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Factors Affecting Preference Responses of the Freshwater Ciliate *Uronema nigricans* to Bacterial Prey

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ABSTRACT. To enhance our understanding of the factors affecting feeding selectivity of bacterivorous protists in aquatic systems, we examined the preference responses of the freshwater ciliate *Uronema nigricans* towards three bacterial prey taxa, *Pseudomonas luteola*, *Serratia rubidaea*, and *Aeromonas hydrophila*. Potential factors influencing the predator–prey contact rate included the previous feeding history of the ciliate and physiological state of bacteria. Preference indexes were obtained from multiple-choice mazes in which ciliates moved preferentially towards alternative bacteria or the prey species on which they had been feeding. *Uronema nigricans* showed differential attraction towards the offered prey types, and these preferences varied as a function of the ciliate feeding history: *U. nigricans* growing on *P. luteola* showed lower preference responses towards the offered bacteria than *U. nigricans* growing on *S. rubidaea*. The bacteria in stationary phase elicited a higher degree of attraction than bacteria in exponential phase, probably due to a higher concentration of carbohydrates in the former. Therefore, this protist will preferentially swim towards bacteria in stationary growth phase, although the degree of this response will be affected by the recent feeding history of the ciliate.

Key Words. *Aeromonas hydrophila*, bacterial physiological stage, bacterivorous protist, feeding history, predator–prey interaction, preference index, *Pseudomonas luteola*, *Serratia rubidaea*.

PATCHY concentrations of dissolved and particulate organic matter in planktonic systems induce the establishment of a non-uniformly distributed bacterial community (Azam, Martínez, and Smith 1993). Chemosensory mechanisms may allow bacteria to locate and colonize the richest microenvironments, giving rise to temporal ''hot spots'' of increased bacterial abundance and growth (Blackburn, Azam, and Hagström 1997; Blackburn, Fenchel, and Mitchell 1998). Owing to these temporal and spatial changes in environmental conditions and nutrient supply, planktonic bacteria likely alternate between growth and starvation-survival stages, demonstrating different and stage-specific physiological characteristics. Growing bacteria are generally relatively large (Iriberri et al. 1987), show high specific uptake rates for organic substrates (Grossart and Simon 1998), and have high cell-specific hydrolytic activities (e.g. Agis et al. 1998). Different growth rates also involve changes in macromolecular cellular composition (Morita 1982).

Both growing and starving bacteria are potential prey for bacterivorous protists. During interactions between the bacterial prey and the protistan predator, preference towards different prey may occur at any of the main stages: approach of the grazer, capture, ingestion, and finally digestion (Jürgens and Matz 2002). Most studies concerning protist selective grazing on bacteria have focused on the ingestion processes (e.g. Sherr, Sherr, and Fallon 1987; Zubkov and Sleigh 1995), and, to a lesser extent, on the capacity to digest and assimilate the ingested prey (e.g. Boenigk et al. 2001). However, information remains scarce on the capacity and mechanisms by which protists locate and move towards bacteria. The accumulation of protists near potential bacterial prey has been commonly observed (Artolozaga et al. 2000; Blackburn and Fenchel 1999), but the factors influencing this behaviour remain unknown. The chemical composition of the prey may be a significant factor since several studies have described positive and negative chemotaxis of protists towards dissolved chemical cues (Sibbald, Albright, and Sibbald 1987; Verity 1991). An additional potential factor is the physiology and recent feeding history of the grazer, which may influence the nature of the response towards alternative prey types. Any of these factors may affect the protist recognition of prey and subsequent accumulation near prey, which in turn would influence contact, ingestion, and digestion of bacteria. Enhancing pre-ingestion recognition abilities would be advantageous for predators in increasing their capability to locate prey and to identify qualitatively different prey items. On a larger scale, such feeding behaviour could directly influence the structure of prokaryotic communities.

