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Differential elimination of enteric bacteria by protists in a freshwater system

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J. IRIBERRI, I. AZÚA, A. LABIRUA-ITURBURU, I. ARTOLOZAGA AND I. BARCINA. 1994. The short-term (1 h) and long-term (3 d) elimination of low and high densities of five enteric bacteria, *Klebsiella pneumoniae*, *Aeromonas hydrophila*, *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus epidermidis*, by flagellate and ciliate protists were measured in a freshwater system. In addition, the two processes, ingestion and digestion, which cause the disappearance of those enteric bacteria as time passes, were quantified.

The results showed that the elimination of these enteric bacteria by protists depends on their initial density, which confirms that the lower the bacterial density the more difficult is their elimination. On the other hand, the short-term and long-term elimination rates of each enteric bacteria were different, and moreover, the order of priority for elimination in the two cases was not the same. *Escherichia coli* showed the highest elimination rate in short-term experiments, while *Aer. hydrophila* disappeared at highest rates in long-term experiments. This different order of priority in the elimination rates and the different digestion rates on the five enteric bacteria by phagotrophic protists indicated that the elimination in time is very much influenced by the digestive capacity on each enteric bacteria of those protists. Thus, the low digestion rates of *Ent. faecalis* and *Staph. epidermidis* by flagellates and ciliates as well as their low disappearance percentages in the long-term experiments confirm that enteric Gram-positive bacteria are eliminated from the aquatic systems at lower rates, because their digestion is difficult.

INTRODUCTION

Urban sewage is one of the most important pollutants in natural freshwaters. The discharge of high quantities of faecal allochthonous micro-organisms to the system leads to imbalances in the microbial dynamics. Moreover, most of these enteric micro-organisms are opportunistic pathogens, e.g. *Escherichia coli* and *Enterococcus faecalis*, usual and abundant inhabitants of human faeces. Other opportunistic pathogens, such as *Klebsiella pneumoniae*, *Aeromonas hydrophila* and *Staphylococcus epidermidis*, should not be underestimated in spite of their lower densities in faeces (Leclerc *et al.* 1977; Oliveri 1982; Bell *et al.* 1983). As a consequence of the discharges freshwater systems may constitute a major public health risk if the water is used for irrigation, fisheries, etc.

The natural habitat of enteric bacteria is the intestine of humans and other animals. When discharged into a natural aquatic system, the organisms find hostile conditions and have to adapt and begin a survival period. During this period the bacteria are affected by several abiotic factors such as light (Barcina *et al.* 1990), temperature (Anderson *et al.* 1983), presence of toxic substances (Grimes *et al.* 1986) or lack of nutrients/starvation (López-Torres *et al.* 1988), and by biotic factors such as protistan grazing (McCambridge and McMeekin 1979, 1980; Barcina *et al.* 1986; García-Lara *et al.* 1991), lysis by bacteriophages (Proctor *et al.* 1988; Bergh *et al.* 1989) and lytic bacteria (McCambridge and McMeekin 1979; Guerrero *et al.* 1986), or competitive processes between bacteria (Mitchell 1968; Jannash 1969). Several works (McCambridge and McMeekin 1979; Fenchel 1982; Gameson 1984; Porter *et al.* 1985; Rassoulzadegan and Sheldon 1986; Sherr *et al.* 1986; McManus and Furhman 1988; García-Lara *et al.* 1991) point to protistan grazing as the main factor that is responsible for bacterial elimination in aquatic systems. Although a number of techniques have been used to measure the grazing activities of planktonic protists (Børsheim 1984; Landry *et al.* 1984; Sherr *et al.* 1986;

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Wikner *et al.* 1986), the most common approach to estimate grazing on bacterial strains is the uptake and/or disappearance of fluorescently labelled bacteria (FLB) (Sherr *et al.* 1987).

The objective of this work was twofold. First, the elimination rates by bacterivorous protists on five enteric bacteria (*E. coli*, *Ent. faecalis*, *Kl. pneumoniae*, *Aer. hydrophila* and *Staph. epidermidis*) usually present in human faeces and therefore abundant in the discharges of urban sewage into aquatic systems were quantified. Second, it was determined if the digestion rates of protists on those bacteria conditioned their long-term elimination rates in the aquatic system.

