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FEMS Microbiology Reviews 20 (1997) 249–259

FEMS MICROBIOLOGY REVIEWS

Effect of flagellates on free-living bacterial abundance in an organically contaminated aquifer

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Abstract

Little is known about the role of protists in the saturated subsurface. Porous media microcosms, containing bacteria and protists, were used to determine whether flagellates from an organically contaminated aquifer could substantively affect the number of free-living bacteria (FLB). When flagellates were present, the 3-40% maximum breakthrough of fluorescently labelled FLB injected into the microcosms was much lower than the 60-130% observed for killed controls. Grazing and clearance rates (3-27 FLB flag-' h-' and 12-23 nl flag-' h-1, respectively) calculated from the data were in the range reported for flagellates in other aqueous environments. The data provide evidence that flagellate bacterivory is an important control on groundwater FLB populations.

Keywords: Subsurface flagellates; Free-living bacterial abundance; Predation; Organically contaminated aquifer

Contents

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PII S0168-6445(97)00011-9

1. Introduction

It is well documented in the literature that protists consume bacteria in freshwater [1,2] and marine environments [3,4] and wastewater treatment plants [5], but little is known about their role in the saturated subsurface. Unlike freshwater and marine environments, the saturated subsurface is typically devoid of active phytoplankton populations and consists primarily of heterotrophic and chemoautotrophic bacteria, fungi, and protists [6-8]. As a result, the microbial loop in aquifers is probably much less complex than the food webs of pelagic systems [911]. In fact, it may approach conditions demonstrated in simple predator prey models [12] and laboratory experiments [13], where predation directly enhances bacterial activity. In models simulating carbon-limited environments, Hunt et al. [121 showed that predation decreased bacterial biomass resulting in a greater uptake rate of the organic substrate per unit bacterial biomass. In nutrient-limited systems, the excretion of nitrogen and phosphorus by protists in turn stimulates bacterial growth [12-161. Marine and freshwater protists graze selectively on bacteria as a function of the prey's size [17-19], growth condition [20,21], species [22,23] and motility [21]. Indeed, estuarine protists, by preferentially consuming those bacteria that are dividing [24,19], control bacterial standing stock abundances [24]. The elevated numbers and greater diversity of protists in organically contaminated aquifers [25,26] compared to pristine sites [27] suggest they may also be responding to increases in bacterial abundance and therefore, may be affecting in situ biodegradation of the pollutants.

Protists, primarily 2-3 ,um flagellates (few amoebae and no ciliates) have been found in an organically contaminated sand and gravel aquifer underlying parts of the towns of Sandwich and Falmouth, MA on Cape Cod in the USA [26,28]. The aquifer contains a contaminant plume 5 km long, I km wide and 23 m deep created by the discharge of 1900 m^3 day-1 of treated wastewater from the Massachusetts Military Reservation onto rapid sand infiltration beds for 50 years. The plume constituents include 1-4 mg I` of dissolved organic carbon (DOC), 0.5 mg I` dissolved oxygen, 60 mg/L nitrate, 2-4 mg/L alkylbenzene sulfonate detergents, and trace amounts of volatile organic compounds such as tri chloroethylene [28]. The plume has been studied extensively since 1984 by the U.S. Geological Survey as part of its Toxic Substances Hydrology Research Program [29].

The goal of our study was to determine whether the protists in the aquifer sediments could substantively affect the number of free-living bacteria (FLB) present, providing evidence that bacterivory may be an important control on groundwater bacterial populations. Ideally, such a study would be undertaken in situ where the attenuation of an inoculum containing labelled FLB could be monitored in the presence and absence of indigenous protists. However, it is difficult to selectively eliminate protists in situ. For example, use of eukaryotic inhibitors (e.g., cycloheximide) would not be satisfactory because they are not 100% effective [4,30,31] and assuring their distribution within the aquifer would be problematic and may compromise groundwater quality.

To avoid these problems, we created laboratory microcosms that mimicked aquifer conditions. Flow-through columns of sieved aquifer sediments were seeded with bacteria and protists isolated from the Cape Cod aquifer. Once stable microcosms of bacteria and protists were established in the sediments, a groundwater injectate containing fluorescently labelled FLB isolated from the aquifer, was allowed to flow through the columns. The dimensionless concentration histories (C/Co) of the fluorescently labelled FLB eluting from the columns were compared to those of a conservative tracer (bromide). The columns were then autoclaved to kill all organisms present (killed control) and the injection of the fluorescently labelled FLB was repeated. The concentration histories of the FLB under the two conditions (aquifer microcosms vs. killed control) were compared to determine the potential impact of the protists on FLB abundance.