An important aspect in the study of feeding behaviour and preferences in protists is the methodological approach. Ideally, different bacterial prey should be simultaneously available to protists, which, after a contact period, will change their swimming behaviour as a function of the characteristics of the offered prey. This differential response may be quantified either as changes in swimming direction (Fenchel and Blackburn 1999) or motility patterns (Ricci, Micelli, and Giannetti 1987), or changes in the protistan density near the prey compared with a control (e.g. using T-mazes, capillary-based techniques, Boyden-type chambers). The latter approaches provide information regarding the consequences of differential protist swimming behaviours, and have been used to show that some planktonic ciliates may use chemosensory behaviour towards attractive or repulsive chemical or bacterial stimuli (Kohidai, Soós, and Csaba 1997; Leick and Helle 1983; Van Houten 1978; Van Houten, Hansma, and Kung 1975; Verity 1988).

In the present study, we investigated to what extent characteristics of the freshwater ciliate *Uronema nigricans*, including its feeding history, and prey characteristics, including different growth stages, influence the preference responses of the grazer as measured by migration and accumulation in multiple-choice mazes.

MATERIALS AND METHODS

Microbial cultures and analysis. Three bacterial species were used: *Pseudomonas luteola* and *Serratia rubidaea*, isolated from the Butron River (43°22′18″N, 3°5′13″W), and *Aeromonas hydrophila*, Strain CECT 839 T, a usual inhabitant of freshwater systems (Hernández and deGarcía 1997), obtained from the Spanish Type Culture Collection (Valencia, Spain). Bacteria were grown in a cereal leaves medium (CLM; Sigma-Aldrich, Madrid,

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Spain) (0.05% w/v) at 20 °C in the dark for 8–15 h to reach exponential growth. To obtain cells in stationary phase, exponentially growing bacteria were filter-concentrated (0.22 μm), washed 3 times with 0.005% (w/v) CLM, and maintained in 0.005% CLM in the dark at 20 °C for 8 days.

Bacteria were counted after acridine orange (AO) staining and filtering on black polycarbonate filters using an epifluorescence microscope (Hobbie, Daley, and Jasper 1977). To measure bacterial biovolumes, AO-stained samples were processed by a semi-automatic image analysis system, VIDS IV (Analytical Measuring Systems, Cambridge, UK). Maximum lengths and widths of at least 100 bacteria were measured. The shape of the three rod-shaped bacteria was equated to a cylinder with two hemispheric ends: bacterial biovolumes (V) were calculated as $V = (L - 0.333W)W^2\pi/4$, where L is the bacterial length and W is the bacterial width. Bacterial carbohydrate was measured as described in Johnson, Burney, and Sieburth (1981). Bacterial protein content was measured as described in Robertson, Williams, and Bada (1987). The carbohydrate or protein content in the 0.2-μm-filtered fractions was subtracted as a blank. Cell-specific concentrations were calculated by dividing protein and carbohydrate contents by the bacterial abundances and their respective biovolumes.

The differences in biovolume between the exponential and stationary phase for each bacterial species were compared using Student's t-test ($P < 0.05$). The chemical compositions of the bacterial prey in the two physiological stages were compared with two-way ANOVA, and specific differences were detected with the post hoc Student–Newman–Keuls procedure corrected for multiple comparisons ($P < 0.05$). Statistics were analysed with SPSS v.15.0 for Windows software (SPSS Inc., Chicago, IL).