MATERIALS AND METHODS

Study site and sampling

All the samples were taken in Butrón River (42° 22′ N 2° 51′ W), a shallow, slow and short river on the North Coast of Spain. Samples were collected from a depth of *ca* 0·5 m in 10 l polypropylene bottles precleaned with diluted HCl, and processed in laboratory within 2 h of sampling.

Preparation of enteric FLB

Enteric fluorescently-labelled bacteria (FLB) were obtained as described by Sherr *et al.* (1987) from two strains of the Spanish Type Culture Collection (Valencia, Spain), *Klebsiella pneumoniae* CECT 517 and *Aeromonas hydrophila* CECT 398, and from three strains of the American Type Culture Collection (Rockville, MD), *Escherichia coli* ATCC 11775, *Enterococcus faecalis* ATCC 19433 and *Staphylococcus epidermidis* ATCC 12228.

The five bacterial strains were grown in nutrient broth to obtain a dense suspension of cells at the end of their exponential growth phase. Each enteric bacterial culture was collected as a pellet after high speed centrifugation (19 149 *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, enteric FLB numbers in each suspension were determined, and 1 ml volumes were placed in cryotubes and stored in the dark at −20°C.

Bacterial and protistan counts

Bacterial numbers were measured by acridine orange epifluorescence direct counting (AODC) (Hobbie *et al.* 1977). Formalin-fixed samples (2% v/v final concentration) were stained with acridine orange (0·01% w/v final concentration) for 2 min, and filtered on 0·2 μm pore size black polycarbonate filters. The wet filters were placed on microscope slides and mounted in low-fluorescence immersion oil. For counting enteric FLB the process was similar but there was no AO staining. The filters were examined under a Nikon epifluorescence microscope, equipped with a filter block B-2A for blue light (EX450 ~ 490 excitation filter, DM510 dichroic mirror and BA520 barrier filter), at a magnification of × 1250. Bacteria or enteric FLB present in at least 30 randomly selected fields were counted.

DAPI-stained preparations for epifluorescence microscopy (Porter and Feig 1980) were used for protistan enumeration. Alkaline Lugol (10 g of I_2, 20 g of KI and 10 g of sodium acetate in 140 ml of distilled water; 0·5% v/v final concentration)–formalin (3% v/v final concentration) (Sherr *et al.* 1988) preserved samples were stained with diamidino-phenylindol (DAPI) (0·2 μg ml^{-1} final concentration) for 7 min and filtered on 0·8 and 3·0 μm pore-size prestained (Irgalan black, 0·2% w/v in 2% v/v acetic acid for 24 h) polycarbonate filters in the case of flagellates and ciliates respectively. The wet filters were placed on microscope slides and mounted in low-fluorescence immersion oil. The filters were examined through a Nikon epifluorescence microscope, equipped with a filter block UV-2B for u.v. light (EX330 ~ 380 excitation filter, DM400 dichroic mirror and BA435 barrier filter), and a filter block B-2A for blue light (see above). Heterotrophic flagellate counts were made under u.v. light at a magnification of × 1250. Autotrophic flagellates were distinguished from heterotrophs by their red autofluorescence under blue light, and were not counted. At least 60 heterotrophic flagellates were counted from each sample. Ciliate counts were carried out at a magnification of × 250, and all the filter surface was examined.

Short-term experiments

Two types of experiments, short-term and long-term, were designed to determine the elimination of enteric bacteria by protists in a freshwater system. Short-term experiments indicated the instantaneous elimination rates of low densities of enteric bacteria, while the long-term experiments allowed the quantification of the disappearance in time of an input of a high density of enteric bacteria.

In the short-term experiments the technique of uptake of monodispersed fluorescently-labelled bacteria (Sherr *et al.* 1987) was used to estimate protistan grazing rates. This technique offers short incubation times, minimal manipulation of water samples, and it allows the determination of specific predation rates for flagellates or ciliates. However, the main drawback is that the heat-killing treatment of the bacterial cells in the process of FLB preparation may alter

the properties of these tracer bacteria (Landry *et al.* 1991), although the degree of alteration of the bacterial surface is unknown.

