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ORIGINAL ARTICLE

A simplified experimental model for clearance of some pathogenic bacteria using common bacterivorous ciliated spp. in Tigris river

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Abstract Bacteria-specific uptake rates of three different protozoan taxa on a pure and mixed bacterial community was studied by means of a simplified and functionally reproducible experimental model. The bacterial species *Shigella flexneri*, *Escherichia coli* and *Salmonella typhi* were isolated and classified from stool samples of patients suffering from diarrhea. *Paramecium caudatum*, *Tetrahymena pyriformis* and *Halteria grandinella*, free living ciliate Protozoans, were isolated and identified from Tigris river water. Pure and mixed (*E. coli* + *S. typhi*), (*E. coli* + *Sh. flexneri*) bacterial cultures were used with each ciliate genera to evaluate the following: predator duplication rate, prey reduction rate, clearance rate and net grazing rate. We used selective lactose fermentation phenomena of enteric bacteria on MacConkey medium for the quantification of bacteria cultural characteristics. The final bacteria concentration was reduced by growing protozoa of 98–99.9 % compared to protozoa-free controls. It showed that *Tetrahymena pyriformis* had the highest duplication rate (4.13 time/day) in both types of cultures (pure and mixed), followed by *Paramecium caudatum* and *Halteria grandinella,* respectively. *Paramecium caudatum* had the highest rate of ingestion in both types of cultures (26×10^3 bacteria/organism/hr) and yielded the longest time required for 90 % bacterial reduction in a pure suspension of *S. typhi* (166 h). Clearance rates of pathogenic bacteria by ciliates ranged between 106 nanoliter/organism/h by *P. caudatum* to *S. typhi* and 1.92 nanoliter/organism/h seen in *T. pyriformis* in (*E. coli* + *S. typhi*) mixed culture. We used aquatic experimental microcosms under controlled conditions to explore bacteria-dependent ciliate growth and examined whether these ciliates could discriminate between equally sized bacterial preys in a mixture.

Keywords Ciliates · Enteric bacteria · Clearance rate · Tigris · Bacterivourous

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Introduction

The interactions of protozoa and bacteria perhaps represent the oldest predator–prey relationships we may study in nature (Jurgens and Gude 1994). Therefore, the understanding of the micro-ecosystem and the relationship between protozoa and disease-causing bacteria within contaminated water has become increasingly important in the context of freshwater purification and pollution control design. Among the most important factors controlling bacterial communities in polluted river water is the ciliates' ability to graze on a variety of bacterial species (Jennifer et al. 2008; Kathol et al. 2009; Ana et al. 2010); they can partly control bacterial standing stock abundance by preferentially removing dividing cells (Simek et al. 1997). Diverse ciliates exhibit different feeding types; some of them up taking suspended bacteria, while some preferably feed on surface-binding bacteria. It appears that different protistan species possess a variety of selection mechanisms which may be important for discriminating between food and non-food objects, rather than between different bacterial strains, (Boenigk et al. 2002) and implies that different bacteria are probably ingested and metabolized at

different rates. This finding is deduced from laboratory studies of protozoa feeding which have shown significant taxon-specific differences in feeding mechanisms and selectivity (Fenchel 1986). The impacts of ciliate grazing on bacterial communities and prey selectivity are based on the complex interplay of several factors. These include grazing differences in the sensitivity of bacterial species to grazing, differences in responses of single bacterial populations to grazing (size flexibility and physiology) (Jan Jezbera et al. 2005), and also other impacts are direct and indirect influence of grazing on bacterial growth conditions (modifying, e.g., substrate supply) and bacterial competition via elimination of competitors. Additionally, other studies have revealed other non-morphological traits such as release of defense metabolites or toxins, changes in prey size, motility, charge and hydrophobicity that affect bacterial vulnerability to protozoan feeding (Pohnert et al. 2007).

However, quantitative studies on the pathogenic bacteria grazing rates by freshwater ciliates are few, because usage of the present means (e.g., microscopy) for the identification of prey types is limited and time-consuming. It is still unknown whether or not human harmful bacteria grazing rates by bacterivorous ciliates respond in a similar manner as natural bacteria grazing rates. Here, we describe studies designed to investigate protistan bacteria uptake of some of the different Enterobacteriaceae species that are routinely found in polluted influents, in semi-natural micro-environments, which are monitored by using differential lactose fermentation on MacConkey agar.

The experiments were designed to test two hypotheses:

(1) Freshwater ciliate assemblages form bacterial grazing morphs in the presence of pathogenic bacteria.
(2) The effects of bacterivorous ciliates are important in the clearance rate of bacterial polluted water.