Uronema nigricans is a scuticociliate common in freshwater and especially in the Butron River where it was isolated (Iriberri et al. 1995). The clonal culture of *U. nigricans* was maintained in the dark on 0.01% (w/v) CLM at 15 °C on a mixed assemblage of freshwater bacteria derived during the isolation of the clone. To analyse the influence of protistan feeding history, the procedure described by Simek, Vrba, and Hartman (1994) was followed to obtain two ciliate cultures: *U. nigricans* fed on *P. luteola* and *U. nigricans* fed on *S. rubidaea*. A well-grown maintenance culture of the ciliate was gravity filtered through 8-μm pore-size cellulose acetate filters and washed 3 times, each time with 20 ml of sterile 0.01% (w/v) CLM. A small volume of this protistan suspension, carrying a very low abundance of accompanying bacteria, was inoculated into a dense suspension of *C. luteola* or *S. rubidaea* grown in 0.05% (w/v) CLM and incubated in the dark at 20 °C until *U. nigricans* reached the stationary growth phase. This replacing procedure was repeated 3 times to ensure the substitution of most of the accompanying bacteria.

For protistan enumeration, the samples were fixed with 2% (v/v) glutaraldehyde (final concentration) and three replicates were placed in a 1-ml Sedgewick Rafter cell (Hausser Scientific, Horsham, PA). Samples were counted using a Nikon Diaphot-TMD inverted microscope (200X) (Nikon Instruments Europe B.V., Amstelveen, The Netherlands) within 24 h after sampling. The specific growth rates by *U. nigricans* growing on *P. luteola* and *S. rubidaea* were calculated from linear regression analysis of ln transformed data within the period of time where protists showed exponential growth.

Selective migration and accumulation of *Uronema nigricans* in the maze assay. Test suspensions were obtained by washing bacteria in exponential or stationary phase 3 times with 0.05% (w/v) CLM onto a 0.2-μm pore filter. Control suspensions (i.e. ciliate with accompanying bacteria) were obtained by gravity filtering 200 ml of the ciliate culture using 8.0-μm pore filters. No ciliate was detected in the filtrates. Bacterial concentrations of test and control suspensions were adjusted to 5×10^6 cells/ml by diluting the suspensions (0.05% (w/v) CLM for test and 0.2-μm filtrate of the protistan culture for controls) or by adding more bacteria. *Uronema nigricans* was cultured for 24 h to obtain cells in exponential growth phase, and concentrations were adjusted to 3,000 cells/ml (ciliates) and 5×10^6 cells/ml (bacteria). Concentrations were adjusted using sterile 0.05% (w/v) CLM, a concentrate of protists obtained by centrifugation (Iriberri et al. 1995) or a concentrate of accompanying bacteria.

The preference responses of *U. nigricans* towards different bacterial prey were determined in multiple-choice mazes by estimation of a preference index (PI). Glass multiple-choice mazes were designed as a central tube (13-mm diam., 60-mm long) with four arms (2.5-mm diam., 10-mm long), each connected to a syringe by a small silicone tube (Fig. 1). The maze was placed horizontally on a stand and the syringes were used to carefully fill three of the four side arms with 200 μl of a test suspension and the fourth with 200 μl of the control suspension, while the central tube was filled with the protistan culture. After 30 min, 1 ml of the central tube and the contents of the arms were each dispensed into separate microfuge tubes, and fixed with 2% (w/v) glutaraldehyde (final concentration). Ten different test suspensions were assayed, and 10–12 replicates were performed for each experiment.

Optimum exposure time was initially examined by running a series of experiments with *P. luteola* for 30, 60, and 90 min (Fig. 2). An exposure time of 30 min was chosen, as this was long enough to obtain a significant difference between test and control arms, but short enough to avoid significant increase of ciliate densities due to cell divisions.

The PI was used to quantify the ciliate response to the prey in the multiple-choice maze, and was defined as $PI = T/(T+C)$, where T equals the mean density of ciliates in the test arms and C equals the density of ciliates in the control arm. A $PI > 0.5$ indicates attraction, or a higher response towards the test suspension than the control suspension. A $PI < 0.5$ indicates repulsion, defined as a lower response towards the test suspension than towards the control suspension, while $PI = 0.5$ indicates that the response of the ciliate towards test and control suspensions did not differ (Van Houten, Hansma, and Kung 1975). A PI significantly differ-

