Digested of bacteria by the freshwater ciliate *Tetrahymena pyriformis*

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ABSTRACT: The digestion of heat-killed/stained *Escherichia coli*, *Pseudomonas aeruginosa*, *Mesorhizobium* sp. and *Staphylococcus aureus* was monitored within one food vacuole passage time in the ciliate *Tetrahymena pyriformis* using the pulse chase technique. Prey digestion proceeded in 2 phases: a digestive phase which lasted ~25 min and a defecation competent phase that showed limited digestive activity and was variable in length. The number of prey cells per food vacuole was found to influence the effectiveness of prey digestion. Complete digestion of the vacuole content was more likely to occur when the number of prey per vacuole averaged ~6 or less. At higher levels, only partial digestion of the vacuole content was recorded and some undigested prey were egested from the ciliate cell. A strain of *Synechococcus* sp. was never digested by this ciliate. Results suggest that bacteria do not necessarily require elaborate mechanisms to evade digestion by protozoa, as possessed by some pathogenic bacteria, but that inefficiency in the protozoan digestive system is all that is required to allow the release of undigested, apparently unharmed prey from their cells. Thus, models on carbon cycling which employ data on protistan ingestion rates alone should consider accounting for digestion efficiency and the subsequent effect of prey concentration, because prey carbon might not always be transferred efficiently to higher trophic levels.

KEY WORDS: Protozoa · Ingestion · Egestion · Phagosome · Feeding-history

INTRODUCTION

Protozoa are important bacterivores in aquatic environments, making a significant contribution to the transfer of bacterial carbon to higher organisms (Azam et al. 1983) and in the regeneration of inorganic nutrients (Johannes 1964). However, this predator-prey relationship is complex as some bacteria can prevent normal digestive processes within protozoan food vacuoles (Abu Kwaik et al. 1998, Strahl et al. 2001), whilst others can undergo the full digestive process and emerge apparently unharmed (Schlümme et al. 1995, 1997, Pickup et al. 2007, Dillon & Parry 2009). This latter phenomenon is poorly understood and may be a result of an active response by bacteria, as it is for pathogens (Abu Kwaik et al. 1998), or selective or inefficient digestion by protozoa (González et al. 1990).

Prey digestion has been assessed using the pulse chase technique (Sherr et al. 1988, González et al. 1990, 1993, Dolan & Coats 1991, 2008, González & Suttle 1993, Iriberri et al. 1994, Dolan & Šimek 1997, 1998, Gunderson & Goss 1997, Jürgens & Šimek 2000, Hammer et al. 2001, Jezbera et al. 2005, Myung et al. 2006). This methodology permits protozoa to be ‘pulsed’ with a fluorescent prey, allowing for the formation of labeled food vacuoles, and then the suspension is ‘chased’ by dilution to significantly reduce further labeled vacuole formation. The number of fluorescent prey/vacuoles within the protozoan cells is then monitored over time to observe vacuole processing and prey loss. The use of indigestible prey allows the total lifespan of vacuoles to be identified, i.e. from vacuole formation to vacuole ‘egestion’: a period known as the vacuole passage time (VPT). As no egestion of vacuolar content occurs within the VPT, any loss of potentially digestible prey during this period is interpreted as being due to digestion only. Beyond the identified VPT, any observed loss of prey might be due to a combination of digestion and egestion.
Fok et al. (1982, 1984) proposed that distinct phases of digestion and non-digestion occur within the VPT of the ciliate *Paramecium caudatum*. The length of the initial digestive phase was found to be constant (~21 min) and was divided into vacuole formation, acidification and condensation, and lysosomal fusion. The defecation competent phase, where vacuoles exhibit no digestion and are waiting to be expelled, was found to be highly variable. Defecation competent vacuoles have also been noted in the raptorial ciliate *Litonotus lamella*, after a digestive phase of 1 h (Verni & Gualtier 1997). These data suggest that the digestive phase might be constant for a given ciliate but that the defecation competent phase is variable, reasons for which have yet to be proposed.

The present study examined the processing of food vacuoles in the freshwater ciliate *Tetrahymena pyriformis*. The length of the VPT was determined from the processing of 2 indigestible prey, i.e. fluorescent microspheres and cells of the freshwater picocyanobacterium *Synechococcus* sp. The ability of this ciliate to digest 4 species of heat-killed, fluorescently-labeled bacterial cells was then evaluated to determine the length of the digestive phase, whether differential digestion of the 4 prey occurred and whether ciliate feeding history affected digestion rates.

**MATERIALS AND METHODS**

**Ciliate and prey maintenance and preparation.** *Tetrahymena pyriformis*: *T. pyriformis* (Culture Collection of Algae and Protozoa [CCAP] 1630/1W) was grown for 3 d in Chalkley’s medium (CCAP) supplemented with live *Mesorhizobium* sp. (formally bacterium B1, Eccleston-Parry & Leadbeater, 1994) at 20°C. Cultures were centrifuged at 185 × g for 10 min, incubated overnight at 20°C and acclimated to 23°C for 2 h prior to experimentation at this temperature. *Mesorhizobium* sp. concentration (‘background bacterium’) was determined by 4’,6-diamidino-2-phenylindole (DAPI) staining and epifluorescence microscopy (UV excitation; BP340-380 nm/LP430 nm filter block) (Porter & Feig 1980).