Materials and methods

Bacterivorous ciliate isolation

Freshwater samples were taken directly from 0.2 m depth in 500 ml 7 cm diameter orifice bottles precleaned with dilute HCl, or by using PUF polyurethane foam as described in (Muqi and Wood 1999), from the pelagic zone of Tigris river, located in the beach of Baghdad University (Jaderyah location), Baghdad, Iraq. Samples were collected from the river at 2–3 days intervals from May to September; the water temperature was 13–20 °C. The samples were processed within 2 h of sampling. Ciliate species used in these experiments were isolated as single cells. After ciliate isolation by the dilution method (Fenchel 1982) and their classification according to (Jahn et al. 1980; Kudo 1954), their culturing occurred in a covered, sterile 500 ml flask containing 300 ml of sterile (15 min, 121 °C, 15 lb/in^2) autoclaved river water, with growing bacteria. Prior to the introduction of protozoans into the grazing experiments, 4–10 days old ciliate cultures grown at room temperature (around 20 °C) were taken from maintenance cultures, gently concentrated by centrifugation and washed five times with 20 ml of autoclaved bacteria-free (0.2-µm-pore-size filters) tap water supplied with antibiotic (penicillin) to remove most of the original bacteria present in the maintenance cultures and to ensure the absence of interfering coliform (no aberrant bacteria colonies observed on MacConkey medium).

Maintenance of bacterivorous protozoans

All protozoa were cultured in bacterised water of the river. Ciliates were cultured and maintained on an uncharacterized assemblage of 0.5–2 µm-long rods. Cocci natural bacteria that isolated from Tigris river water grew as suspensions, maintained by their culturing on yeast extract agar and incubating at 25 °C for 48 h to reach the exponential phase. These bacteria were inoculated into ciliate vessel, to maintain protozoan life, before doing experiments.

Pathogenic bacteria strain isolation

150 stool samples of patients suffering from diarrhea who went to the Central Child Hospital and Central Health Laboratory in Baghdad from October 2000 to May 2001 were processed to isolate O157:H7 Strain of *E. coli, S. typhi* and *Sh. flexneri.* The bacterial species were diagnosed by using physical characteristics, Epi20E system and biochemical and serological tests. Pathogenic bacteria suspensions were maintained by growing on nutrient broth (Difco Laboratories) at 37 °C.

Experimental design

Diarrhea-causing species of bacteria were harvested at exponential culture phase, centrifuged (5,900×*g*, 20 min), washed and suspended in 10 ml of 0.45 µm filtered Tigris river water in 30 ml screw-capped tubes in duplicate (initial average bacterial concentration, 3×10^6 to 6×10^6 cells/ml). Culturing of autoclaved or roughly filtered river water was compared with 0.45 µm filtered water samples on MacConkey medium to check the efficacy to exclude the putative effect of endogenous coliform. There were no significant differences (data not shown). To reduce the count of indigenous bacteria in ciliate inocula, 40 ml of protozoan suspension was washed with autoclaved river

water + antibiotics and centrifuged three times for 10 min at 2,000–5,000 rpm according to their size. The pellets were carefully overlaid with 3 ml autoclaved river water, counted with a Buerker counting chamber and then re-suspended to reach the desired concentration.

Ciliates were added to 30 ml vessels as 10 ml ciliate culture suspension and allowed to acclimate for 3–4 h prior to prey introduction. Then, pure and mixed prepared bacterial suspensions were inoculated into the vessel and incubated at 25 °C with shaking (30 times/min) for up to 384 h.

Protozoan grazing evaluation in pure and mixed bacterial feed

In pure culture, prey (bacteria) and predator (bacterivorous protozoan) counting was done by sampling in triplicate according to a time series ranging from 0 to 384 h (depending on the grazer species). Aliquots of 1 ml were collected at each 0, 24, 48, 72, 120, 144, 168, 192, and 384 h time point after the time of prey addition. The number of non-grazed living bacteria as well as control bacteria count was monitored as distinctive colonies on the Enterobacteriaceae selective medium (MacConkey agar) by culturing under experimental conditions with time. The predators were enumerated by slide chamber counting under light microscope. To estimate individual protozoan grazing, we divided the uptake rates of bacteria (evaluated by MacConkey culture) by ciliate abundance (monitored by hematocytometer).

For evaluation of nonspecific grazing, the particular ciliate culture was inoculated with *E. coli, S. typhi or Sh. flexneri* singly and colony forming unit (CFU) numbers were determined directly by counting the lactose-fermented colonies (*E. coli* is a lactose fermenter and has pink color colonies on MacConkey agar), when incubated at 37 °C for 24 h.

An assay of species-selective grazing in mixed cultures was done, by ciliate exposure to certain concentrations of two bacteria combinations (*E. coli* + *S. typhi*) or (*E. coli* + *Sh. flexneri*). Bacterial mixtures were added to bacterivorous organism's suspension vessel and incubated at 25 °C for the aforementioned time series. At each time point, a particular volume was removed from the reactor vessel for direct protozoan count by cytometer slide counting, while mixed enteric bacteria were enumerated by their differential lactose fermentation on MacConkey agar (*E. coli* have pink colonies, while *S. typhi* or *Sh. flexneri* are non-lactose fermented and have white colonies).