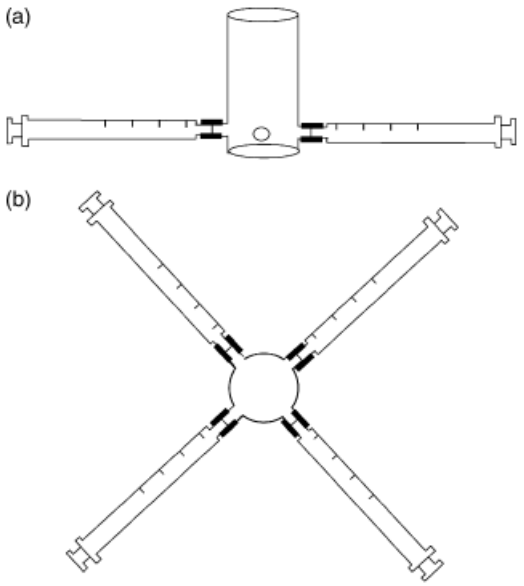

Fig. **1.** Schematic drawing of the experimental setup: side (**A**) and upper (**B**) views of the maze connected to the four syringes.

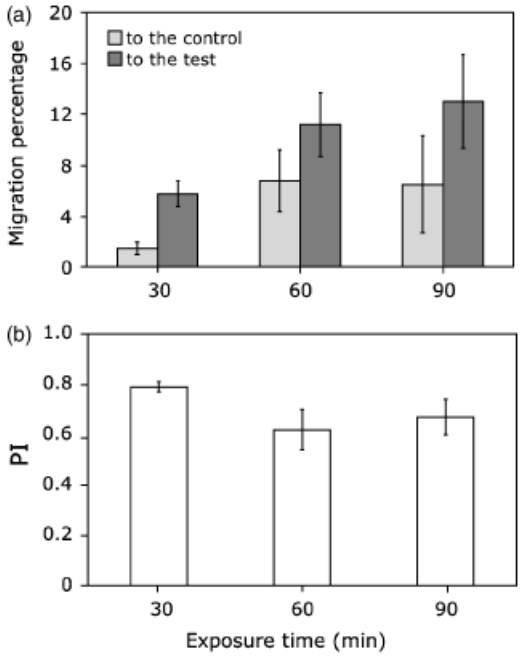

Fig. **2.** (**A**) Migration percentages (± SE) of the ciliate *Uronema nigricans* to alternative bacterial prey and to control bacteria upon which they were feeding at the time of the test. (**B**) Preference indexes (PI ± SE) obtained for the three exposure times assayed (30, 60, and 90 min).

ent from 0.5 was detected with Student's *t*-test ($P<0.05$), and detection of any significant difference in PI values among the ten experiments was performed with one-way ANOVA. Analysis of variance contrast was then used to analyse the influence of feeding history on ciliate preference for alternative or usual prey. For each feeding history, the influence of prey species and physiological state were analysed with two-way ANOVA. Significant differences among mean values was considered at $P<0.05$. Statistical analysis was performed with SPSS v. 15.0 for Windows software.

RESULTS

Both bacteria supported ciliate growth to an approximately similar extent. After 32 h, ciliate density reached 6,660/ml when fed on *P. luteola* and 7,500/ml when fed on *S. rubidaea*. Specific growth rates were also similar: 0.167/h on *P. luteola* and 0.168/h on *S. rubidaea*.

The biovolume of *P. luteola*, *S. rubidaea*, and *A. hydrophila* was 1.5–2.5-fold lower in stationary conditions compared with growth conditions ($P<0.05$) (Table 1). Significant differences in protein and carbohydrate concentrations were detected both at the species and the physiological stage levels ($P<0.01$) (Table 1). The concentrations of carbohydrates in *P. luteola* and *S. rubidaea* were similar and significantly lower ($P<0.05$) than that observed with *A. hydrophila* (Table 1). The protein concentrations were significantly higher ($P<0.05$) for *S. rubidaea* than for *P. luteola* and *A. hydrophila*, which were both similar (Table 1). In all three bacterial species, both the carbohydrate and protein concentrations were significantly higher ($P<0.008$) during the stationary stage than in the exponential growth stage, ranging between a 2.1- to 4.7-fold difference for the carbohydrate concentration and 2.5- to 4.7-fold difference in the protein concentration (Table 1).

