Grazing of a *Tetrahymena* sp. on Adhered Bacteria in Percolated Columns Monitored by In Situ Hybridization with Fluorescent Oligonucleotide Probes

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Predation of attached *Pseudomonas putida* mt2 by the small ciliate *Tetrahymena* sp. was investigated with a percolated column system. Grazing rates were examined under static and dynamic conditions and were compared to grazing rates in batch systems containing suspended prey. The prey densities were 2 × 10⁶ bacteria per ml of pore space and 2 × 10⁹ bacteria per ml of suspension, respectively. Postigestion in situ hybridization of bacteria with fluorescent oligonucleotide probes was used to quantify ingestion. During 30 min, a grazing rate of 1,382 ± 1,029 bacteria individual⁻¹ h⁻¹ was obtained with suspended prey; this was twice the grazing rate observed with attached bacteria under static conditions. Continuous percolation at a flow rate of 73 cm h⁻¹ further decreased the grazing rate to about 25% of the grazing rate observed with suspended prey.

A considerable proportion of the protozoans fed on neither suspended bacteria nor attached bacteria. The transport of ciliates through the columns was monitored at the same time that predation was monitored. Less than 20% of the protozoans passed through the columns without being retained. Most of these organisms ingested no bacteria, whereas the retained protozoans grazed more efficiently. Retardation of ciliate transport was greater in columns containing attached bacteria than in bacterium-free columns. We propose that the correlation between grazing activity and retardation of transport is a consequence of the interaction between active predators and attached bacteria.

Flagellates and small ciliates can control bacterial densities in many ecosystems (2). Protozoan bacterivory has been shown to reduce bacterial numbers and also to enhance bacterial activity (33) and is therefore accepted as a pivotal process in microbial food webs. Protozoan feeding has been studied intensively in pelagic habitats, but little information is available for porous systems (4, 6, 18, 21).

Although interstitial biofilms are a typical habitat for protozoans, the effect of protozoans on biofilm performance and biofilm detachment has been described only qualitatively (16, 19, 29). Protozoan feeding rates on biofilm bacteria have not been directly quantified previously. A protozoan food preference for attached bacteria has been demonstrated in several studies (5, 37, 38), but there is also evidence that adhesion has a protective effect (5, 11). These contradictory results reveal the complex but largely unknown role of attachment in food availability. The influence of interstitial water flow on bacterial predation also has only scarcely been evaluated, although such water flow could potentially control grazing efficiency. It has been proposed that interstitial flow reduces protozoan feeding, since it may cause abrasion of surface-associated tectic predators (28). In pelagic systems, hydrodynamic forces have been shown to influence the availability of suspended bacteria for various protozoan species (24, 36).

Protozoan bacterivory can be quantified by determining individual grazing rates. Processing of bacteria inside food vacuoles (34) has been monitored, and nongrazing subpopulations (23) have been identified. However, in most attempts to determine uptake of bacteria the workers used analogs of native prey (35, 41, 44) or enzymatic tracers (40, 43) to quantify vacuole contents. These approaches suffer from possible selectivity against or in favor of the artificial prey (23, 27, 35). The restrictions of such methods are even more obvious in porous habitats because of the predominance of attached bacteria, which are difficult to simulate by prey analogs (38). It is evident that the adhesion behavior of native bacteria cannot be mimicked with heat-killed, fluorescently labeled cells. Although several studies demonstrated that protozoan grazing occurs in sediments, they were not able to quantify grazing on bacteria embedded in biofilms (3, 8, 14, 17, 26). Hence, the use of unstained, viable prey is necessary to determine grazing rates in porous environments.

In this study, we investigated predation on bacteria attached to glass beads in a well-defined column system by the ciliate *Tetrahymena* sp. under lotic (percolated) and lentic (static) conditions. We describe below a new application of in situ hybridization with oligonucleotide probes. Hybridization of ingested bacteria inside the food vacuoles of the predators allowed the feeding rates on viable prey to be determined.