Parameter calculation

1. The percentage of grazing rate was calculated according to the equation:

$$\begin{aligned}\%\ \text{grazing rate} &= 100 \\ &\times [\text{CFU on control plate (without the predator)}] \\ &- [\text{CFU on test plate (with the predator)}] \\ &/[\text{CFU on control plate (without the predator)}].\end{aligned}$$

2. Daily duplication rate (time/day) $R = \frac{\log B - \log A}{\log 2}$ where A is the number of ciliates on day1 and B the number of ciliates on day 2 (Curds and Vandyke 1966).
3. Ingestion rate (bacteria/ciliate/h) $I.R = \frac{No - Nt}{t(Hr)} \times \frac{1}{Np}$ where $N0$ is the prey (bacteria) count at zero time, Nt the prey count at (t) time and Np the count of bacterivorous ciliates (Carrias et al. 1996).
4. Clearance rate (nanoliter/ciliate/h) $CR = IR/Ci \times 10^6$ (CR: clearance rate, IR: ingestion rate, Ci: concentration of prey; Pfister and Arndt 1998).

Statistical analysis

All presented results were analyzed as experimental points, obtained in triplicate and indicated as means of significance levels that were determined by Student's t test analysis at 95 % confidence interval (CI). The analysis was done using Graph-pad Prism 5.0 software. P value ≤ 0.05 was considered as the statistical difference between groups in all the tests performed.

Results

The use of MacConkey agar (Enterobacteriaceae selective medium) yields a sufficiently strong and distinguishable approach, which allows a relatively precise quantification of the targeted bacterial cell uptake by the studied bacterivorous protists.

Growth and food requirements of ciliates in pure culture

Pathogenic bacteria sustained the growth rate and duplication of the studied ciliates clearly (see Fig. 1). The addition of pathogenic bacteria to the ciliate organism culture vessel resulted in the expected fast development of protozoan numbers into logarithmic growth state, especially during the first 2 days of the experiment. *S. typhi* was the most ingested species than others in their pure culture, followed by its shared culture with *E. coli*. The importance of bacteria for ciliate growth ranged from very relevant (as for *Tetrahymena pyriformis)* to slightly low dependency, (*Halteria grandinella*), which may reflect the variable bacterial nutritional values, such as vital amino acids, provided to ciliates.