In enteric FLB uptake experiments, the initial densities of enteric FLB added ranged between $2{\cdot}1 \times 10^5$ and $11{\cdot}6 \times 10^5$ enteric FLB ml^{-1}, with a mean value of $6{\cdot}7 \times 10^5$ enteric FLB ml^{-1}, which represented 12% of total bacteria (planktonic bacteria plus enteric FLB). The assays to measure enteric FLB uptake by flagellates and ciliates were carried out separately in 3 l flasks, which were previously acid-washed and copiously rinsed with deionized water. After enteric FLB were added the samples were hand-shaken for 10 s to randomize the distribution of enteric FLB. The flasks were incubated for 1 h at *in situ* temperature in the dark and hand-shaken each 30 s. During the first 20 min 20 ml subsamples for flagellates and 50 ml subsamples for ciliates were taken at 2 min intervals, and at 5–10 min intervals during the next 40 min. Subsamples were preserved with alkaline Lugol-formation (see above) and stored for 2 weeks at most, at 2°C in the dark until microscopical processing.

The experiments to determine digestion of enteric FLB were carried out according to the experimental design described by Sherr *et al.* (1988). A 10-fold dilution with filtered (0·2 μm) river water containing non-labelled bacteria in the same density of natural bacterioplankton diminishes the possibility of the protists meeting an enteric FLB, and therefore protistan uptake of enteric FLB decreases to very low (usually undetectable) levels. In these conditions it is possible to follow the decrease in numbers of FLB inside the protists in time, that is, the protistan digestion rates on enteric FLB. A linear decrease in the number of enteric FLB inside the flagellates and the ciliates as time passed was observed.

These experiments, carried out with the water subsamples used in the ingestion experiments, were conducted in 3 l flasks which were previously acid-washed and copiously rinsed with deionized water. Two hours after the beginning of the ingestion experiment, 150 ml and 250 ml of water were diluted with 1350 ml and 2250 ml of 0·2 μm pore size filtered river water in the case of flagellates and ciliates respectively. A concentrate of bacterioplankton, obtained by tangential flow filtration was added to these volumes of water in order to achieve a bacterial density similar to that recorded for river water. Blanks were included to control the uptake of enteric FLB at those low densities for the digestion experiments. These blanks consisted of river water diluted 10-fold (see above) to which a suspension of bacterioplankton plus enteric FLB was added to achieve the same densities as in the digestion experiments. Subsamples were taken, preserved and stored as in the ingestion experiments. From both experimental and control flasks 40 ml subsamples for flagellates and 100 ml subsamples for ciliates were taken at 2 min intervals during the first 20 min, and at 5 min intervals during the next 60 min. Subsamples were preserved and stored as described above.

For microscopical processing, subsamples were DAPI-stained, filtered, and filters mounted for immediate epifluorescence microscopical observation as described above for protistan counts. The filters were first observed under u.v. light at a magnification of $\times 1250$ in the case of flagellates, and of $\times 250$ in the case of ciliates. When a protist was located the incident light was changed to blue light, which allowed the enteric FLB contained inside to be counted. At least 60 flagellates and 100 ciliates were inspected in each subsample.

Enteric FLB ingestion and digestion rates were obtained from the slopes, determined by regression analysis ($n > 6$) of the plots of enteric FLB numbers inside the protist *vs* time. The digestion rate of enteric FLB per-protist was corrected for enteric FLB uptake after dilution. These rates were compared by the F test for the difference between the absolute regression coefficients (Sokal and Rohlf 1969).

Enteric FLB eliminated in 1 h by each community of protists were determined by multiplying the enteric FLB ingested per flagellate or per ciliate per h (obtained from regression analysis) by the abundance of flagellates or ciliates respectively. For each community of protists the disappearance percentage of each enteric FLB was calculated by dividing the number of enteric FLB eliminated per community per h ($\times 100$) by the initial abundance of enteric FLB added. Total percentage of elimination of enteric FLB by protists was determined from the sum of the disappearance percentages for flagellate and ciliate communities. These percentages were compared by the Student t test (Sokal and Rohlf 1969) after their transformation into arcsine square roots, in order to make them more heteroscedastic.