**MATERIALS AND METHODS**

**Organisms and culture conditions.** The hymenostomatide ciliate *Tetrahymena* sp. (9) was isolated from sediment from a Swiss prealpine river, Neckar River (7). Small volumes (<100 µl) of pore water containing *Tetrahymena* sp. and indigenous bacteria were transferred into 50 ml of sterile mineral medium containing (per liter) 2.86 g of Na₂HPO₄·H₂O, 1.46 g of KH₂PO₄, 0.1 g of MgSO₄·7H₂O, 1 g of NH₄NO₃, and 0.05 g of Ca(NO₃)₂·4H₂O. The medium was amended with 0.87 g of sodium benzoate per liter as the sole source of energy and organic carbon. During repeated transfer into fresh medium, *Tetrahymena* sp. grazed on indigenous bacteria and outcompeted all other protozoans, as determined by microscopy. Clonal polyxenic cultures of *Tetrahymena* sp. were obtained by
isolating a single individual that was transferred into 50 ml of medium. The body sizes of 64 organisms were measured microscopically. The average length and width of the ellipsoidal cells were 33.2 ± 7.8×21.4 ± 4.4 μm, respectively, corresponding to an average body volume of 8,900 ± 5,340 μm³. Cultures containing ciliates and indigenous bacteria were started 3 to 9 days before the grazing experiments were started. Fifty-milliliter portions of medium in 100-ml Erlenmeyer flasks were each inoculated with 100 μl of a stock culture, and the preparations were incubated on a rotary shaker at 25°C and 100 rpm.

_Pseudomonas putida_ mt2 (42), which has been used in several studies on bacterial adhesion (15, 31, 32), was independently cultured overnight in 500-ml portions of the mineral medium described above in 1-litre Erlenmeyer flasks on a rotary shaker at 25°C and 100 rpm. The mean cell volume, 0.73 μm³, was calculated on the basis of microscopic measurements.

### Preparation of cell suspensions

Cultures of _P. putida_ mt2 were harvested by centrifugation and washed twice in phosphate-buffered saline containing (per liter) 4.93 g of NaCl, 0.29 g of KH₂PO₄, and 1.56 g of K₂HPO₄ (PBS1). One part of the concentrate was used for predation experiments with suspended bacteria, and another part was resuspended in PBS1 and adjusted to an optical density at 280 nm of 0.5. This corresponded to a concentration of 9×10⁹ cells ml⁻¹, which was the influent bacterial concentration used for column loading.

In order to reduce the concentration of indigenous bacteria in suspensions of _Tetrahymena_ sp., 40-ml culture samples were centrifuged for 10 min at 8,000×g. The supernatants were removed, and the pellets were each carefully overlaid with 3 ml of PBS1. Within 3 h, a considerable fraction of the _Tetrahymena_ sp. cells swam from the pellets into the supernatants and simultaneously emptied their food vacuoles. The supernatants were pooled, the protozoans were counted with a Buerker counting chamber (Faust AG, Schaffhausen, Switzerland), and the suspension was diluted with PBS1 to a final density of 20,000 cells ml⁻¹. This procedure reduced the concentration of indigenous bacteria to less than 4×10⁷ cells ml⁻¹. The suspensions of _Tetrahymena_ sp. were used for grazing experiments within 2 to 3 h.

### Adhesion of bacteria

Cells of _P. putida_ mt2 were attached to glass beads by the method of Rijnarts et al. (31), which has been shown to result in nearly uniform distribution of bacteria. Briefly, water-filled columns (length, 10 cm; inside diameter, 1 cm), each with a glass frit at the lower end, were filled with glass beads (diameter, 0.45±0.50 mm). The total pore space, 2.7 ml (corresponding to a porosity of 0.34), was determined gravimetrically. Suspensions of _P. putida_ mt2 cells were added to the vertical downflow columns with a peristaltic pump (Ismatec, Glattbrugg, Switzerland) at a hydraulic flow rate of 1 ml min⁻¹. The total volume of each system, which included the pumping tube, the pore space, and a small tube at the outlet, was 3.9 ml. The residence time of the conservative tracer NaCl in the system was 12 min. During percolation of _P. putida_ mt2 through the glass, irreversible adhesion of the effluent was below the detection limit, which confirmed that irreversible adhesion of _P. putida_ mt2 to the glass had occurred (31).