**Indigestible prey:** Suspensions of carboxylated fluorescently-labelled microspheres (yellow, 0.49 or 0.92 µm ± 0.01 [SE] diameter or blue 0.48 µm ± 0.01 diameter) (Fluoresbrite™ Polysciences) were prepared in the freshwater ciliate *T. pyriformis* culture. The mixture was diluted 1:1000 in Chalkley’s medium supplemented with live ‘invisible’ *Mesorhizobium* sp. at 3.01 × 10⁷ cells ml⁻¹ to replace the initial bacterial and fluorescent marker prey concentrations. The fluorescent prey was thus decreased (<0.1% of total prey) and this reduced further uptake by the ciliate. Sub-samples were removed at defined time intervals (200 µl pre-dilution and 1 ml post-dilution) and fixed in 1% (final v/v) ice-cold glutaraldehyde. Cells were viewed with epifluorescence microscopy (magnification ×1250), and even though glutaraldehyde possesses an inherent autofluorescence (Sanders et al. 1989), prey were easily visualised within the food vacuoles. Between 20 and 30 ciliate cells were examined for each replicate sample and the number of fluorescent vacuoles cell⁻¹ recorded. Controls were included to monitor the level of residual ingestion of fluorescent prey after the chase, following González et al. (1993). Non-exposed *T. pyriformis* cultures were incubated with fluorescent prey and live *Mesorhizobium* sp. at equivalent concentrations to those present in the test suspensions after dilution. Sub-samples were removed, fixed and the number of labeled food vacuoles cell⁻¹ was enumerated in the same way as in the test cultures.

**Multiple pulse chase experiments.** To observe the order in which food vacuoles are processed inside...
Synechococcus sp. or blue microspheres at 2 \times 10^5 \text{ particles ml}^{-1} to allow formation of blue primary food vacuoles. The culture was then diluted 10-fold into Chalkley’s medium containing yellow microspheres at 2 \times 10^6 \text{ particles ml}^{-1} for 5 min to allow formation of yellow secondary food vacuoles. At 10 min, the culture was diluted 1:40 into Chalkley’s medium containing live Mesorhizobium sp. at 2 \times 10^6 \text{ cells ml}^{-1} to produce tertiary ‘invisible’ vacuoles. The persistence of yellow and blue vacuoles was monitored using 2 methods. (1) Fixed method: sub-samples were removed and fixed with ice-cold glutaraldehyde (1% final v/v) at predetermined intervals. Between 20 and 30 cells in each replicate were examined for the presence of yellow and/or blue vacuoles using epifluorescence microscopy (magnification \times1250). (2) Live monitoring: from previous experiments, the start of egestion was expected at some point 30 min after the initial feed, so at 30 min, 20 \mu l of the culture was placed on a microscope slide with 10 \mu l of 10% Pluronic F-68 solution (Sigma). Carefully, 3% alginic acid sodium salt (Fluka) was added to slow ciliate motility and cause egested food vacuoles to ‘cling’ to some of the ciliates’ cytoproct. Pluronic F-68 prolonged the life-span of cells within the viscous alginate (Hellung-Larsen et al. 2000). The cells were observed with epifluorescence microscopy (magnification \times500) to deduce the order in which vacuoles were egested from cells and whether 2 vacuoles fused and were ‘egested’ simultaneously.

**Digestion experiments.** Pulse-chase experiments were performed using different concentrations of DTAF-stained *Escherichia coli* K12, *Mesorhizobium* sp., *Pseudomonas aeruginosa* and *Staphylococcus aureus*. In each case, controls employing an equivalent concentration of an indigestible prey ran alongside, to identify the length of the VPT. Cultures were pulsed for 5 min then diluted 1:200 in Chalkley’s medium supplemented with live unstained counterpart. Microspheres and *Synechococcus* sp. cells were replaced with *Mesorhizobium* sp. Samples were removed, fixed and 20 to 30 cells per replicate were viewed with epifluorescence microscopy as described above. Both the number of prey cell$^{-1}$ and number of vacuoles cell$^{-1}$ were recorded. Controls were included to monitor the level of residual ingestion after dilution.

**Effect of ciliate feeding history.** *Tetrahymena pyriformis* was pre-fed either live *Mesorhizobium* sp., *Synechococcus* sp. or blue microspheres at 2 \times 10^5 \text{ particles ml}^{-1} for 5, 15 and 30 min prior to performing a pulse-chase experiment with DTAF-stained *Mesorhizobium* sp. Those populations fed with *Mesorhizobium* sp. were considered well fed for 5, 15 and 30 min and would contain different numbers of food vacuoles containing live digestible prey prior to the pulse chase experiment. Those fed with *Synechococcus* sp./microspheres were considered starved for 5, 15 and 30 min and would contain different numbers of food vacuoles containing live/inert indigestible prey. A *T. pyriformis* culture, which had not been pre-fed and would contain the least number of food vacuoles prior to experimentation, acted as the control. Each of the pre-fed populations (with their pre-existing food vacuoles), were immediately subjected to dilution (1:10) into Chalkley’s medium containing fluorescent *Mesorhizobium* sp. at 2 \times 10^7 \text{ cells ml}^{-1}, together with non-fluorescent *Mesorhizobium* sp. to replace the background bacteria (1.35 \times 10^7 \text{ cells ml}^{-1}). After 5 min, the cultures were diluted (1:50) in Chalkley’s medium supplemented with live *Mesorhizobium* sp. at 3.47 \times 10^7 \text{ cells ml}^{-1}. The persistence of the fluorescent *Mesorhizobium* sp. was monitored over time and the digestion rate (prey vacuole$^{-1}$ min$^{-1}$) was deduced within 1 VPT, with the VPT being defined as the time the first pre-existing fluorescent food vacuole (containing microspheres or *Synechococcus* cells) was lost from the cells.