2. Methods and materials

2.1. Column construction

Glass Chromaflex (Kontes; Vineland, NJ, USA) chromatography columns (0.3 m long, 4.8 cm inside diameter (ID)) were packed with 0.5-1.0 mm (grain size) sieved sediments collected from the Cape Cod

site using a hollow stem auger drill to bring them to the surface. A packing method developed by Johnson [32] was used which achieved good uniformity. Dried (37*C) sediments were added to the columns in 2-3 cm lifts that were compacted with a tamping rod. The columns were sealed with flow-adapter end caps whose polyethylene bed supports were replaced with stainless steel wire mesh (mesh = 40 and 325; Small Parts, Miami, FL, USA) because it is less hydrophobic and should have a lower tendency to sorb organic colloids [331. The columns were attached to a constant head system consisting of a 4 1 glass reservoir connected to a I I glass Erlenmeyer flask that provided the constant head. A volume of 500 ml of injectate remained in the tightly stoppered Erlenmeyer flask at all times to maintain a constant head. Both the reservoir and constant head flask were on magnetic stir plates to insure their contents were well mixed. The injectate was fed to the columns through 3.1 mm (ID) tygon tubing. The flowrate was controlled by a 30 cm long piece of 0.64 mm ID AutoAnalyzer tubing (Cole Parmer, Chicago, IL, USA) connected in line with the 3.1 mm ID tubing using twist connectors. Stainless steel tee valves, located just upstream of the columns, allowed influent sampling and introduction Of C02 during the column saturation procedure. The columns were run in an upflow mode (10º from horizontal) on top of a laboratory bench (temperature= 19-23ºC). Column effluent could be collected directly or using an Eldex (San Carlos, CA, USA) model UIA fraction collector set to advance at 8 min intervals.

2.2. Injectate spikes and column inocula

2.2.1. Injectate spike

The FLB injected into columns were isolated from the porewater of sediment cores taken at site S318 (12 m below land surface (bls)) at the Cape Cod site. Bacteria that formed distinct white opaque colonies on streak plates of nutrient agar were used to prepare the spike. They were diluted with sterile water to form a slurry of - I *x* 101 bacteria ml-I and then stained with 15 ,ul ml-1 of 4.3 ,uM (final conc.) DAPI (4',6-diamino-2-phenylindole, Sigma, St. Louis, MO, USA) [34]. They were not fixed with glutaraldehyde prior to staining.

2.2.2. Column inocula

Core samples were retrieved from S318 (12 m bls) at the Cape Cod site using a wireline piston corer [351 and the method outlined in Bunn [36]. To prepare the microbial inoculum for the columns, ~ 1 g of core material or 5 ml of liquid from an existing culture was placed in a sterile (15 min, 121ºC, 15 psig) I I jar containing 500 g of 0.5-1.0 mm sterile (90 min, 15 psig), sieved sediments and 125 ml of sterile 4% Cerophyl-Prescott (CP) media [34]. The porous media culture was incubated at room temperature for 4-5 days before FLB and protistan abundances were at their maxima (~10^6 and 10^4 ml^{-1}, respectively). Liquid was removed from the porous media cultures by placing the tip of a 5 ml pipettor into the sand and aspirating sample. The liquid was placed in the Erlenmeyer flask of the constant head column system.

2.3. Column saturation and inoculation

After the entire column and constant head system was sterilized (121ºC, 15 psig, 90 min), it was allowed to cool to room temperature and all tubing was connected aseptically to the appropriate containers. Filter-sterilized (0.45 µm C02 from a pressurized cylinder was connected to the system with a tee valve and allowed to flow through the column for 90 min at 0.3 ml h-' to displace any air. Then 1.5 1 of 0.005 M CaS04 was supplied to the column via the constant head system. The CaS04 was displaced by sterile distilled water. Once this saturation procedure was completed, the reservoir was filled with 4 1 of autoclaved 4% CP medium, and the constant head flask was filled with 500 ml of the porous media inoculum containing flagellates and bacteria. A flow of 1-2 ml min^{-1} was established and continued for 4 h. The 500 ml constant head flask was then replaced with an identical one containing 500 ml of sterile 4% CP. The flow of sterile 4% CP was continued for - 30 days. During this period, the system tubing, reservoir and flask were resterilized every 2-3 days to prevent contamination of the 4% CP feed. The effluent from the aquifer microcosm columns was monitored every 3-4 days for protists (abundance and size). It took 20 days for the effluent protistan abundance and size to achieve steady state.