Assays to study protozoan grazing in columns. _A. tetrahydromena_ sp. suspension was pumped into 35 columns, including 3 columns without bacteria, for 13 min. In the 32 columns containing bacteria, the infiltrating protozoans had a chance to feed on attached bacteria. Protozoans were transported for 3 min through the pumping tubes. Thus, the grazing times in the beds of the columns during infiltration ranged from 10 min for the protozoans entering first to 0 min for the protozoans entering last. To calculate average grazing rates, a mean grazing time of 5 min during infiltration was assumed.

Following the infiltration period, the columns were subjected to three different flow protocols. This allowed us to determine the influence of different flow periods and static conditions on the grazing rates. In five columns, the flow was continued immediately with PBS1 containing no protozoans. Three effluent fractions were obtained; these fractions contained the protozoans leaving the columns 0 to 17, 17 to 28, and 28 to 31 min after the end of the infiltration period. The first and third fractions (designated samples C1 and D1, respectively) (Table 1) were used to determine grazing rates. In 16 columns (including the 3 columns without bacteria) the flow was stopped for 10 min. Five of these columns were immediately sacrificed by emptying their contents into flasks containing 2.7 ml of fixation buffer (see below) and the protozoans designated sample b2. Percolation of the remaining 11 columns was continued with cell-free PBS1. Effluent samples were obtained at 3.7-min intervals for 22 min after percolation was started again. The second and sixth samples were used to determine grazing rates; these samples were designated samples b3 and D3, respectively. The grazing experiments performed with all three flow modes

### Table 1. Grazing rates and vacuole formation for _Tetrahymena_ sp. preying on suspended or attached bacteria

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Sample</th>
<th>T1 (min)</th>
<th>T2 (min)</th>
<th>T3 (min)</th>
<th>T1, T2, T3 (min)</th>
<th>No. of replicates</th>
<th>Columns or batches</th>
<th>Individuals</th>
<th>Grazing rate (no. of bacteria individual⁻¹ h⁻¹)</th>
<th>Vacuole vol (μm³ individual⁻¹)</th>
<th>No. of vacuoles</th>
<th>Grazing rate (no. of bacteria individual⁻¹ h⁻¹)</th>
<th>Vacuole vol (μm³ individual⁻¹)</th>
<th>No. of vacuoles</th>
<th>Active grazers only (%)</th>
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<td>a</td>
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<td>0</td>
<td>5</td>
<td>154</td>
<td>13 ± 24</td>
<td>1.1 ± 1.7</td>
<td>27 ± 28</td>
<td>4.2 ± 1.7</td>
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</tr>
<tr>
<td>a2</td>
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<td>113</td>
<td>3,045</td>
<td>370 ± 375</td>
<td>5.1 ± 5.7</td>
<td>4,575</td>
<td>557 ± 329</td>
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<td>665 ± 286</td>
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<td>110</td>
<td>618</td>
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<td>7.4 ± 5.9</td>
<td>861</td>
<td>629 ± 411</td>
<td>10.2 ± 4.4</td>
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<tr>
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<td>15</td>
<td>5</td>
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<td>299 ± 313</td>
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<td>35</td>
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<td>30</td>
<td>35</td>
<td>5</td>
<td>49</td>
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<td>21</td>
<td>36</td>
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<td>319 ± 262</td>
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<td>d3</td>
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<td>30</td>
<td>21</td>
<td>56</td>
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<td>53</td>
<td>228</td>
<td>155 ± 197</td>
<td>4.1 ± 4.9</td>
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<td>305 ± 173</td>
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<td>31</td>
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<td>27</td>
<td>354</td>
<td>155 ± 168</td>
<td>5.8 ± 8.7</td>
<td>598</td>
<td>262 ± 139</td>
<td>8.5 ± 9.3</td>
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<tr>
<td>e2</td>
<td>5</td>
<td>10</td>
<td>22</td>
<td>37</td>
<td>7</td>
<td>84</td>
<td>888</td>
<td>400 ± 407</td>
<td>5.3 ± 4.2</td>
<td>1,243</td>
<td>559 ± 377</td>
<td>7.4 ± 3.0</td>
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<td>e3</td>
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<td>30</td>
<td>22</td>
<td>57</td>
<td>7</td>
<td>126</td>
<td>317</td>
<td>219 ± 231</td>
<td>6.0 ± 4.8</td>
<td>407</td>
<td>282 ± 226</td>
<td>7.7 ± 4.0</td>
<td>78</td>
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</table>