**Interpretation of data.** Graphs were plotted of prey cell$^{-1}$ and vacuoles cell$^{-1}$ against time (min) and linear regression analysis of the increasing slopes between time (t) = 0 and the point of dilution yielded ingestion rates and vacuole formation rates, respectively. The length of VPT was deduced from the onset of the loss of the indigestible particles from the cells. Prey egestion rates were determined from linear regression analysis of the decreasing slope of indigestible particles cell$^{-1}$ beyond the VPT. Vacuole ‘egestion’ rates were also determined this way, although strictly speaking, vacuoles are not egested from the cells (only their contents), as vacuole membrane is recycled to make new vacuoles (Allen & Fok 1980).

In digestion experiments, for each ciliate cell, the number of prey cell$^{-1}$ was divided by the number of vacuoles cell$^{-1}$ to give the number of prey vacuole$^{-1}$, and the rate of digestion was calculated from the linear decline in prey vacuole$^{-1}$ from the point of dilution (5 min) to 25 min. Although rates of digestion can be expressed as loss of prey cell$^{-1}$, its use to evaluate differential digestion is only valid if the different prey give rise to equivalent numbers of vacuoles. If some prey promotes higher numbers of vacuoles, then higher rates of digestion may be calculated, and this is misleading as it is a result of simultaneous digestion in all the vacuoles. The loss of prey vacuole$^{-1}$ provides a better indication of differential digestion between prey types, but these data are difficult to correct for residual ingestion. Generally a high dilution minimises residual ingestion, but each experiment must be considered on its own merit.

The overall fate of enclosed prey within food vacuoles was characterised as being (1) not digested (ND),
(2) partially digested (PD) or (3) totally digested (TD) within 1 VPT, using the following set of criteria, respectively: if there was no loss in both prey cell–1 and vacuoles cell–1, the vacuole contents were considered to be ND. If there was a loss in prey cell–1, but no loss of vacuoles cell–1, this indicated that the vacuole contents were PD and the number of fluorescent food vacuoles remained constant due to the presence of some undigested fluorescent prey within them. A loss of both prey cell–1 and vacuoles cell–1 indicated the vacuole contents were TD, and because of this, food vacuoles became ‘invisible’.

All linear regression analyses were performed on each triplicate data set using SPSS statistics package 15.0. These rates were then averaged to produce mean rates. Differences between means were detected by one-way ANOVAs and further identified by post-hoc Tukey’s test or Student’s t-test.

RESULTS

Processing of food vacuoles

The nature of food vacuole processing in *Tetrahymena pyriformis* was investigated using indigestible prey so the full life-span of the vacuoles could be monitored. Fig. 1 shows an example of the results obtained for this ciliate feeding on either yellow microspheres or *Synechococcus* sp. cells (as described in ‘Materials and methods’). During the pulse, vacuole formation rates were 0.231 ± 0.005 (SE) and 0.444 ± 0.017 vacuoles cell–1 min–1 for microspheres and *Synechococcus* sp. cells respectively, which are significantly different (p = 0.003). At 20 min, the culture was diluted 1:1000 into Chalkley’s medium containing ‘invisible’ *Mesorhizobium* sp. Although this significantly reduced the uptake of residual fluorescent prey, data were still corrected (Fig. 1 dotted lines). The number of fluorescent vacuoles cell–1 remained relatively constant for a further 10 min (Fig. 1 vertical hashed line) before the onset of linear egestion. The VPT was thus identified as being 30 min; however, VPTs did differ between experiments and ranged from 25 to 55 min. Because of this, all digestion experiments included an indigestible prey to identify the length of the VPT.

Linear egestion (Fig. 1 dotted lines) suggests that food vacuoles are processed in an orderly manner, and this was confirmed in the multiple-chase experiment. Fig. 2a shows the percentage of ciliate cells containing (1) no fluorescent vacuoles, (2) blue vacuoles only, (3) yellow vacuoles only or, (4) both blue vacuoles and yellow vacuoles, throughout the experiment. The ciliates

![Fig. 1. *Tetrahymena pyriformis*. Processing of vacuoles containing non-digestible prey. Prey were incubated at a concentration of 2 × 10^7 prey ml–1. Arrow shows 1:1000 dilution. Vacuole passage time: time when vacuole number starts to decreases linearly (vertical hashed line). Both observed (unbroken lines) and corrected data for residual ingestion (broken lines) are shown. Error bars: SE](image)

![Fig. 2. *Tetrahymena pyriformis*. Multiple pulse chase experiment. Ciliates were incubated with blue microspheres for 5 min, followed by a 1:10 dilution and pulsed with yellow microspheres. At t = 10 min, the culture was diluted 1:40 and fluorescent prey replaced with *Mesorhizobium* sp. (a) % ciliates containing only blue vacuoles, only yellow vacuoles, both blue vacuoles and yellow vacuoles or those devoid of fluorescent vacuoles. (b) persistence of blue, yellow and total vacuoles over time (error bars: SE)](image)
were initially fed with blue microspheres for 5 min and ~90% of the cells ingested this prey, forming blue primary vacuoles (Fig. 2a). At 5 min, the culture was diluted in Chalkley’s medium containing yellow microspheres and by 10 min, 85% of the cells contained both yellow secondary vacuoles and blue primary vacuoles (Fig. 2a). At 10 min, the culture was diluted 1:40 with live *Mesorhizobium* sp. to produce ‘invisible’ tertiary vacuoles, and although these contained mainly *Mesorhizobium* sp. cells, they did contain a low level of fluorescent prey due to residual ingestion following the chase. However, these vacuoles were easily identified and their persistence in the cell was not monitored.