Long-term experiments

These experiments were performed to assay the removal, over a 4 d period, of enteric FLB added in higher densities than in the short-term experiments. In these long-term experiments the initial densities of enteric FLB ranged between $34{\cdot}5 \times 10^5$ and $73{\cdot}1 \times 10^5$ enteric FLB ml^{-1}, with a mean value of $52{\cdot}7 \times 10^5$ enteric FLB ml^{-1}, which represented 78% of total bacteria (planktonic bacteria plus enteric FLB). This type of experiment implies a higher manipulation of the water samples as incubation periods are longer, but in turn it allows the inclusion of the ingestion and digestion of prey in the elimination process as time passes.

These experiments were conducted in 250 ml flasks which were previously acid-washed and copiously rinsed with deionized water. Enteric FLB were added to 200 ml of

water samples with and without natural microbiota and then incubated in the dark with shaking (100 rev min^{-1}) at *in situ* temperature. Samples without natural microbiota were obtained by filtering river water on 0·2 μm pore size filters. Ten ml subsamples were taken at 12 h intervals, preserved with alkaline Lugon-formalin (see above) and stored at 2°C in the dark until microscopical processing. Enteric FLB were enumerated as described above. After 3 d, enteric FLB disappearance percentages were calculated as the difference ($\times 100$) in enteric FLB abundance between the beginning and the end of the experiment divided by the initial abundance of enteric FLB.

RESULTS

The densities of planktonic bacteria in Butrón River were between $1{\cdot}43 \times 10^6$ and $4{\cdot}85 \times 10^6$ bacteria ml^{-1}. For protists the densities of flagellates were between 297 and 1181 flagellates ml^{-1} and ciliates between 2·5 and 36·5 ciliates ml^{-1}.

Short-term experiments

The capacity of the protists to eliminate (ingest) enteric FLB was different for each bacterial strain assayed. The number of enteric FLB eliminated in 1 h in these experiments (Table 1) ranged from $1{\cdot}67 \times 10^3$ *Kl. pneumoniae*-FLB ml^{-1} to $3{\cdot}78 \times 10^3$ *E. coli*-FLB ml^{-1}. These values represented mean disappearance percentages ranging from 0·18% for *Ent. faecalis* to 0·71% for *E. coli*, whose mean disappearance percentage was significantly higher ($P < 0{\cdot}05$) than those obtained for *Ent. faecalis* and *Aer. hydrophila* (0·33%).

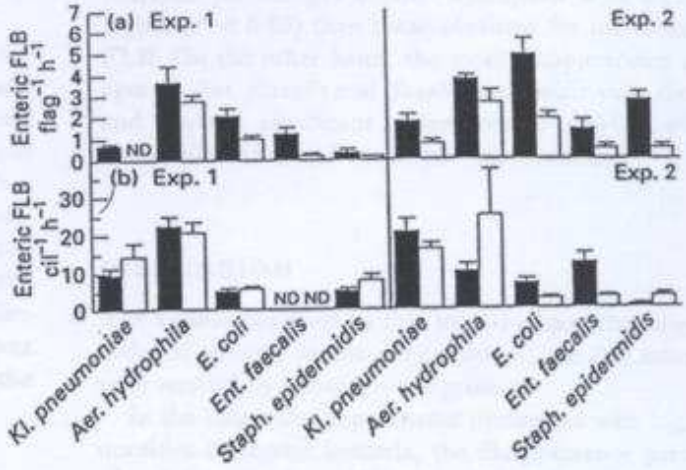

Fig. 1 Ingestion (■) and digestion (□) rates of (a) flagellates and (b) ciliates on the five enteric fluorescently-labelled bacteria (FLB) in the short-term experiments

The flagellates were the community mainly responsible for the elimination of enteric FLB, as these protists were more abundant than ciliates. However, the ingestion rates of enteric FLB on a per-protist basis were higher for ciliates (Fig. 1), with the only exception of the prey *Staph. epidermidis* in the second experiment.