The PI values were significantly different from 0.5 ($P<0.05$) in 80% of the cases, indicating that *U. nigricans* was able to discriminate between the prey on which it was growing (control suspension) and the alternative prey (test suspension) (Table 2). One-way ANOVA showed significant differences in PI among the 10 different predator–prey combinations ($P<0.001$). Analysis of the effect of the protistan feeding history revealed that the PI values of the ciliates fed on *P. luteola* were significantly lower ($P<0.05$) than the PI of ciliates fed on *S. rubidaea*. Additionally, in considering if the feeding history influenced the degree of preference between the usual and alternative prey, we hypothesized that protists fed on a bacterial species may show higher PI values for usual prey than for alternative prey. ANOVA contrast analysis revealed that there were no significant differences ($P>0.05$) in the PI towards usual or alternative prey for the two feeding histories.

The physiological stage of the bacterial prey clearly affected the PI for ciliates with either feeding history. *Uronema nigricans* showed significantly higher PI values ($P<0.04$ in the two cases: when growing in *P. luteola* and when growing in *S. rubidaea*) for bacterial cells in the stationary stage than for bacteria in the exponential growth stage (Fig. 3).

Table **1.** Cell biovolume and protein and carbohydrate concentration values (mean ± SE) of bacteria in growth and in stationary conditions.

		Bacterial physiological stage	
	Bacterial species	Exponential growth	Stationary
Biovolume (μm^3/cell)[a]	*Pseudomonas luteola*	0.381 ± 0.100	0.211 ± 0.050
	Serratia rubidaea	0.404 ± 0.080	0.164 ± 0.050
	Aeromonas hydrophila	0.254 ± 0.020	0.172 ± 0.010
Carbohydrate ($\times 10^{-9}$ glucose $\mu mol/\mu m^3$)[b]	*P. luteola*	0.26 ± 0.08	0.55 ± 0.15
	S. rubidaea	0.34 ± 0.07	0.86 ± 0.33
	A. hydrophila	0.54 ± 0.27	2.51 ± 0.89
Protein ($\times 10^{-10}$ glycine $\mu mol/\mu m^3$)[b]	*P. luteola*	0.62 ± 0.54	1.77 ± 0.90
	S. rubidaea	3.92 ± 1.49[c]	9.85 ± 3.05
	A. hydrophila	0.65 ± 0.32	3.05 ± 1.01

[a] $n = 100$; [b] $n = 12$; [c] $n = 10$.

DISCUSSION

The behaviour of the freshwater ciliate *U. nigricans* in its approach towards different bacterial prey was quantified by the PI,

Table **2.** Preference indexes (PI) showed by *Uronema nigricans* with a recent feeding history on *Pseudomonas luteola* and on *Serratia rubidaea* towards the three bacterial prey in different physiological stage.

			PI			
Feeding history	Physiological stage	Bacterial species	Mean	SE	*n*	Response
Pseudomonas luteola	Growing	*P. luteola*	0.66	0.04	9	+
		S. rubidaea	0.55	0.07	9	o
		Aeromonas hydrophila	0.85	0.01	9	+
	Stationary	*P. luteola*	0.71	0.05	12	+
		S. rubidaea	0.68	0.02	12	+
		A. hydrophila	0.88	0.01	9	+
Serratia rubidaea	Growing	*P. luteola*	0.67	0.04	10	+
		S. rubidaea	0.53	0.06	11	o
	Stationary	*P. luteola*	0.79	0.04	12	+
		S. rubidaea	0.94	0.02	11	+

Symbols for the response of the ciliate mean: o, indifference; +, attraction ($P<0.05$, Student's *t*-test).