The vacuole profile of the ciliate population (~80% both, 15% yellow only, 5% none, Fig. 2a) and the number of fluorescent vacuoles cell⁻¹ (Fig. 2b) remained relatively unaltered until 40 min. We take this to indicate the end of the VPT and the start of egestion sometime between 40 and 50 min. At this point, the % cells containing both vacuole colours started to decrease while those containing only yellow vacuoles increased, suggesting that blue were being ‘egested’ and that some cells, which originally contained both vacuole colour types, now only contained yellow vacuoles (Fig. 2a). This pattern continued until the % cells with no fluorescent vacuoles began to increase sometime between 80 and 90 min, indicating that yellow vacuoles were now being ‘egested’, some 40 min after the first release of blue vacuoles. The VPT for the younger yellow vacuoles was therefore 75 to 85 min: 35 min longer than the VPT of the older blue vacuoles (40 to 50 min). This shows that vacuole ‘egestion’ was an orderly process and that the yellow vacuoles were queuing behind the blue vacuoles before they could be ‘egested’ from the cytoproct.

Another feature common to all the experiments was that vacuole ‘egestion’ rates were slower than their corresponding formation rates (e.g. Fig. 1, Table 1), due to the cells not being in steady-state within batch culture experiments. Vacuole ‘egestion’ rates were also variable with initial prey concentration, provided the vacuoles contained the same prey type, i.e. microspheres (Table 1), where the average vacuole ‘egestion’ rate was 0.046 ± 0.026 (SE) vacuoles cell⁻¹ min⁻¹. However, ‘egestion’ rate can vary if the vacuoles contain different prey and the ‘egestion’ rate of microsphere-containing vacuoles (0.049 ± 0.002 vacuoles cell⁻¹ min⁻¹) was significantly lower than the ‘egestion’ rate of *Synechococcus* sp.-containing vacuoles (0.137 ± 0.004 vacuoles cell⁻¹) (Fig. 1); the reason for this variation is currently unknown. Even so, vacuole content did not appear to affect the duration of the VPT, as all experiments have yielded equivalent VPTs when both microspheres and *Synechococcus* cells are used (e.g. see Figs. 1 & 5a).

**Defining the length of the digestive phase within food vacuoles**

Fig. 3 shows an example of a *Tetrahymena pyriformis* pulse-chase experiment that employed yellow microspheres (to define the VPT) and 2 potentially digestible bacteria (*Staphylococcus aureus* and *Pseudomonas aeruginosa*). The 1:200 dilution at 5 min reduced further ingestion of fluorescent prey, and residual ingestion did not yield rates which significantly changed the data, so the data were not corrected for (as in Fig. 1). The VPT was 40 min in this experiment (Fig. 3a), so the loss of digestible prey was only monitored between 5 and 40 min, when egestion was known not to be occurring. Prey loss cell⁻¹ was not

<table>
<thead>
<tr>
<th>Microsphere concentration (prey ml⁻¹)</th>
<th>Microsphere diameter (µm)</th>
<th>Ingestion rate (prey cell⁻¹ min⁻¹)</th>
<th>Vacuole formation rate (vacuole cell⁻¹)</th>
<th>Prey egestion rate (prey cell⁻¹ min⁻¹)</th>
<th>Vacuole egestion rate (vacuole cell⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 × 10⁶</td>
<td>0.49</td>
<td>ND</td>
<td>0.12 ± 0.01</td>
<td>ND</td>
<td>0.03 ± 0.00</td>
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<tr>
<td>4.5 × 10⁶</td>
<td>0.49</td>
<td>ND</td>
<td>0.23 ± 0.01</td>
<td>ND</td>
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<td>7.3 × 10⁶</td>
<td>0.49</td>
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<td>0.29 ± 0.02</td>
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<td>1.0 × 10⁷</td>
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<td>ND</td>
<td>0.25 ± 0.03</td>
<td>ND</td>
<td>0.09 ± 0.01</td>
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<td>1.0 × 10⁷</td>
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<td>2.58 ± 0.32</td>
<td>0.41 ± 0.05</td>
<td>0.12 ± 0.01</td>
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<td>2.0 × 10⁸</td>
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<td>0.64 ± 0.03</td>
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</tr>
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</table>
uniform; there was an initial linear loss of both *P. aeruginosa* and *S. aureus* until 25 or 30 min, respectively (Fig. 3a). Beyond this, no loss of prey was evident. After ~25 min, we consider the vacuoles to be inactive and awaiting egestion from the cell (Phase 2, Fig. 3). Thus, all future calculations of prey digestion rate in this study used only data from the chase to the end of the digestive period (25 min), so the chase occurred early (at 5 min) to allow 20 min worth of data for linear regression analysis.

**Effect of ciliate feeding history**

*Tetrahymena pyriformis* was pre-fed either live *Mesorhizobium* sp. (digestible and non-fluorescent), *Synechococcus* sp. or microspheres (both indigestible but fluorescent) for 5, 15 and 30 min prior to a pulse chase experiment where the digestion of fluorescent *Mesorhizobium* sp. cells was monitored. The control culture was not pre-fed. The length of the pre-feeding period gave rise to different numbers, and contents, of pre-existing food vacuoles in the ciliate cells prior to the pulse-chase experiment, but only those containing fluorescent prey could be enumerated (Table 2). A 5 min pre-feed gave rise to 1.5 to 2.2 pre-existing food vacuoles cell–1, while 30 min yielded 5.2 to 6.5 pre-existing food vacuoles cell –1 (Table 2). The time at which the first of these fluorescent food vacuoles was lost from the cells yielded the VPT, i.e. 55 min. This was also considered to be the VPT of cells pre-fed with invisible *Mesorhizobium* sp. cells.