2.4. Experimental protocol

A schematic of the experimental design is provided in Fig. 1. Groundwater was collected from Well S314-51 at the Cape Cod site, which is also located near the infiltration beds, using a stainless steel submersible pump (Keck Geophysical Instruments, Okemos, MI, USA) equipped with teflon tubing. The well was screened (250 gm slot width) over a 0.6 m interval, the midpoint of which was 15.5 m b1s. The pump was lowered into the 5.0 cm ID PVC monitoring well until it was ~ 1 m below the water's surface which was located at 10.4 m b1s. Three well volumes of water were pumped from the well to remove standing water and replace it with fresh groundwater. Forty liters of groundwater was collected, chilled, and returned to our University of New Hampshire laboratory where it was filtered (GF/C followed by 0.45 ,um filtration) and sterilized in 4 1 aliquots (121ºC, 15 psig, 90 min). Four days prior to conducting an experiment, the aquifer microcosm feed was switched to the sterilized Cape Cod groundwater. This caused little change in the effluent protistan abundance or size because the 4% CP medium had a similar DOC (3-4 mg C/L) and pH (6.0).

On the day of the experiment, 4.5 1 of sterile Cape Cod groundwater containing 200 mg Br/L` was spiked with DAPI-stained (4.3 ,uM FLB to achieve a final concentration in the range of 3×10^5 -6×10^6 FLB ml-'. This solution was placed in a sterile reservoir and constant head flask and attached to the aquifer microcosm column using new sterilized tubing. The flow of this injectate was started and continued for 800 min. During this period, the microcosm effluent was collected by the fraction collector. Effluent samples were monitored for bromide and DAPI-stained FLB. The aquifer microcosm column was then disconnected from the system and sterilized (121ºC, 15 psig, 90 min). Subsequently, it was reattached to the resterilized constant head system and the entire saturation protocol ($C0_2$, $CaS0_4$, distilled water) was repeated. A flow of sterilized groundwater was re-established for ~ 24 h and a second injection of DAPI-stained FLB was made into the killed control column and conducted for 800 min.

2.5. Microbial enumeration

Effluent samples, taken during the 30 days while the column was developing a microbial population, were monitored for protists using an epifluorescent direct count method. Samples were fixed for 15 min with 10% filtered-sterilized glutaraldehyde buffered with 0.1 M cacodylic acid (sodium salt; pH 7.0) (final glutaraldehyde concentration = 1% (v/v)). Protists were stained with DAPI (300 ,ul/ml) for 10 min. (N.B., samples being analyzed for DAPI-stained FLB from the injectate could be filtered without further staining.)

A 12 port filtration apparatus equipped with 25 mm 0.45 ,uM Metricel' backing filters (Gelman Sciences; Ann Arbor, MI, USA) was used to filter all microbial samples. DAPI-stained FLB and protists were collected on 0.2 μm and 0.8 μm, black, polycarbonate membrane filters, respectively. The vacuum used for protistan filtration was :5 12.5 mmHg to prevent rupturing cells. The filters were air dried and placed on a glass slide containing one drop of low fluorescence (Cargille A) immersion oil. A glass coverslip was placed on top. Slides were stored ≤2 months at 4ºC in the dark prior to counting. Sterile water blanks were monitored with every batch of samples.

Fields on the filter were examined for protists at 400 *x* magnification and fluorescently labelled FLB

Table 1
Flagellate concentrations in the column effluents prior to the start of the injection experiments

Column	Condition	Flagellate concentration (# ml^{-1})	Percent of flagellate population at given size	
			2–3 μm	3–4 μm
I	Protists present	1.8×10^4 (2.5×10^2)[a]	96	4
	Killed control	NA		
II	Protists present	3.2×10^4 (1.1×10^3)	77	23
	Killed control	NA		

[a]Standard error of the mean.