The ingestion rates of flagellates were twice or even higher than the respective digestion rates, with the exception of *Aer. hydrophila* (Fig. 1). On the other hand, in the

Table 1 Absolute value and percentages of enteric fluorescently-labelled bacteria (FLB) disappeared per h (short-term experiments) and during a 3 d period (long-term experiments)

	Short-term experiments (1 h)		Long-term experiments (3 d)	
Strains	(10^3 enteric FLB ml^{-1})	%	(10^5 enteric FLB ml^{-1})	%
Klebsiella pneumoniae CECT 517	1·67 (±1·08)*	0·29 (±0·18)	26 (±9)	34 (±8)
Aeromonas hydrophila CECT 398	3·77 (±1·04)	0·33 (±0·02)	53 (±0)	86 (±4)
Escherichia coli ATCC 11775	3·78 (±2·33)	0·71 (±0·01)	17 (±9)	45 (±2)
Enterococcus faecalis ATCC 19433	2·10†	0·18†	8 (±3)	15 (±7)
Staphylococcus epidermidis ATCC 12228	0·83 (±1·53)	0·50 (±0·22)	10 (±7)	29 (±0)

CECT, Colección Española de Cultivos Tipo; ATCC, American Type Culture Collection.

* Mean value (±S.E.) ($n = 2$).

† Values obtained in one experiment.

case of ciliates the ingestion rates were not always higher than digestion rates. It is noticeable that for *Aer. hydrophila* in experiment 2, the ciliates showed a digestion rate value more than twice the ingestion rate.

There were different digestion rates for each of the five enteric FLB assayed, and these differences were statistically significant ($P < 0.05$) in 50% of the comparisons. It should be pointed out the high digestion rates of flagellates and ciliates on *Aer. hydrophila*, which were significantly higher ($P < 0.05$) than the digestion rates on the other enteric bacteria with the exception of *Kl. pneumoniae*. On the other hand, the digestion rates of flagellates and ciliates on the two Gram-positive cocci, *Ent. faecalis* and *Staph. epidermidis*, were similar and low. They were significantly lower ($P < 0.05$) than for the other enteric bacteria in 63% of the comparisons.

Long-term experiments

Figure 2 shows the evolution in time of *Aer. hydrophila*-FLB abundance in the absence (Fig. 2, line A) and presence (Fig. 2, line B) of natural microbiota in the first long-term experiment. This figure is representative of the evolution of the enteric FLB disappearance in time in the long-term experiments. Enteric FLB did not disappear in the absence of natural river microbiota, while in the presence of the natural microbiota, the disappearance of FLB was evident. After the third day of the experiments, the disappearance of enteric FLB became much slower.

In these long-term experiments differences were also observed between the disappearance percentages of the bacterial strains studied. The number of enteric FLB eliminated in a 3 d period (Table 1) varied from 8×10^5 *Ent. faecalis*-FLB ml^{-1} up to 53×10^5 *Aer. hydrophila*-FLB ml^{-1}. These values represented disappearance percentages ranging from 15% to 86% respectively. The mean disappearance percentages of *Aer. hydrophila* were significantly higher ($P < 0.05$) than those obtained for the other enteric FLB. On the other hand, the mean disappearance percentages on *Ent. faecalis* and *Staph. epidermidis* were the lowest, and showed significant differences ($P < 0.05$) with *Aer. hydrophila* and *E. coli* in the two experiments.

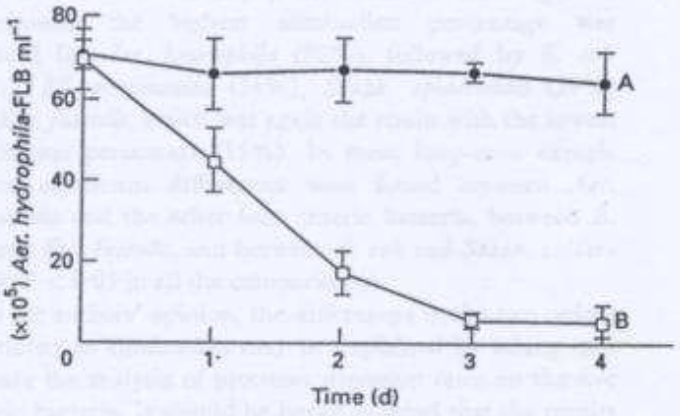

Fig. 2 Representative plot (*Aeromonas hydrophila* fluorescently-labelled bacteria (FLB), first long-term experiment) of evolution in time of enteric bacteria in absence (A) and presence (B) of natural microbiota in the long-term experiments

DISCUSSION

The results obtained in this work indicate that there is an order of priority in the elimination of the five enteric bacteria studied by bacterivorous protists.