These pre-fed cells were immediately pulsed for 5 min with fluorescent *Mesorhizobium* sp. cells, chased and then digestion rates (prey vacuole –1 min–1) determined over a 25 min period (Fig. 4: begins at 5 min, the point of dilution). The first point to note is that the 3 pre-feeding regimes did not alter the rate at which food vacuoles were formed (Table 2), but vacuoles of pre-fed cells contained significantly more fluorescent *Mesorhizobium* sp. cells than non pre-fed cells (control) (Fig. 4, Table 2). Secondly, no matter what the ciliate had been pre-fed with or the duration of the pre-feeding, there was an immediate loss of fluorescent *Mesorhizobium* sp. within the food vacuoles (Fig. 4) suggesting that newly formed food vacuoles are immediately presented with digestive machinery, i.e. fusion with acidosomes and lysosomes. Thirdly, the digestion rates of fluorescent *Mesorhizobium* sp. were equivalent in all ciliate cultures except for those pre-fed with an indigestible prey for 30 min (Table 2). These were significantly higher (p ≤ 0.01) due to a ‘burst’ of rapid digestion over the first 5 min (Fig. 4a,b), suggesting that an elevated level of digestive machinery was being presented to new vacuoles after 30 min of starvation.
Tetrahymena pyriformis was presented with 6 different prey (2 indigestible and 4 potentially digestible) (Fig. 5a) and the ingestion rates for all prey were equal, except for Synechococcus, which was ingested at a significantly faster rate (p < 0.009) (Table 3). The 1:200 dilution at 5 min reduced further ingestion of fluorescent prey, and residual ingestion did not yield rates which significantly changed the data. The number of microspheres and Synechococcus cells ciliate⁻¹ remained level between 5 and 30 min (Fig. 5a) indicating a VPT of 30 min. The number of bacteria vacuole⁻¹ declined linearly from 5 to ~25 min (Fig. 5b), and the rates of digestion for Escherichia coli K12, Mesorhizobium sp. and Pseudomonas aeruginosa (Gram negative bacteria) were not significantly different from each other but were significantly faster than for Staphylococcus aureus (Gram positive bacterium) (p < 0.05), which was hardly digested at all (Table 3).

### Table 2. Tetrahymena pyriformis. Ingestion and digestion rates ± SE of DTAF-stained Mesorhizobium sp. (fluorescently-labeled bacterium: FLB) following 10 different pre-treatments. Fluorescent bacterium was incubated with the ciliate at 2 × 10⁷ prey ml⁻¹ for 5 min before dilution. *p < 0.05: significantly different from control (no pre-feeding). ND: not determined

<table>
<thead>
<tr>
<th>Initial prey type</th>
<th>Incubation time (min)</th>
<th>No. of vacuoles cell⁻¹</th>
<th>Time of observed egestion (min)</th>
<th>No. of FLB ingested cell⁻¹ in 5 min</th>
<th>No. of FLB vacuoles cell⁻¹ in 5 min</th>
<th>No. of FLB vacuole⁻¹</th>
<th>Initial rate of FLB loss (prey vacuole⁻¹ min⁻¹)</th>
</tr>
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<tr>
<td>Synechococcus</td>
<td>30</td>
<td>6.5 ± 0.3</td>
<td>55</td>
<td>20.2 ± 1.5*</td>
<td>2.3 ± 0.1</td>
<td>8.88 ± 0.5*</td>
<td>0.161 ± 0.005*</td>
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<td>Synechococcus</td>
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<td>55</td>
<td>17.6 ± 1.2*</td>
<td>2.5 ± 0.1</td>
<td>7.01 ± 0.5*</td>
<td>0.127 ± 0.013</td>
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<td>Synechococcus</td>
<td>5</td>
<td>2.2 ± 0.1</td>
<td>55</td>
<td>22.0 ± 1.1*</td>
<td>2.9 ± 0.2</td>
<td>7.65 ± 0.1*</td>
<td>0.135 ± 0.016</td>
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<td>Microspheres</td>
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<td>7.02 ± 0.2*</td>
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<td>Microspheres</td>
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<td>2.5 ± 0.1</td>
<td>5.74 ± 0.2*</td>
<td>0.112 ± 0.013</td>
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<tr>
<td>Mesorhizobium</td>
<td>30</td>
<td>ND</td>
<td>ND</td>
<td>13.8 ± 0.9</td>
<td>2.5 ± 0.2</td>
<td>5.33 ± 0.1*</td>
<td>0.082 ± 0.007</td>
</tr>
<tr>
<td>Mesorhizobium</td>
<td>15</td>
<td>ND</td>
<td>ND</td>
<td>16.2 ± 0.5*</td>
<td>2.9 ± 0.2</td>
<td>5.40 ± 0.2*</td>
<td>0.081 ± 0.005</td>
</tr>
<tr>
<td>Mesorhizobium</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>14.0 ± 0.7</td>
<td>2.5 ± 0.2</td>
<td>5.50 ± 0.3*</td>
<td>0.098 ± 0.005</td>
</tr>
<tr>
<td>No treatment</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>10.7 ± 0.7</td>
<td>2.7 ± 0.2</td>
<td>3.64 ± 0.2</td>
<td>0.090 ± 0.003</td>
</tr>
</tbody>
</table>

### Classification of prey digestibility

Tetrahymena pyriformis was presented with 6 different prey (2 indigestible and 4 potentially digestible) (Fig. 5a) and the ingestion rates for all prey were equal, except for Synechococcus sp., which was ingested at a significantly faster rate (p < 0.009) (Table 3). The 1:200 dilution at 5 min reduced further ingestion of fluorescent prey, and residual ingestion did not yield rates which significantly changed the data. The number of microspheres and Synechococcus sp. cells ciliate⁻¹ remained level between 5 and 30 min (Fig. 5a) indicating a VPT of 30 min. The number of bacteria vacuole⁻¹ declined linearly from 5 to ~25 min (Fig. 5b), and the rates of digestion for Escherichia coli K12, Mesorhizobium sp. and Pseudomonas aeruginosa (Gram negative bacteria) were not significantly different from each other but were significantly faster than for Staphylococcus aureus (Gram positive bacterium) (p < 0.05), which was hardly digested at all (Table 3).