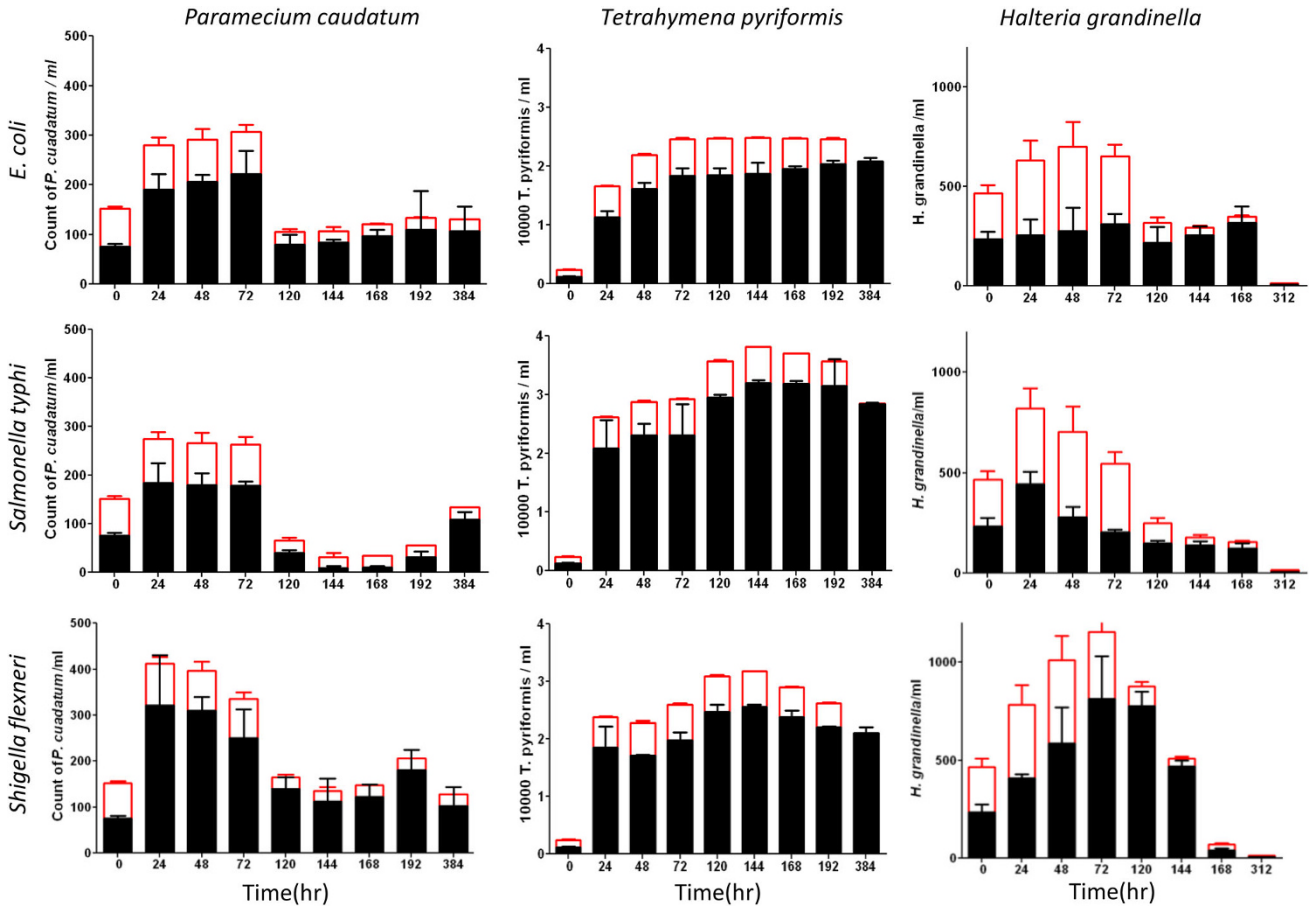

Fig. 1 The growth rate of studied protozoan organisms related to their pathogenic bacteria consumption. Bacteria presence as food source is clearly associated with protozoa growth enhancement. Bacteria *E. coli, Salmonella typhi* and *Shigella flexneri* (*black colored histogram*) in comparison with ciliate growth rate without bacteria (*red outlined colored histogram*); the *bar* represents the values of standard deviation ± SD. Note the significant sustaining ciliate proliferation by pathogenic bacteria introduction

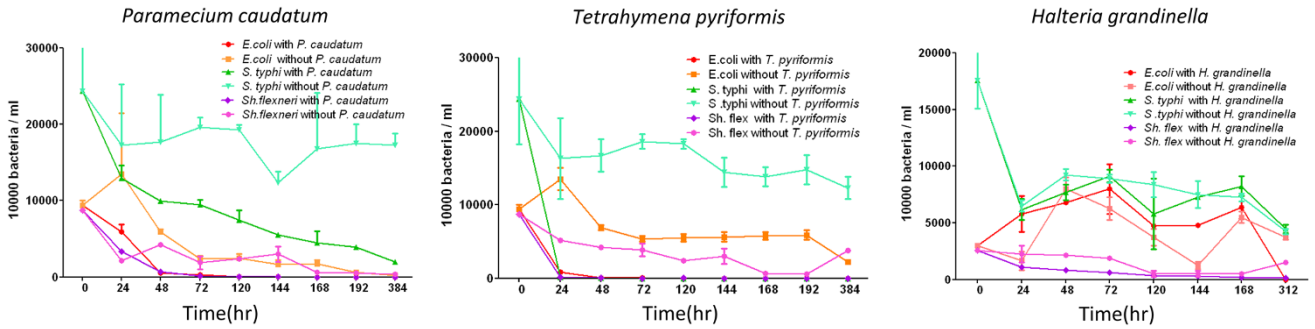

Fig. 2 The consumption rate of pathogenic bacteria. *Orange, light green and purple colored curves* indicate *E. coli, Salmonella typhi* and *Shigella flexneri* counts, respectively, when cultured alone without a ciliate predator. *Red, green and violet colored curves* represent *E. coli, S. typhi* and *Sh. flexneri* counts, respectively, when cultured with identified ciliate species. All ciliates species are bacterivorous grazing efficiently pure bacteria and reduce their count greatly, suggesting ciliate investment as biological control of pathogenic polluted water purification

The highest prey concentration was 10^8 bacteria/ml. However, maximum ingestion rates were attained by all grazers at 10^7 bacteria/ml. The minimum prey concentration that was ingested was 10^3 bacteria/ml (see Fig. 2). *S. typhi* had the highest maximum ingestion rate, attaining 26×10^3 bacteria/ciliate/h at a prey concentration of 10^8 bacteria/ml by *Paramecium caudatum*. The lowest prey concentration, while still inducing bacterial uptake, was

observed in 1.3×10^3 bacteria/ml for *Sh. flexneri* by *Tetrahymena pyriformis.* Consumption of prey was not observed for *Paramecium* sp. below 1×10^4 bacteria/ml, where bacteria were ingested at 3×10^3 cells/ciliate/h.

At low bacterial concentrations (6×10^6 bacteria/ml), the rates of ingestion were markedly reduced to between 1/2 and 1/3 of the maximal levels (data not shown). We examined ciliate grazing by comparing and quantifying the proportions of bacterial prey concentration versus prey ingested by protists. Figure 5b shows the results of ingestion experiments of three ciliates species feeding on both pure and mixed bacterial strains (*S. typhi* + *E coli)* or (*Sh. flexneri* + *E. coli)*, monitored by lactose fermentation. In parallel with increasing ciliate numbers, bacteria dropped sharply under the influence of grazing. For example, the inoculation of *Tetrahymena pyriformis* with *Sh. flexneri* caused a bacterial decrease from 4.38×10^7 to 1.3×10^3 cells/ml, in contrast with increasing ciliate numbers during the first 4 days, and then both remained relatively stable. *T. pyriformis* abundance increased sharply to 2500 cells/ml (see Figs. 1, 2 and data not shown).

Growth and food consumption of ciliates in mixed culture

A maximum number of ciliate organisms accompanied with mixed bacterial count decline were observed with all studied ciliates except *Halteria grandinella*. The time of maximum growth ranged from 48 to 192 h for *H. grandinella* and *T. pyriformis*, respectively (see Fig. 3). *T. pyriformis* did not ingest bacteria under a density of 10^2 cell/ml.Ciliates showed fast exponential growth and reached up to 2.83×10^4 cells/ml, by *T. pyriformis.* During the experiment the mean individual grazing rate was up to 1.3×10^4 bacteria/ciliate/h, by *Paramecium caudatum* in the first bacterial mixture.