- Sample types were distinguished as follows: sample type a, protozoans feeding on suspended bacteria in batch systems; sample type b, protozoans recovered from samples of columns with effluent; sample type c, protozoans that elucidated immediately; sample type d, protozoans with retarded elution; sample type e, protozoans retained in eluted columns.
- The total grazing time in a column (t_tota) included an infiltration period (t_1), a no-flow period (t_2), and a mean elution period (t_3).
- Active grazers were defined as individuals which contained at least one food vacuole.
were stopped after elution by sacrificing the columns and pouring their contents into small flasks (samples e1, e2, and e3). Protozoans from sacrificed columns were recovered by gently shaking the flasks and removing 2-ml-subsamples. Preliminary experiments showed that all fixed protozoans were recovered by this procedure. All samples were fixed immediately with an equal volume of fixation buffer (4.4% [wt/vol] formaldehyde and 150 μl of 1 M NaOH per liter in buffer containing [per liter] 7.60 g of NaCl, 1.25 g of NaH₂PO₄, and 0.41 g of Na₂HPO₄ [PBS2]). Two-milliliter portions were then placed in Eppendorf vials and stored at 4°C overnight. The protozoan densities of all samples were determined with a Nageotte counting chamber (Faust AG).

Assay to study protozoan grazing in bacterial suspensions. In addition to the column experiments, grazing also was investigated in batch experiments. Six-milliliter portions of the suspension of *Tetrahymena* sp. used in the column experiments were transferred into glass tubes. At the same time that grazing was started in the columns, 100- to 300-μl portions of the washed *P. putida* mt2 concentrate were added to a final concentration of ca. 2.0 × 10⁸ cells ml⁻¹. The tubes were manually shaken every 5 min to keep the cells in suspension. Samples (0.8 ml) were taken at zero time and after 10, 30, and 60 min (samples a1 to a4) and occasionally 120 min after the bacteria were added. They were fixed with the same volume of 4.4% fixation buffer and stored before they were used for in situ hybridization.

In situ hybridization. Two milliliters of each sample of protozoans was washed twice in PBS2 by using a table centrifuge (10 min, 16,000 × g). The pellets were each resuspended in 0.3 ml of PBS2, an equal volume of ethanol was added, and

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**FIG. 1.** In situ hybridization of oligonucleotide probe EUB 338 with *P. putida* subjected to predation by *Tetrahymena* sp. (a) Grazing ciliates (bright light). (b) Cells before predation (green excitation). (c through f) Cells 5, 30, 60, and 250 min, respectively, after predation of a suspension containing 5 × 10⁸ bacteria ml⁻¹. (g) DAPI staining (blue excitation). (h) Same microscopic field as microscopic field in panel g but with green excitation of EUB 338. Bars = 30 μm. Panels b through h are shown at the same magnification. Adobe Photoshop was used to capture the images.
the samples were stored at −20°C. For in situ hybridization of ingested bacteria, the eubacterial 5′-fluorescein-linked oligonucleotide probe EUB 338 (MWG Biotech, Ebersberg, Germany) was used. Thawed samples were centrifuged and resuspended in 20 to 500 μl of PBS2, depending on the protozoan density. Drops (10 μl) were placed on agarose-coated glass slides. The slides were air dried for 30 min at 45°C to attach the protozoans, and then the residual agarose was removed by treatment for 9 min with increasing aqueous concentrations of ethanol (50, 70, and 100%). Hybridization was performed at 45°C for at least 2 h in 10 μl of buffer (0.9 M NaCl, 20 mM Tris-HCl, 0.05% sodium dodecyl sulfate, 30% formamide) containing an oligonucleotide probe at a concentration of 67 ng μl−1. The hybridization chambers used were plastic vials that contained a piece of absorbent paper soaked with hybridization buffer to prevent drying of the slides. The hybridization mixture was removed, and sample plots were covered for 20 min at 45°C with another 10 μl of hybridization buffer and for 10 min at 4°C with distilled water. In some cases the samples were counterstained for 10 min with 0.0001% (wt/vol) DAPI (4′-6-diamidino-2-phenylindole-dihydrochloride). Finally, the samples were treated with 8 μl of anti-fading solution (Citrafluor Ltd., London, United Kingdom).