### Rates of bacterial prey digestion

Fig. 6 shows examples of the persistence of fluorescent prey ciliate⁻¹ and fluorescent vacuoles ciliate⁻¹ from the chase (at 5 min) to the end of the VPT, which was 35 min (Fig. 6 a,c) or 50 min (Fig. 6b), as determined with microspheres (data not shown). Fig. 6a
shows that *Synechococcus* sp. cells ciliate\(^{-1}\) and fluorescent vacuoles ciliate\(^{-1}\) remained constant throughout the VPT, indicating that this prey was ND during one VPT. Fig. 6b shows that *Mesorhizobium* sp. cells ciliate\(^{-1}\) declined, but the number of fluorescent vacuoles ciliate\(^{-1}\) remained constant, indicating that the vacuolar contents were only PD within 1 VPT. Finally, Fig. 6c shows a loss in both *Mesorhizobium* sp. cells ciliate\(^{-1}\) and fluorescent vacuoles ciliate\(^{-1}\), indicating TD of vacuolar contents within 1 VPT. In the latter 2 examples, the ingestion of a single prey species resulted in 2 classifications, i.e. PD and TD. The main difference between these 2 experiments was that at 5 min, the ciliate food vacuoles in one system (Fig. 6b) contained ~11 prey while those in the other system (Fig. 6c) contained ~5. The effect of prey vacuole\(^{-1}\) on digestibility was examined further using this classification scheme (Fig. 7), and results suggest that there is increased likelihood that all vacuole-enclosed cells are digested if the average number of prey vacuole\(^{-1}\) is ~6 or below. Despite various attempts with very high concentrations of *Pseudomonas aeruginosa*, food vacuole content never exceeded ~6 cells per vacuole, therefore, complete digestion always occurred. Conversely, attempts to achieve <6 *Synechococcus* sp. cells vacuole\(^{-1}\) failed, so this prey type was always recorded as never being digested.

**DISCUSSION**

Protozoa are known to be major grazers of bacteria in aquatic environments, yet only factors that affect prey ingestion have been thoroughly examined to date (Montagnes et al. 2008). Far less is known about the post-ingestion phase even though some studies have indicated that differential digestion might be just as influential as selective ingestion in shaping the nature of bacterial communities (Sherr et al. 1988, González et al. 1990, 1993, Iriberri et al. 1994).

The present study examined the post-ingestion phase of *Tetrahymena pyriformis* and proposes that food vacuole processing is orderly and that egestion proceeds at a defined, linear rate from a single site, i.e.

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![Fig. 5. *Tetrahymena pyriformis*. Loss of prey within 1 vacuole passage time. Initial concentration of all prey types = 2 \(\times\) 10\(^8\) prey ml\(^{-1}\). At 5 min, suspensions were diluted 1:200. Digestion is identified between the point of dilution and the start of microsphere and *Synechococcus* sp. egestion at 30 min (hashed lines). (a) Number of prey ciliate\(^{-1}\), (b) shows decline of prey vacuole\(^{-1}\) from 5 min. Error bars: SE](image)

<table>
<thead>
<tr>
<th>Prey</th>
<th>Biovolume (µm(^3))</th>
<th>Ingestion rate (prey cell(^{-1}) min(^{-1}))</th>
<th>Average prey vacuole(^{-1}) at 5 min</th>
<th>Vacuole formation rate (vacuole cell(^{-1}) min(^{-1}))</th>
<th>Average vacuole cell(^{-1}) at 5 min</th>
<th>Prey loss (prey vacuole(^{-1}) min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> K12</td>
<td>0.20 ± 0.03</td>
<td>6.63 ± 0.19</td>
<td>33.17 ± 0.97</td>
<td>1.15 ± 0.01</td>
<td>5.77 ± 0.13</td>
<td>0.116 ± 0.010</td>
</tr>
<tr>
<td><em>Mesorhizobium</em> sp.</td>
<td>0.17 ± 0.01</td>
<td>7.42 ± 0.16</td>
<td>37.09 ± 0.81</td>
<td>1.15 ± 0.03</td>
<td>5.75 ± 0.13</td>
<td>0.114 ± 0.010</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>0.27 ± 0.01</td>
<td>6.59 ± 0.56</td>
<td>32.97 ± 2.82</td>
<td>1.18 ± 0.05</td>
<td>5.88 ± 0.14</td>
<td>0.091 ± 0.015</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>0.31 ± 0.02</td>
<td>8.09 ± 0.28</td>
<td>40.35 ± 2.69</td>
<td>0.93 ± 0.03</td>
<td>4.63 ± 0.13</td>
<td>0.024 ± 0.012</td>
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<tr>
<td>Microspheres</td>
<td>0.41 ± 0.02</td>
<td>6.81 ± 0.41</td>
<td>34.05 ± 2.07</td>
<td>0.65 ± 0.01</td>
<td>3.27 ± 0.13</td>
<td>–</td>
</tr>
<tr>
<td><em>Synechococcus</em> sp.</td>
<td>0.74 ± 0.02</td>
<td>10.07 ± 0.53*</td>
<td>55.20 ± 3.66</td>
<td>1.07 ± 0.05</td>
<td>5.35 ± 0.15*</td>
<td>–</td>
</tr>
</tbody>
</table>