In the long-term experiments performed with high initial densities of enteric bacteria, the disappearance percentages after 3 d varied from 15% (8×10^5 FLB ml^{-1}) in the case of *Ent. faecalis* up to 86% (53×10^5 FLB ml^{-1}) in the case of *Aer. hydrophila*. In the short-term experiments, carried out with low densities of enteric bacteria, the disappearance percentages ranged from 0·18% ($2{\cdot}10 \times 10^3$ FLB ml^{-1}) in the case of *Ent. faecalis* up to 0·71% ($3{\cdot}78 \times 10^3$ FLB ml^{-1}) for *E. coli*.

In the two types of experiments, short-term and long-term, the disappearance of enteric bacteria previously heat-killed and stained with a fluorochrome were studied, i.e. enteric FLB and not live bacteria. It should be indicated that Landry *et al.* (1991) found that the marine zooflagellate *Paraphysomonas vestita* showed higher grazing rates when fed live bacteria than when the prey was heat-killed bacteria. This phenomenon was not quantified in our experiments, so it is possible for the disappearance rates of live enteric bacteria to be higher than the values obtained with heat-killed bacteria. On the other hand, the techniques used in the two types of experiments were different. The disappearance values in the short-term experiments were estimated from the sum of elimination rates by flagellate and ciliate bacterivorous protists obtained from the technique described by Sherr *et al.* (1987), while in the long-term experiments (see Materials and Methods) the effect of the whole microbiota of the river on each enteric species was quantified. It is considered that the results on enteric bacterial elimination obtained in the two types of experiments may be compared, as flagellate and ciliate protists are known to be the most important bacterivorous protists in aquatic environments (Azam *et al.* 1983; Sieburth 1984; Porter *et al.* 1985; Sherr *et al.* 1987; Fenchel 1988), and in addition, the blanks showed the lack of importance of enteric bacterial disappearance due to viral lysis (Proctor *et al.* 1988; Bergh *et al.* 1989), bacterial lysis (McCambridge and McMeekin 1979; Guerrero *et al.* 1986), or even the loss of fluorescence of the enteric FLB as time passes.

In comparisons of the results obtained with high and low densities of enteric bacteria (taking into account the different incubation periods, 3 d *vs* 1 h), and studying the evolution of the enteric bacterial disappearance in time (Fig. 2), it was observed a noticeably higher capacity of river protists to eliminate high densities than low densities of enteric bacteria. Therefore, these results confirm the positive effect of prey density on the grazing rates by the community of bacterivorous protists, at least in the particular case of the five enteric bacterial prey studied. This relationship has been observed by a number of authors working with bacterioplankton (Jackson and Berger 1985; Sherr *et al.* 1988; Choi and Peters 1992) and with particular bacterial strains. Therefore, as Coleman (1964) working with *Escherichia coli*, Danso and Alexander (1975) with *Rhizobium*, and Mallory *et al.* (1983) with *Salmonella typhimurium* and *Klebsiella pneumoniae* observed, grazing by planktonic protists depends on prey density, and the decline rate of prey abundance diminishes with lower prey densities.

A fundamental aspect of this study is the differential elimination of the five enteric bacteria. The results showed the existence of differential elimination of bacteria by protists, as well as a different order of priority in that elimination, in the two types of experiments. In the short-term experiments, the high elimination of *E. coli* (0·71%) stands out compared to the values obtained for *Staph. epidermidis* (0·50%), *Aer. hydrophila* (0·33%) and *Kl. pneumoniae* (0·29%), while *Ent. faecalis* showed very low values (0·18%). From these results, it could be established that there were significant differences between *E. coli* and *Ent. faecalis*, and between *E. coli* and *Aer. hydrophila* ($P < 0·05$ in both cases), while there were not significant differences between *Aer. hydrophila*, *Kl. pneumoniae* and *Staph. epidermidis* ($P > 0·05$ in all cases). However, in the long-term experiments the highest elimination percentage was detected for *Aer. hydrophila* (86%), followed by *E. coli* (45%), *Kl. pneumoniae* (34%), *Staph. epidermidis* (29%) and *Ent. faecalis*, which was again the strain with the lowest elimination percentage (15%). In these long-term experiments, significant differences were found between *Aer. hydrophila* and the other four enteric bacteria, between *E. coli* and *Ent. faecalis*, and between *E. coli* and *Staph. epidermidis* ($P < 0·05$ in all the comparisons).