Although we still do not know the reasons for the slow *H. grandinella* growth, we decided to include this data set as an example of lower pathogenic bacteria grazing by the well-known freshwater bacterivorous ciliate that has bad grazing in comparison with obvious bacterivorous effect of others, in spite of their good duplication rate. For example, *E. coli* abundance decreased from 1.5×10^7 to 0.9×10^7cells/ml during the experiment period due to this ciliate organism.

Mixed bacteria count was reduced sharply under ciliate grazing pressure, approximately by all of the studied ciliates beginning from the second day (see Fig. 4).

Ciliate grazing resulted in a 1000- to 10000-fold decrease of mixed bacterial numbers within 2 days. *E. coli* + *S typhi* abundance dropped from 4.38×10^7 to 1.25×10^4 and from 1.03×10^8 to 8.8×10^4cell/ml, respectively, while *E. coli* + *Sh. flexneri* decreased from 4.38×10^7 to 6.3×10^4 and from 1.63×10^7 to 7.5×10^3 cell/ml, respectively, during just 2 days. Ciliate sp. grew constantly for 3–8 days before the expected decline in their count (Fig. 1).

Bacterial abundances in the control, predator-free vessel remained almost stable during the experiment, with slightly higher numbers (on average 2×10^5 cells/day/ml).

Comparison of ciliate bacterivory characteristics

Rate of duplication

Comparisons of ciliate division times in pure and mixed bacterial culture revealed an inverse relationship between the rate of replication and size of ciliates. Therefore, *T. pyriformis* ciliate was the fastest doubling organism among studies of ciliates (4.13 times/day) in mixed (*S. typhi* + *E. coli*) suspension (see Fig. 5a), followed by *P. caudatum* and *H. grandinella*. *P. caudatum* and *T. pyriformis* ciliates had a better duplication in the mixed than in the pure bacterial suspensions, while *H. grandinella* did not develop in mixed culture, but grew and multiplied only in the pure *Shigella* suspension (1.2 times/day).

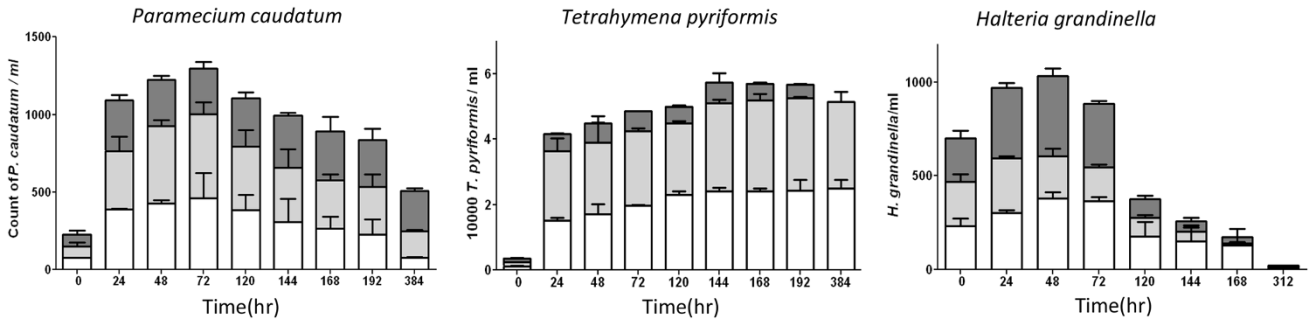

Fig. 3 The growth rate of the studied bacterivorous ciliates when cultured alone without bacteria (*heavy gray colored histogram*), feeding on (*E. coli, and S. typhi*) mixture (*light gray colored histogram*) or ingesting of (*E. coli and Sh. Flexneri*) a mixture (*white colored histogram*). Note that the first mixture evoked all ciliate duplication except *H. grandinella* in comparison with the second one