*p < 0.05

Table 3. *Tetrahymena pyriformis*. Comparison of ingestion, vacuole formation and digestion rates, as prey loss vacuole\(^{-1}\) min\(^{-1}\) (± SE) for the ciliate feeding on 6 prey types (at 2 \(\times\) 10\(^8\) prey ml\(^{-1}\)) and chased at 5 min with a 1:200 dilution. Prey loss vacuole\(^{-1}\) are net values after correcting for non-digestible prey loss over 25/30 min.
the cytoproct, for a given particle at a given temperature. Previous studies on prey/vacuole loss from protozoan cells have recorded both a linear decline (Sherr et al. 1988, González et al. 1990, 1993, González & Suttle 1993, Hammer et al. 2001, Myung et al. 2006) and an exponential decline (Dolan & Coats 1991, Dolan & Šimek 1997, 1998, Jürgens & Šimek 2000, Jezbera et al. 2005), with the latter implying that food vacuoles fuse together and are egested simultaneously (Dolan & Coats 1991). Although vacuolar fusion has been observed in *Euplotes woodruffi* (Dolan & Coats 1991) and *E. vannus* (Dolan & Coats 2008), we have never observed this in *T. pyriformis*. In addition, the multiple chase experiment showed that vacuoles were ‘egested’ in the same order they were formed, suggesting that vacuole processing is orderly and there is no ‘short circuiting’ of the system. We do however acknowledge that loss of prey/vacuoles cell⁻¹ may sometimes appear exponential, and propose that this is due to inherent variation in the feeding rates of individual cells within a given population: a feature reported in other short-term feeding studies (Dolan & Šimek 1997, Jürgens & Šimek 2000, Dolan & Coats 2008). For example, if by the time of the chase, 50% of the ciliate population contained 2 vacuoles and 50% contained 4 vacuoles, and if each cell ‘egested’ vacuoles in a linear manner, the rate of decline in vacuoles cell⁻¹ for the population as a whole would be linear until all ciliate cells had ‘egested’ 2 vacuoles. After this, only 50% of the population would continue to ‘egest’ the remaining 2 vacuoles; thus, the average loss rate of vacuoles cell⁻¹ for the population as a whole would be halved, giving the impression of an exponential decline. Thus, orderly, ‘linear’ egestion could give rise to both graphical forms. Orderly processing could also explain why the VPT of *T. pyriformis* cells varied between experiments: a feature which has been observed in other studies (Fok et al. 1982, 1984, Fok & Shockley 1985). Although ciliate cultures were prepared in a consistent manner prior to experimentation, there could still be inherent variation with regards to the number of pre-existing food (invisible) vacuoles present within the cells. Thus, the observed variation in VPT might be governed by the extent to which fluorescent vacuoles (in the pulse-chase experiment) have to queue behind invisible pre-existing vacuoles before they are ‘egested’.

Studies on *Paramecium caudatum* have shown that the defecation competent phase is variable whilst the digestive phase is fixed at 21 min (Fok et al. 1982, 1984, Fok & Shockley 1985). The present study concurs, with *Tetrahymena pyriformis* exhibiting a constant digestive phase of ~25 min, yet possessing a variable VPT. Our prey digestion rates were therefore calculated during this 25 min digestive period, but other studies have monitored prey loss after a prolonged pulse period, and observations have probably been made during times that exceeded both the digestive phase and the VPT of the protozoan concerned (Sherr et al. 1988, González et al. 1990, 1993, Dolan & Coats 1991, González & Suttle 1993, Dolan & Šimek 1997, 1998, Gunderson & Goss 1997, Jezbera et al. 2005, Myung et al. 2006). Their observed prey losses might therefore be due to a combination of both digestion and egestion. For example, Gunderson & Goss (1997) moni-

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**Fig. 6.** *Tetrahymena pyriformis*. Prey classification depending upon prey and associated vacuole loss during vacuole passage time (VPT). (a) No digestion of prey (*Synechococcus* sp.) during a 35 min VPT. (b) Partial digestion of *Mesorhizobium* sp. during a 50 min VPT. (c) Total digestion of *Mesorhizobium* sp. during a 35 min VPT. Error bars: SE.
tored the disappearance of fluorescent *Escherichia coli*-containing food vacuoles from *Tetrahymena* sp. cells after a 3 h pulse phase at 25°C. They observed a more rapid ‘loss’ of vacuoles within the first 30 min, followed by a slower rate that continued for 90 min. We can now interpret this first rate to be a result of both digestion and egestion of the prey, while the second rate is probably due to egestion only. This not only supports our findings of a ~25 min digestive phase in *Tetrahymena* sp. food vacuoles, but also demonstrates that true digestion rates, and the duration of the digestion phase, can be determined from such data provided the frequency of data points is high enough, or the digestive phase is long enough, to discern the 2 rates. Using this rationale, we could, for example, propose that the digestive phase within food vacuoles of *Halteria grandinella* is ~1 h based on the data presented in Fig. 2A by Jürgens & Šimek (2000). This is slower than the 21 and 25 min recorded for *P. caudatum* (Fok et al. 1982, 1984) and *T. pyrifomis* (the present study), but experiments were performed at different temperatures (16, 25 and 23°C, respectively), and temperature has been shown to affect the rate of food vacuole formation (Lee 1942) and digestion/egestion (Sherr et al. 1988). Temperature was maintained at 23°C for all experiments during the present study.