In the authors' opinion, the differences in the two orders of priority in elimination may be explained by taking into account the analysis of protistan digestion rates on the five enteric bacteria. It should be borne in mind that the results of the two types of experiments may be comparable with respect to the communities responsible for the elimination (see above), but the results concerning instantaneous elimination (short-term experiments) reflect differential elimination due to the ingestion process on bacteria (and possibly to the immediate egestion process also). By contrast, the long-term elimination includes ingestion and digestion of bacteria, the latter differing according to prey type. In this respect, González *et al.* (1990) detected lower values of protistan digestion rates on *Ent. faecalis* than on *E. coli*, which could explain the higher persistence of *Ent. faecalis* in aquatic systems.

The digestion rates of the flagellates, the protists mainly responsible for the elimination of those enteric bacteria, and of some ciliates were lower than the ingestion rates (Fig. 1). This indicates that the enteric bacterial elimination in time will be highly influenced by the digestion rates on those enteric bacteria. Therefore, the fact that not *E. coli* but *Aer. hydrophila* became the enteric bacteria most easily eliminated in long-term experiments may be due to the very high digestion rates of flagellates and ciliates on *Aer. hydrophila* when compared with *E. coli* and the other enteric bacteria. Regarding the values obtained for the elimination of *Staph. epidermidis* in long-term experiments, the digestion rates of flagellates and ciliates on this strain were also the lowest. The change in the order of elimination could also be explained by a possible adaptation of protists to graze upon a given type of prey in the long-term experiments. In the authors' opinion, 3 d does not seem to be a long enough period to enhance this phenomenon, but if so, this adaptation coincides with the easy digestion of the bacterial strain by the protists.

Therefore, these results reveal the importance of the digestion processes by the bacterivorous protists in order to establish a priority in the elimination of enteric bacteria in time in an aquatic system.

The order of elimination of enteric bacteria in time in aquatic systems is considerably important from the point of view of public health. With respect to *Aer. hydrophila*, it should be noted that this organism is present in human faeces in relatively small numbers. These low proportions, as well as the quick elimination by the protists of the system, indicate that its hazard is low when sewage is dumped into fluvial systems. On the contrary, *Ent. faecalis* should be considered. This opportunistic pathogen related with subacute endocarditis and urinary tract infections (Bergey 1986) is present in large numbers in human faeces and, as observed above, its elimination by bacterivorous protists is slow and difficult. Therefore, it may become highly abundant in the aquatic systems where urban sewage is discharged, and a situation with a potential health risk may be created. Moreover, taking into account the fact that *Ent. faecalis* is used as an indicator of the presence of pathogens, the phenomena reported appear highly relevant because it could distort the relative abundance of indicator organisms making interpretation of water quality data difficult.

Finally, the Gram-negative rod-shaped bacteria examined in this work were eliminated easier than the

Gram-positive coccoid-shaped bacteria. The elimination of Gram-negative bacteria at the end of 3 d ranged from 34% for *Kl. pneumoniae* to 86% for *Aer. hydrophila*, while in the case of Gram-positive cocci, it ranged between 15% and 29% for *Ent. faecalis* and *Staph. epidermidis*, respectively. A possible explanation for these results also lies in the more difficult digestion by protists of the Gram-positive studied bacteria (Fig. 1). As indicated by Taylor and Berger (1976) and Nilsson (1987), the difficult digestion of Gram-positive bacteria would be due to the complexity of the bacterial cell wall. Moreover, if the fact that the bacterioplankton is mainly constituted by Gram-negative rod-shaped bacteria is taken into account, it is possible for the bacterivorous protists, flagellates and ciliates to adapt to grazing upon this type of prey, as was already indicated by ZoBell and Upham in 1944.

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