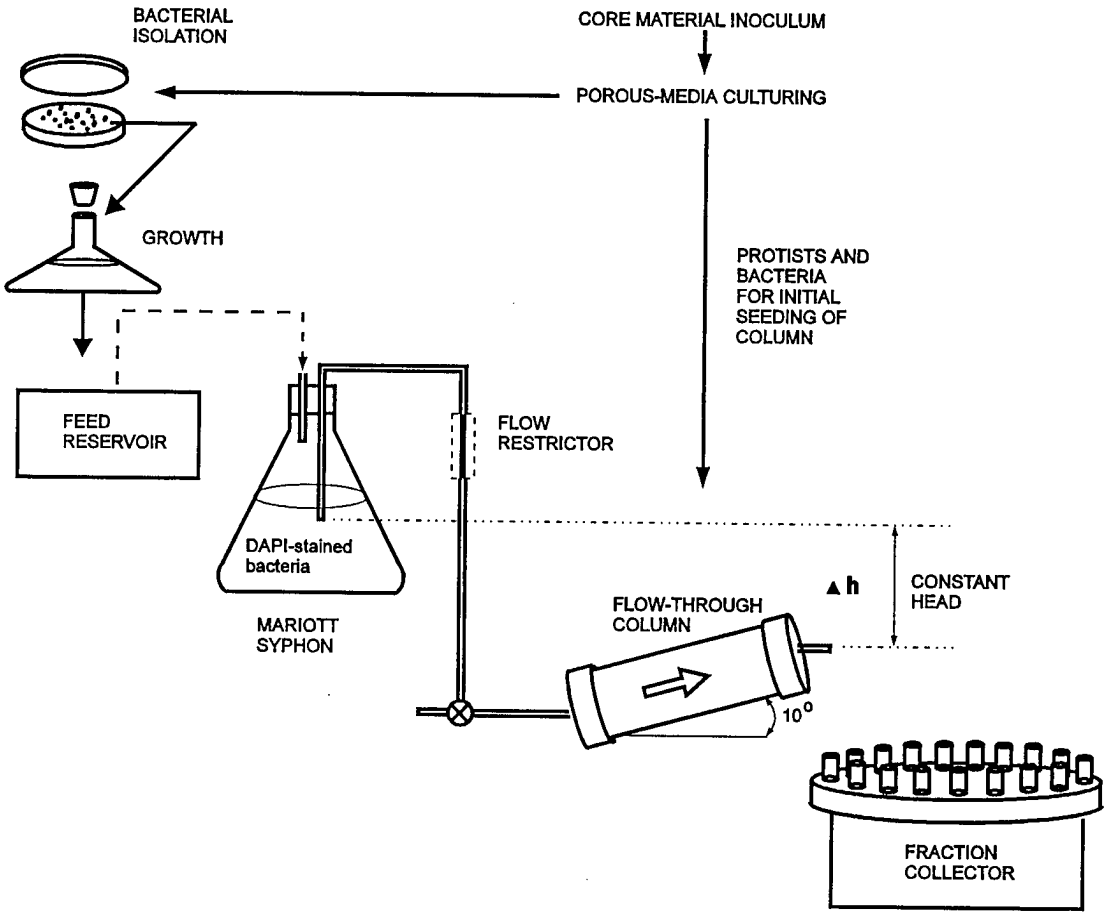

Fig. 1. Experimental protocol for assessing removal of labelled groundwater bacteria by aquifer protists using a flow through column of aquifer sediments.

at 1000 x magnification using a Nikon Optiphot epifluorescence microscope equipped with a 400 nm dichroic mirror, a 300-380 nm excitation filter and a 420 nm barrier filter. Fields were randomly chosen until > 100 organisms were counted with a minimum of 7 fields counted per slide. When concentrations of FLB or protists necessitated counting > 20 fields to achieve a total count of > 100 organisms, a scanning enumeration technique was used [36]. This employed a metal brace developed for the microscope that allowed the objective to travel a known distance (11.4 mm) across the filter. Location of each scan on the filter was chosen using a random number table [37]. Three scans were made of each filter.