The duration of these digestive phases may appear short, but data suggest that for *Tetraymena pyriformis* at least, newly formed food vacuoles are immediately presented with biochemical machinery for the digestion of prey. This agrees with the work of Mueller et al. (1965) who also found evidence for immediate digestion in *T. pyriformis* vacuoles, together with *Paramecium multilicronucleatum*, and with Peck & Hausmann (1980) and Hausmann (2002) working with *Pseudomo-

![Graph](image-url)

**Fig. 7. Tetrahymena pyriformis.** Effect of prey vacuole –1 on the extent of digestion within the food vacuole. Prey classified as either totally digested, partially digested or not digested. A higher number of prey vacuole –1 resulted in less complete digestion for each prey type with the exception of Synechococcus sp. which was not digestible. Error bars: SE.

The intensity of the presented digestive machinery, as measured by the digestion rate of *Mesorhizobium* sp. by *T. pyriformis*, appeared to be independent of ciliate feeding history except when cells were pre-fed an indigestible prey for 30 min. In these cases, there was a ‘burst’ of rapid digestion of *Mesorhizobium* sp. over the first 5 min after the chase, suggesting that an elevated level of digestive machinery was being presented to new vacuoles after a period of induced starvation. There are 2 hypotheses that might explain how this was achieved.

1. *There is evidence that the social amoeba Dictyostelium discoideum* (Souza et al. 1997, Rupper et al. 2001) and macrophages (Oh & Swanson 1996) can detect vacuolar content and make adjustments to vacuole trafficking, so in the present study digestive machinery might have been immediately re-directed from the vacuoles containing indigestible prey to those newer ones containing digestible prey.

2. *Fok & Paeste (1982) showed that levels of enzyme activity in 2 Paramecium spp. were increased under nutrient stress; thus, T. pyriformis may have up-regulated digestive enzyme activity in response to the prolonged feeding on indigestible prey.*

Both theories are currently being studied with *T. pyriformis*, but whatever the mechanism, results imply that feeding history might significantly influence food vacuole processing and digestive efficiency. It would have been interesting to compare how other pre-feeding regimes, such as using different bacterial prey or an axenically-grown ciliate culture, might have affected vacuole processing, but although important, this was not in the remit of the current study and constitute future work.

When non-starved *Tetrahymena pyriformis* was presented with a range of prey, the 3 Gram negative bacteria were digested at equivalent rates. However, this may be an artefact of the method used to prepare these prey as they were all heat-killed and stained, making them more equivalent. Although Sherr et al. (1987) showed that stained prey could sustain protozoan growth rates equal to their unstained counterparts, other work has challenged this assumption (Landry et al. 1991, Parry et al. 2001), and stained *Staphylococcus aureus* cells have been shown to be more difficult to digest than their live counterpart in *Paramecium* sp. (Mehlis & Hausmann 1990). It is fully anticipated that if the prey species tested here had been live, differential digestion might have been recorded, as demonstrated by Jezbera et al. (2005). However, for the present study, the use of heat-killed
bacteria has allowed an evaluation of the duration of the digestive phase in *T. pyriformis* and the ciliate’s ability to digest bacteria in the absence of an active bacterial response to resist digestion.

Only 1 Gram positive bacterium was included in this study (*Staphylococcus aureus*), but we could speculate that reduced digestion, compared to Gram negative strains, is attributed to differences in cell wall structure as suggested by Nilsson (1987), González et al. (1990) and Iriberri et al. (1994), with Gram positive cell walls being thicker than those of Gram negative cells. *Synechococcus* cells, although Gram negative, have even thicker cell walls (Goleczi 1977) and this may have contributed to their resistance to digestion, not only in *T. pyriformis*, but also in amoebae (Pickup et al. 2007, Dillon & Parry 2009). Vacuoles containing <6 *Synechococcus* sp. cells vacuole⁻¹ proved difficult to achieve, but if this had been possible, it may have changed the overall outcome of digestion, as observed with the other bacterial prey. With these, results suggest that if the number of prey vacuole⁻¹ remained below a certain threshold (~6), the enclosed bacteria were more likely to be digested. Above this threshold, digestion was less efficient and intact bacterial cells could be egested from the cell.

This latter observation demonstrates that bacterial cells do not necessarily require elaborate mechanisms to evade digestion in protozoan food vacuoles, as possessed by some pathogenic bacteria (e.g. Abu Kwaik et al. 1998). Inefficiency in the protozoan digestive system might be all that is required to allow the release of un-digested, ‘apparently unharmed’ prey from their cells. However, these emergent prey might possess different characteristics to their un-ingested counterparts, which would cause them to interact differently within the microbial food web. For example, emergent cells of pathogenic bacteria such as *Legionella pneumophila* possess an increased level of pathogenicity (Cirillo et al. 1994) and increased resistance to antimicrobials (Barker et al. 1992, 1995). Emergent cells of the non-pathogenic *Escherichia coli* K12 have been shown to possess different genotypes due to increased levels of conjugation in *Tetrahymena pyriformis* food vacuoles (Schlimme et al. 1997). Recently, it has been hypothesized that the acidic nature of protozoan food vacuoles induces temperate phage within lysogenised enter the lytic cycle; thus, emergent cells would be in the irreversible process of cell lysis (Clarke 1998, Parry et al. 2006). These few examples show that interactions between protozoa and prey, at the food vacuole level, are complex. They deserve further investigation to elucidate how protozoan predators might enhance the survival, replication and distribution of bacteria in situ and contribute to bacterial succession and evolution. In addition, the potential inability of protozoa to digest every prey cell ingested has implications for carbon cycling, and those mathematical models that employ data on protistan ingestion rates alone should consider accounting for digestion efficiency and the subsequent effect of prey concentration, as prey carbon might not always be transferred efficiently to higher trophic levels.

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