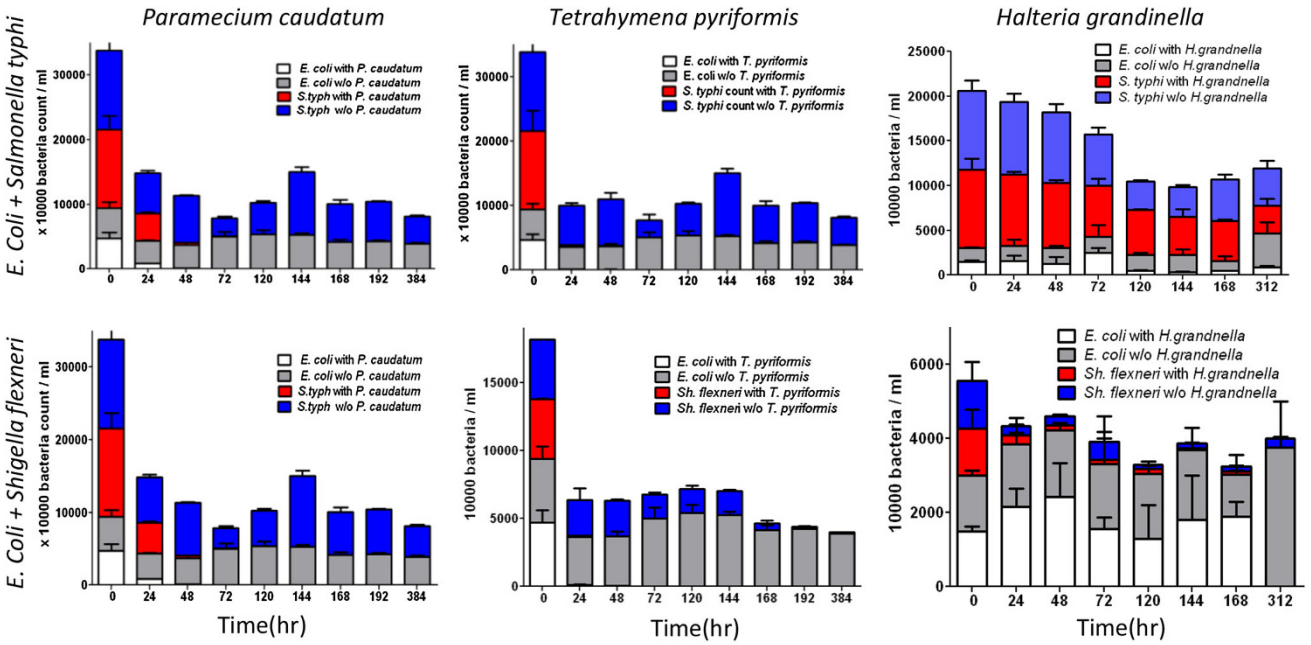

Fig. 4 The bacterivorous ciliate consumption rate of pathogenic bacteria mixtures (*E. coli* + *S. typhi*) and (*E. coli* + *Sh. flexneri*). *Blue and red histograms* represent the indicated first and second bacteria mixture counts, respectively, in the control tube. *Light gray colored and white colored histograms* present the first and second bacteria mixture counts, respectively, when cultured with bacterivorous ciliates. Especially in the first mixture, all bacterivorous ciliates efficiently reduce mixed bacteria, except H. grandinella

Ciliate consumption rate of bacteria

Paramecium caudatum had the greatest rate of bacteria ingestion (26×10^3 bacteria/ciliate/h) in this study in the pure *Salmonella* suspension, with the *Salmonella* species being more prone to be eaten by ciliates, whether in the pure or mixed suspension. (Fig. 5b) followed by *E. coli*, while *Shigella* was the lowest consumed and preferable bacteria in this study.

The high rate of consumption of *S. typhi* was probably due to their high initial density in comparison with the others. All studied bacteria were negative for Gram staining and taxonomically belonged to the same family (Enterobacteriaceae). They have similarity in many phenotypic features except motility. Potential selection of *Salmonella* may be a result of positive chemotaxis of ciliates toward the protein (flagellin) that is composed of a large number of peripheral flagella or because flagellin is essential for the growth of several ciliate species, the reason *Salmonella* has a greater nutritional value than (for example) the *Shigella* species.

Even in mixed bacterial culture, *S. typhi* + *E. coli* was the most attractive mixture to graze by different species of ciliates, except *H. grandinella* that had the least appetite for pathogenic bacteria, with the one exception of *Sh. Flexneri* bacteria in pure culture.

The consumption rate was proportional to the size of the organism. *Paramecium* was the largest ciliate included in this study and it was the most bacterivorous, followed by *Tetrahymena* species that comes after it in the size.

Ciliate clearance rate

Pure bacterial culture was cleared at the highest rate by *Paramecium*. It was able to filtrate higher amount of water that containing all three types of bacteria (80, 106 and 97.4 nanoliter/ciliate/h, respectively; see Fig. 5c). *T. pyriformis* consumed *E. coli* at a considerably higher rate than *S. typhi* and *Sh. flexneri* (34, 2 and 2 nanoliter/ciliate/h), respectively, in pure culture. In mixed bacterial culture, also *Paramecium* had the highest bacterial ingestion rate, especially for *E. coli* and *shigella* mixture (96.4 nanolitre/ciliate/h), coming before *T. pyriformis* and *H. grandinella*, respectively. Our data here indicated clearance rate less than in previously documented experiments for some ciliates (Dolan 1991; Simek et al. 1995). in which lower bacterial densities were used.

The percentage of net ciliate grazing

In pure bacterial culture, *T. pyriformis* ciliate achieved the highest percentage of net grazing rate for all studied bacteria types, followed by *P. caudatum* and *H. grandinella*. The grazing of *Salmonella* was higher than the remaining two species, when grazed by *Paramecium* and *Tetrahymena*. Unusually, the grazing of *Sh. flexneri* was the