Table 2
Concentrations of fluorescently labelled FLB in the injectate

Column	Condition	Injectate FLB concentration (# ml^{-1})	FLB size
I	Protists present	2.7×10^5 (2.1×10^4)[a]	1–2 μm rods
	Killed control	5.0×10^5 (1.2×10^5)	1–2 μm rods
II	Protists present	1.2×10^6 (2.0×10^4)	1.5–3 μm rods
	Killed control	5.9×10^5 (5.9×10^4)	1.5–3 μm rods

[a]Standard error of the mean.

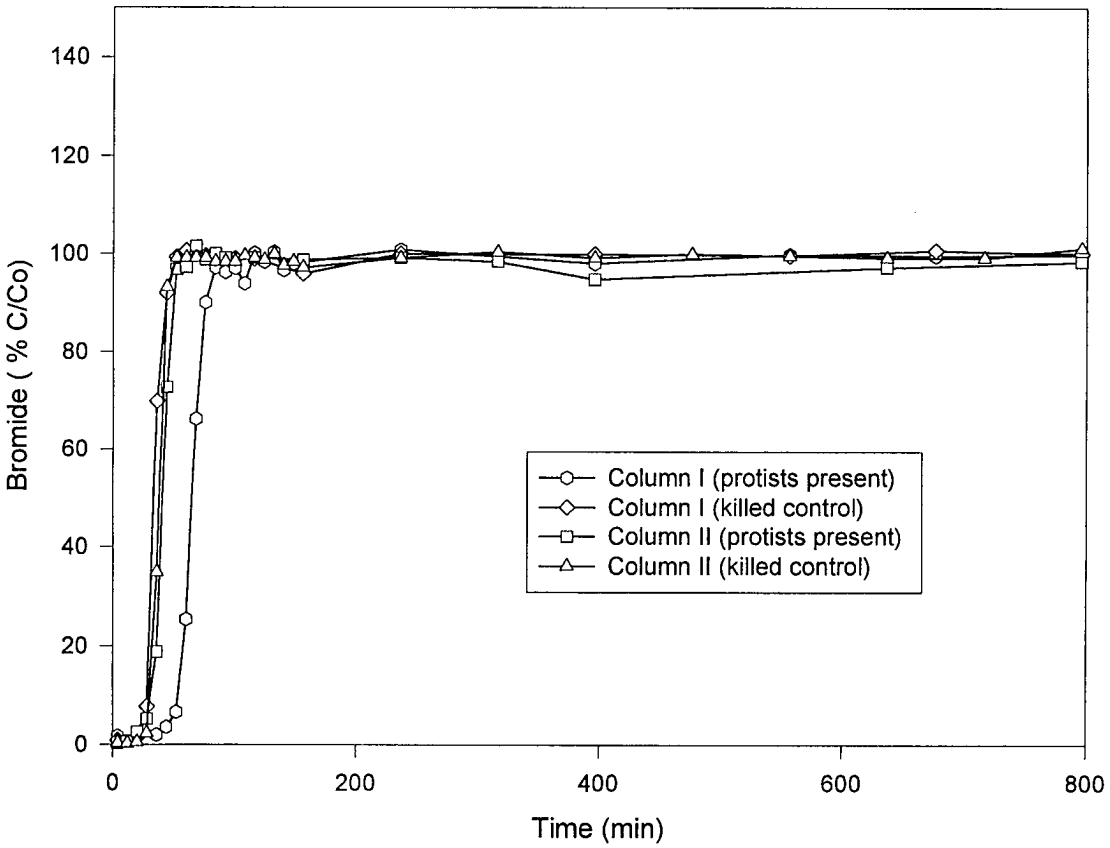

Fig. 2. Bromide breakthrough for all injection experiments.

2.6. Bromide

Bromide was analyzed using an ion selective meter equipped with halide and single junction reference electrodes (Orion Research; Boston, MA, USA). 1, 10, 100 and 1000 mg *1-1* standards were used to prepare the calibration curve (slope=-54 to -60 mV log (mg/L) $^{-1}$). Ionic strength adjustor (5 M $NaN0_3$) was added to all samples as recommended by the electrode manufacturer. A readback and blank were processed every 10 samples to monitor for drift.

