanence of bacteria which are smaller in size can be expected. The remaining ciliates will selectively graze the allochthonous bacteria, and therefore the presence of different morphotypes and the size of these subcommunities will be decisive in determining the maintenance or disappearance of the allochthonous bacteria in the aquatic system.

ACKNOWLEDGEMENTS

This work was supported by grant UPV EB 033/95 from the University of the Basque Country to A.L. and J.I. and grants MEC PB97-0635 from the Spanish Government and PIGV 98–0025 from the Basque Government to J.I.

REFERENCES

Barcina, I., Lebaron, P. and Vives-Rego, J. (1997) Survival of allochthonous bacteria in aquatic systems: a biological approach. *FEMS Microbiology Ecology* **23**, 1–9.

Carlough, L.A. and Meyer, J.L. (1991) Bacterivory by sestonic protists in a southeastern blackwater river. *Limnology and Oceanography* **36**, 873–883.

Epstein, S.S. and Shiaris, M.P. (1992) Size-selective grazing of coastal bacterioplankton by natural assemblages of pigmented flagellates, colorless flagellates and ciliates. *Microbial Ecology* **23**, 211–225.

Fernández-Leborans, G. and Novillo, A. (1996) Protozoan communities and contamination of several fluvial systems. *Water Environment Research* **68**, 311–319.

Foissner, W. and Berger, H. (1996) A user-friendly guide to the ciliates (Protozoa, Ciliophora) commonly used by hydrobiologists as bioindicators in rivers, lakes and waste waters, with notes on their ecology. *Freshwater Biology* **35**, 375–482.

González, J.M., Iriberri, J., Egea, L. and Barcina, I. (1990) Differential rates of digestion of bacteria by freshwater and marine phagotrophic protozoa. *Applied and Environmental Microbiology* **56**, 1851–1857.

Hobbie, J.E., Daley, R.J. and Jasper, S. (1977) Use of Nuclepore filters for counting bacteria by epifluorescence microscopy. *Applied and Environmental Microbiology* **33**, 1225–1228.

Iriberri, J., Ayo, B., Santamaría, E., Barcina, I. and Egea, L. (1995) Influence of bacterial density and water temperature on the grazing activity of two freshwater ciliates. *Freshwater Biology* **33**, 223–231.

Iriberri, J., Ayo, B., Unanue, M., Barcina, I. and Egea, L. (1993) Channelling of bacterioplanktonic production toward phagotrophic flagellates and ciliates under different seasonal conditions in a river. *Microbial Ecology* **26**, 111–124.

Iriberri, J., Azúa, I., Labirua-Iturburu, A., Artolozaga, I. and Barcina, I. (1994) Differential elimination of enteric bacteria by protists in a freshwater system. *Journal of Applied Bacteriology* **77**, 476–483.

Jürgens, K. and Güde, H. (1994) The potential importance of grazing-resistant bacteria in planktonic systems. *Marine Ecology Progress Series* **112**, 169–188.

Patterson, D.J. and Hedley, S. (1992) *Free-Living Freshwater Protozoa. A Colour Guide*. Wolfe Publishing Ltd, Aylesbury, England.

Porter, K.G. and Feig, Y.S. (1980) The use of DAPI for identifying and counting aquatic microflora. *Limnology and Oceanography* **25**, 943–948.

Sanders, R.W., Porter, K.G., Bennett, S.R. and DeBiase, A.E. (1989) Seasonal patterns of bacterivory by flagellates, ciliates, rotifers and cladocerans in a freshwater planktonic community. *Limnology and Oceanography* **34**, 673–687.

Sherr, B.F., Sherr, E.B. and Fallon, R.D. (1987) Use of monodispersed flurescently labeled bacteria to estimate *in situ* protozoan bacterivory. *Applied and Environmental Microbiology* **53**, 958–965.

Sherr, B.F., Sherr, E.B. and Rassoulzadegan, F. (1988) Rates of digestion of bacteria by marine phagotrophic protozoa: temperature dependence. *Applied and Environmental Microbiology* **54**, 1091–1095.

Simek, K., Bobková, J., Macek, M. and Nedoma, J. (1995) Ciliate grazing on picoplankton in a eutrophic reservoir during the summer phytoplankton maximum: a study at the species and community level. *Limnology and Oceanography* **40**, 1077–1090.

Small, E.B. and Lynn, D.H. (1985) Phylum Ciliophora Doflein, 1901. In *An Illustrated Guide to the Protozoa* ed. Lee, J.J., Hutner, E.H. and Bovee, E.C. Lawrence, Kansas, USA: Society of Protozoologists, Allen Press.

Vaqué, D., Pace, M.L., Findlay, S. and Lints, D. (1992) Fate of bacterial production in a heterotrophic ecosystem: grazing by protists and metazoans in the Hudson Estuary. *Marine Ecology Progress Series* **89**, 155–163.