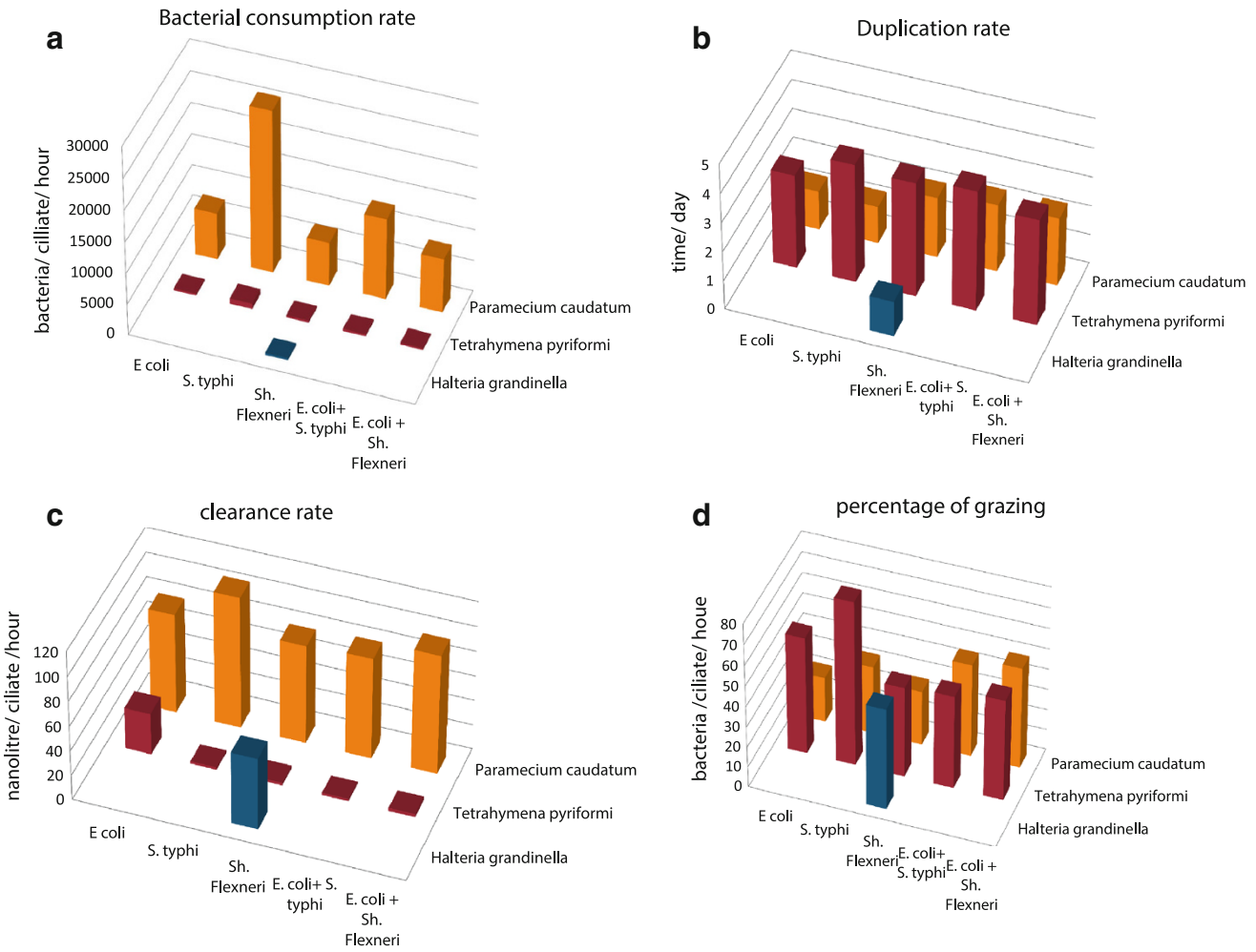

Fig. 5 The cultural features of the studied ciliates presented as **a** duplication rate, **b** prey reduction (consumption) rate, **c** clearance rate and **d** net grazing rate. Pure and mixed enteric bacteria enhance growth of ciliate organisms, shorten their duplication time and finally elevate net grazing rate. On the contrary, the presence of bateriovorous ciliate leads to prey count reduction and increase in bacteria clearance rate. Generally, all studied ciliate preferred *S. typhi* in the first mixture and *E. coli* in the second one

highest by *H. grandinella* only (see Fig. 5d). In the mixed bacterial culture, also *T. pyriformis* and *P. caudatum* achieved the highest rates of net grazing, while *H. grandinella* did not achieve any grazing net rate in the mixed culture.

In general, the rate of grazing in the second mixture (*Sh. flexneri* + *E. coli*) is higher than that of the first mixture *(S. typhi* + *E. coli).*

What is the reason for significant reduction of ciliate net grazing calculation? The loss of bacteria in the control vessels may be accelerated by unrecognizable reasons that leads to underestimation of values obtained by subtraction the rate of reduction of bacteria by ciliate organism in comparison with control vessel count (bacteria without ciliate). Generally, the results of net grazing agree with the previously published results (Drift et al. 1977; Warren 2001) that show theoretically ciliates' ability to remove all enteric bacteria from sewage, but there are other mechanisms that make their contribution up to 50 % of elimination, such as antagonism and competition (Sherr and Sherr 1987). However, it was ensured that ciliates alone were responsible for 100 % of the total protozoan bacterivory of bacteria.

Discussion

The ciliate grazing impact of bacteria (especially pathogenic bacteria) is essential to evaluate their role in autorecovery of polluted water, such as in effluent of domestic wastes. Hence, the purpose of this study was to measure the initial ingestion rates of three species of bacterivorous ciliates over a wide range of prey concentrations of three human pathogenic bacteria species.