The protozoans were observed with an Olympus model BH 2 microscope at a magnification of ×1,250 by using an interference filter set at a wavelength of 495 nm (type BP 495 exciter filter; type 515 IF barrier filter; type DM505 dichroic mirror; Olympus AG).

Calculation of grazing rates. Counting single bacteria inside the food vacuoles was not feasible since the bacteria were too densely packed. Therefore, great numbers were inferred from the vacuole volumes. The diameters of the generally spherical vacuoles were measured with a calibrated grid projected onto a slide. Up to 30 protozoans from each sample were analyzed in this way. Grazing rates (q) (in number of bacteria per individual per hour) were calculated by using the ratio of the total food vacuole volume of a grazer (Vp) (in cubic micrometers) to the volume of a bacterial cell (Vb) (in cubic micrometers) at a concentration of 67 ng μl−1 (in number of bacteria per individual per hour) were calculated by using the ratio of the total food vacuole volume of a grazer (Vp) (in cubic micrometers) to the volume of a bacterial cell (Vb) (in cubic micrometers) at a concentration of 67 ng μl−1. A bacterial concentration of 2.0 × 108 cells ml−1 (mean ± standard deviation) of the influent concentration. The bacterial concentrations, after individual columns were loaded, ranged from 1.28 × 108 to 2.55 × 108 cells ml−1 of pore space−1. After the initial breakthrough, the protozoan densities in the effluents of columns with attached bacteria ranged from 7 to 13% of the influent concentrations, regardless of whether the flow had been stopped for 10 or 30 min. A total of 19% ± 16% of all protozoans were eluted during the experiments. When the eluted columns were sacrificed, another 46% ± 18% of the retained protozoans were recovered, whereas 36% ± 25% of the protozoans were lost. The total recovery rate, 64% ± 24%, equals the recovery rate of 64% ± 22% obtained for the protozoans from the columns which were sacrificed before elution. This indicates that the loss of protozoans occurred during infiltration, possibly in the peristaltic pump. In bacterium-free columns, the level of retention of protozoans was much lower. While 50% ± 8% of the individuals appeared in the effluent, only 12% ± 3% were retained in the columns and 38% ± 13% were lost.

Visualization of ingested bacteria. Food vacuoles inside Tetrahymena sp. were clearly visible after hybridization of the ingested bacteria (Fig. 1). The number and volume of the spherical vacuoles increased with grazing time. The diameters of individual vacuoles ranged from 1.5 to 8.0 μm. Intact shapes of ingested bacteria could still be observed more than 35 min after ingestion. After 2 h of grazing, fluorescent label was visible inside the predator cytoplasm, indicating that egestion of the vacuole contents had occurred. Staining with DAPI resulted in weak blue fluorescence of the ingested bacteria.

Grazing activities. Table 1 summarizes the grazing activities of Tetrahymena sp. on suspended and glass-associated bacteria. At the beginning of the experiments, the grazers contained almost no ingested bacteria (Table 1, sample a1). Indigenous bacteria were not ingested, as shown by the controls without added food. The highest grazing rate was observed after 10 min of predation in batches containing suspended bacteria (sample a2). Subsequently, the grazing rate declined rapidly, as shown by the insignificantly larger vacuole volumes after 30 and 60 min of grazing (samples a3 and a4). The fraction of active grazers increased only slightly from 66% after 10 min to 72% after 60 min. In order to directly compare a complete Tetrahymena sp. population feeding on suspended cells, some columns were sacrificed before elution (samples b1 and b2). The fractions of grazing individuals in the column system were slightly lower than the fraction of grazing individuals in the bath system, and the absolute grazing rates were considerably reduced when the prey was glass associated prey (compare samples b2 and a3).