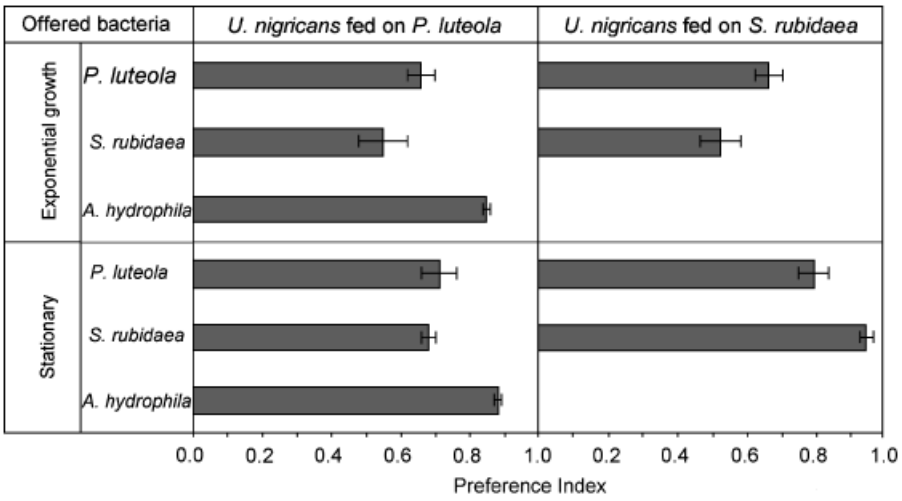

Fig. **3.** Preference indexes of the ciliate *Uronema nigricans* under each feeding history towards the three bacterial species in exponential growth and stationary phase.

an indicator of the relative migration of the grazer towards a prey compared with the migration of the grazer towards the prey on which it was grown. These protists were able to move quickly (1–1.5 mm/s) to localize resource gradients and to accumulate around prey patches (Fenchel and Blackburn 1999). Under conditions of prey density and temperature similar to those used in this work, *U. nigricans* showed bacterial ingestion rates as high as 500 cells/protist/h (Iriberri et al. 1995). We selected an exposure time of 30 min in order to allow not only prey detection and subsequent changes in the swimming behaviour of the ciliate, but also to permit direct contact between prey and grazers, enabling ingestion, digestion, and even release of bacterial debris. Here, we detected PI values higher than 0.5 in 80% of treatments, indicating that in most of the experimental treatments, *U. nigricans* was able to detect different prey types and, by changing its swimming behaviour, to move towards, accumulate around, and remain in close proximity to bacterial prey different from that with which it was grown upon.

Importantly, this response varied significantly as a function of grazer characteristics, such as feeding history, as well as prey characteristics, such as the bacterial species and its physiological state. In these experiments, the abundance of prey was kept constant as the satiated or starved nutritional state of the protists can affect their selective response (e.g. Boenigk et al. 2001). Both bacteria supported the growth of *U. nigricans* similarly, indicating that *P. luteola* and *S. rubidaea* represent good prey options for this ciliate. However, the PIs of the ciliates fed on *P. luteola* were significantly lower than the PIs of the ciliate fed on *S. rubidaea*, suggesting that preculturing the ciliate on specific bacteria affected its ability to exhibit preferences. In contrast, no differences were found for each feeding history when PIs towards alternative and usual prey were analysed, showing that the preference response to bacteria was not modulated by the feeding history of the ciliate. One possible explanation for these results may be the presence of different chemicals in the co-cultures of *U. nigricans* with either *P. luteola* or *S. rubidaea*. Hartz, Sherr, and Sherr (2008) observed that up to five chemicals were able to inhibit the chemosensory response of marine ciliate *Uronema* sp. without affecting its feeding rates on bacteria. These differing chemical environments might have altered some ciliate surface receptors involved in behavioural shifts, such as changes in swimming rate or prey recognition (Kung and Saimi 1982; Wootton et al. 2007).