Our data showed that ingestion rates of different ciliate organisms increased with prey density and the uptake of three bacterial species ranged from 10^3 to 10^5 bacteria/ciliate/h, in 10^6–10^8 bacteria/ml range concentration. It can be argued that this is not real within the range of pathogenic bacteria concentrations, typically reported for river water. However, bacterial concentrations can reach much higher concentrations at times or in areas of high productivity (Santos et al. 2007). Prey size is well documented as the most important property which influences ingestion and selection, but in our study we used three enteric bacterial species, characteristically similar, as targets to prevent size-selective grazing from taking place. For selectivity testing, we used highly concentrated bacteria in almost all experiments, because it has been well documented that increased selectivity occurs after acclimation to higher bacterial concentrations ($\sim 10^7$ cells/ml). There is no direct evidence that under typical natural conditions with relatively low prey abundance, ciliates do select for or against certain bacterial species or groups and the discrimination may decrease at lower concentrations (Boenigk et al. 2001). Therefore the uptake rates of ciliates might be modulated by prey concentration decline. Decreased ingestion rates at a certain prey density suggests a possible prey concentration threshold, below which the ciliate's ability to graze human pathogenic bacteria diminishes. Other characteristic features such as surface charge, mobility or chemical composition of cell wall may be involved in differential digestion of bacteria, as Gram-negative bacteria are generally digested more rapidly than Gram-positive cells (Ronn et al. 2002).

Theoretically, in contrast with natural bacteria, the pathogenic strains are not capable of balancing grazing losses, and growth rates are likely to be present in natural assemblages, due to unsuitable environmental conditions, resulting in almost complete eradication of human pathogenic strains.

Studies of taxon-specific bacterivory have shown that among pelagic ciliates, the most important consumers of bacteria in both marine and freshwaters are often small oligotrichs (Sherr and Sherr 1987; Simek et al. 1995; 1996; Thouvenot et al. 1999) such as the genus Halteria. The species *H. grandinella* has been identified as an abundant bacterial consumer and is common and widespread in several meso- and eutrophic lakes and freshwater ponds (Simek et al. 2000).

In our study, ciliate *H. grandinella* was the lowest consumer of pathogenic bacteria, partially because this small filter-feeding ciliate is an omnivorous species which is able to efficiently feed on a wider prey size spectrum from 0.5 to 5 μm, covering heterotrophic and autotrophic pico- and nanoplankton in its diet (Jürgens and Simek 2000). Therefore, they did not depend on living bacteria only, but detritus also serve as potential food source or it is negatively influenced by other suspended non-grazable particles.

The main food uptake mechanism of the studied ciliates was surface dwelling, which permits ingestion rates of ciliates to be higher in sediment-associated bacteria than those in suspension. This is because the growth of bacteria attached to surfaces presents localized areas of high prey density allowing ciliates to ingest efficiently and reduce searching time and amount of effort spent on capture of prey as opposed to filtering prey from suspension, allowing energy to be conserved for other metabolic processes.

Each protozoan species shows highest clearance rates for a distinct bacteria species, and for larger ciliates this range may even change with the size of the predator during its life cycle (Fenchel 1980). The bacteria may play an important role in the development and growth of ciliates: positively (for example) by releasing some peptides that are required for suppression of ciliate encystment (Yukari Otani and Tatsuomi Matsuoka 2010) or negatively as some prey types exhibit behavioral defensive strategies, such as exopolymer formation, aggregation, prey stickiness and electrostatic or hydrodynamic factors generation (Montagnes et al. 2008) that minimize prey contact with predator. With some ciliates, the bacterial count increased slightly at the end of the time series. This may due to high rates of survived bacteria that released into the environment through food vacuoles exocytosis would be more likely to be recovered as CFUs by plating either vacuoles or bacteria released from vacuoles; this in addition to fact of regeneration of nutrients such as nitrogen and phosphorus by the grazers might have stimulated bacteria growth.

The difference in the ciliate selection of bacteria may be based on prey characteristics and predator physiological status. Therefore, we used the ciliates at logarithmic growth phase due to low nutrition state during constancy phase (Decamp et al. 1999), as the size of protozoan organisms varied greatly according to the feeding rate and ciliate ingestion rate depending on the size (Pelegri et al. 1999). The anaerobic conditions created by heavy bacterial growth may be a reason for decrease in ciliate *H. grandinella* count. There are some ciliates such as *T. periformes* that devour each other upon reaching certain density as well as cause the accumulation of some toxic metabolic substances that reduce both ciliate growth rate and their bacterial prey ingestion. Also, the physical contact of ciliates or reluctance behavior may limit their count. Ciliate organic debris may provide suitable matter and place for bacteria multiplication protect them from bacterivous ciliates and affecting the conditions in the accounts of prey-predator (Caron 1991). So we took into account as much as possible the conditions that ensured the stability of bacterial density during the experiment time.

In this study, we attempted to directly measure protozoan food preferences employing lactose sugar bacterial fermentation as indicator. The results obtained from this study suggest the possibility of using this method as a qualitative and quantitative measure of ciliate predation of Enterobacteriaceae family members that differ in this manner. In future, we will use different sugars for further analysis of protozoan preferences, especially when assessing other enteric bacteria species.

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