Clearly different grazing rates were observed with the protozoans that were immediately eluted with the flowing liquid (samples c1 through c3), the protozoans that passed through the columns with a delay (samples d1 through d3), and the protozoans that remained in the columns even after prolonged elution (samples e1 through e3). Very low grazing rates and fractions of active grazers of only 15 to 18% were obtained with the conservatively transported protozoans, and the intermediate no-flow period (samples c1 through c3) did not have much influence. The mean number of vacuoles and the total vacuole volumes of immediately eluted active grazers were similar to those of individuals in the column influents (compare samples c1 through c3 with sample a1). The fraction of active grazers was considerably smaller than the fraction in the column influents, indicating that selective retention of feeding organisms occurred. Clearly higher grazing rates were observed for protozoans which were transported with a delay. These organisms grazed for 35 min during continuous percolation (sample d1), but grazed more efficiently when static conditions were provided for 10 min (sample d2). When the flow was stopped for 30 min (sample d3), the total vacuole volume of active grazers did not increase further, and the fraction of active grazers stayed nearly constant.

The grazing rates and the numbers of vacuoles of retained organisms increased considerably when a no-flow period of 10 min was used (compare samples e1 and e2). A prolonged no-flow period did not result in further increases. The mean vacuole volumes even decreased when the no-flow period was extended to 30 min before elution and protozoan recovery (sample e3). This may have been due to digestion that led to a reduction in the vacuole volume. Generally, the fraction of actively grazing individuals was as high as the fraction obtained with suspended prey (compare samples e1 through e3 with samples a1 through a4). It should be noted, however, that the percentages of active grazers in the column systems refer to only the ca. 65% of the individuals which could be recovered.

DISCUSSION

In situ hybridization. Postingestion in situ hybridization is a powerful method for monitoring ciliate predation on viable bacteria. We have demonstrated that this method is valuable in predation experiments performed with attached bacteria, since...
it overcomes a possible drawback of prelabeling of prey, the influence of the labeling procedure on bacterial viability, and adhesion behavior. In situ hybridization with specific oligonucleotide probes would offer the additional possibility of investigating the prey preference of predators. Binding of fluorescently labeled oligonucleotides to bacterial target RNA has been used previously to characterize endosymbionts in anaerobic ciliates (1), to examine natural assemblages of protists (20), and to monitor flagellate grazing on bacterial suspensions (30) or biofilms (16).

Direct counting of incorporated bacteria was not feasible since food vacuoles are three-dimensional, densely packed organelles. We therefore decided to infer grazing rates from vacuole volumes. It is obvious that premature processing of vacuoles could lead to underestimates of ingested bacteria. In the present experiments, the shapes of intact single bacteria could still be distinguished inside the food vacuoles after 35 min of grazing, which is the duration of continuous uptake of bacteria by Tetrahymena sp. (10, 25). Typically, distinct phases of prey ingestion, digestion, and defecation can be observed (34). After 1 h of grazing, the vacuole number became constant and the vacuole volumes decreased, which indicated that digestion was beginning. This was accompanied by the appearance of fluorescent label in the ciliate cytoplasm. Hence, vacuole volume appears to be an adequate measure of the number of ingested bacteria in grazing experiments less than 1 h long.

**Availability of attached bacteria.** The attached mode of living can protect bacteria from predation (5, 11), but may also increase grazing rates on attached bacteria were 1 order of magnitude lower (3). Our grazing rates of up to 880 attached bacteria individual\(^{-1}\) h\(^{-1}\) in river sediments have been calculated on the basis of protozoan growth and reductions in bacterial numbers (5). Our grazing rates of up to 880 attached bacteria individual\(^{-1}\) h\(^{-1}\) are the first values obtained for a clearly defined porus system. Our maximum grazing rate corresponds to a cleared collector surface of 3.420 \(\mu\)m\(^2\) individual\(^{-1}\) min\(^{-1}\) when a uniform distribution of bacteria is assumed. This is an area slightly larger than the cell surface of the ciliate. Tetrahymena sp. lacks characteristics of surface-associated tectic protozoans and seems to be adapted to a pelagic environment. However, it is able to feed on attached prey, although less efficiently than on suspended prey.

**Transport and grazing behavior of Tetrahymena sp. in a porous medium.** Water flow is an important factor for protozoan predation in porous systems (13). Presumably, grazers reach sites with elevated numbers of bacteria by passive transport and grazing behavior. Insects lack characteristics of surface-associated tectic protozoans and seem to be adapted to a pelagic environment. However, it is able to feed on attached prey, although less efficiently than on suspended prey.

**References**