The ciliates showed a higher degree of attraction for stationary phase compared with exponentially growing bacteria. The three bacteria were smaller in stationary than in growth conditions by 1.5- to 2.5-fold, but were also more concentrated in protein (between 2.5- and 4.7-fold) and carbohydrate (2.1- to 4.7-fold). Thus, a decrease in size was related to a higher concentration of biomass components, in agreement with results obtained by Simon and Azam (1989) and Gundersen et al. (2002). The cell size of the growing bacteria was not too large to be ingested, as judged from a previous study on a mixed community of scuticociliates from the Butron River (Ayo et al. 2001), which were able to efficiently ingest rod-shaped bacteria greater in size than those used in the present study. Ciliates have been reported to be able to discern among prey within a mixed community (Hamels et al. 2004; McCormick 1991), to actively reject unattractive prey (Taniguchi and Takeda 1988), and to discriminate between live and dead prey (Landry et al. 1991) or different strains of the same microalgae species (Wolfe, Steinke, and Kirst 1997).

Our results support the notion that the higher preference of ciliates for stationary phase bacteria may not be based on size but more likely on their chemical composition. In this respect, a positive relationship between the carbohydrate concentration (normalized as $1+\log$ glucose $\mu mol/\mu m^3$) and PI can be expressed as a significant linear regression:

$$\mathrm{PI} = 3,285 + 0.31(1 + \log \text{carbohydrate concentration})$$
$$[n = 10,\ P = 0.031,\ R^2 = 0.46]$$

This relation was not observed when the bacterial protein content was considered ($P = 0.87$, $R^2 = 0.004$). In spite of limitations, such as the small numbers of bacterial species, physiological states, and food histories analysed, this relationship provides evidence that the capacity of ciliates for approaching and accumulating near bacterial prey depends on the particular chemical composition of the prey. When prey biovolumes are similar, it may be beneficial for the ciliates to feed on bacteria with higher carbohydrate content, because digestion of these bacteria would provide a higher amount of easily assimilable energetic compounds. Boenigk et al. (2001) found that the fate of ingested particles for three heterotrophic nanoflagellates seemed to depend mainly on their biochemical characteristics. In this work the offered bacteria were carefully washed to eliminate any chemi-

cal that was not closely linked to the cell surface. However, there are other aspects of the washed bacterial prey, in addition to their carbohydrate content, that may have influenced the swimming behaviour of the predators and that were not addressed by Boenigk et al. (2001). Among them, we can cite differential mobility of the prey, specific composition of cell surface, or even a high production and release rate of chemical cues that would induce chemotactic responses in the predators.

In summary, the model freshwater ciliate *U. nigricans* distinguished bacterial prey according to their physiological state and preferentially accumulated near cells in the stationary phase. With similar-sized prey, the preference to migrate to and accumulate nearby appears to be related to bacterial carbohydrate concentrations or other unknown biochemical factors. The differential approach behaviour towards different prey has ecological implications: more importance must be given to the approach stage in the protist–bacteria interaction, a step regulated by the feeding history of the grazer, by the chemical characteristics of the prey, and potentially also by released dissolved compounds. Aquatic ecosystems are characterized by their heterogeneity in the spatial and temporal distribution of their components. Patches, microcolonies or bacterial prey aggregates differ depending on the physiological situation or specific composition, resulting in being more or less attractive for bacterivores and conditioning either remaining in the patch or rejection and subsequent relocation for new food sources. More information is needed concerning the protist–bacteria interaction before we reach a precise understanding and are able to make predictions of protistan grazing on bacteria, a process fundamental to understanding energy and matter fluxes through the microbial trophic web.

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