3. Results and discussion

All of the protists observed in the aquifer microcosm effluents were flagellates. Their concentrations 'in the effluent of Columns I and 11 just prior to the start of the injection experiments (Table 1) were stable and similar to those found near the infiltration beds at the Cape Cod site (1.1.-3.2 x 10^4 flagellate ml^{-1}). The size distribution of the flagellates was different for the two columns (Table 1). In the Cape Cod aquifer, ≥ 80% of the flagellates are 23 ,um and < 20% are in the range of 3-5 µn. The limiting pore throat diameters (> 70 µm assuming close packing of the 0.5 mm grains) in the 0.5-1.0 mm (grain size) columns were more than large enough to accommodate 3-4 µm flagellates. It has been postulated that in situ, the 2-3 µm flagellates predominate because particles in this size range are most readily transported in the aquifer [38] and because of the scarcity of available bacteria [39]. In the aquifer microcosms, flagellates of 3-4 µm were transported because of the low buoyant densities (1.021.03) [40], the short travel path (30 cm column), and the larger more uniform grain size distribution.

Table 3
Time to maximum breakthrough for bromide and fluorescently labelled FLB

Column	Condition	Flow (ml min^{-1})	Time interval (min) to initial $C/C_{o\ max}$	
			Bromide	FLB
I	Protists present	1.7	108–116	116–156 (40%)[a]
	Killed control	2.9	44–52	36–76 (124%)
II	Protists present	2.7	60–68	4–76 (3%)
	Killed control	2.8	44–52	36–116 (102%)

[a]% C/C_o of FLB at given time.

The concentration of fluorescently labelled FLB in the injectate for each experiment (Table 2) was somewhat below the range found near the infiltration beds at Cape Cod (10^6-10^7 FLB ml^{-1}) [41]. In addition, because the FLB isolates from S318 were grown on nutrient agar prior to use in the experiments, they tended to be slightly larger or in the upper range of the sizes commonly found in the aquifer (0.2-1.6 μm average 0.5 μm [42].

The flow regime remained fairly constant during the period where breakthrough of bromide and fluorescently labelled FLB occurred. However, the flow in the Column I aquifer microcosm was significantly lower (Q = 1.7 ml/ min) than that in the corresponding killed control (Q = 2.9 ml/min). In contrast, the flows during the two runs with Column 11 were similar (Q=2.7 and 2.8 ml/ min). The bromide breakthrough curves for all of the experiments (Fig. 2) indicated that the columns were all performing hydraulically in a similar fashion and operating as plug flow reactors. The time to maximum bromide breakthrough for the three runs with similar flows was similar (Table 3) occurring in the range of 42-68 min. This was slightly lower or in the range of the predicted theoretical breakthrough of 65 min. The breakthrough for the Column I aquifer microcosm was later because of its lower flowrate. However, breakthrough did occur near the time predicted by theoretical calculations based upon pore volumes (112 min).

The breakthrough of the fluorescently labelled FLB in both runs of Column I and Column 11 (Figs. 3 and 4) occurred shortly after the bromide (Table 3) indicating that they were slightly retarded. This is sometimes observed in field transport studies (e.g., Ref. (381). When flagellates were present, the maximum breakthrough of FLB observed in Column I was 40% of influent concentration, but only 3% in Column 11, suggesting some column to column variability. However, in both columns, FLB appeared

Table 4
Grazing and clearance rate estimates for flagellates from our column experiments and the literature

Column	Grazing rate[a] (FLB $flag^{-1}$ h^{-1})	Clearance rate[a] (nl $flag^{-1}$ h^{-1})	FLB size (μm)	Flagellate sizes[b]
I	3	12	1–2	96% 4–14 μm^3 4% 14–33 μm^3
II	27	23	1.5–3	77% 4–14 μm^3 23% 14–33 μm^3
Literature[c]				
River [1]	1.1–90.4	0.2–8.9	–	–
Lakes [2,45]	2–181	0.2–44	–	–
Marine [4]	5.2–27.4	1.4–4.3	–	–
Estuarine [3,46]	–	1.7–3.2	–	–

[a]Calculated using average bromide breakthrough times (Table 3) as contact time.
[b]Assuming flagellates are spherical shapes.
[c]Literature values represent a range of experiments conducted by several researchers. They represent a variety of temperatures and sizes of bacteria and protists, but only include experiments with fluorescently labelled bacteria. The data were summarized in Barcina et al. [1].

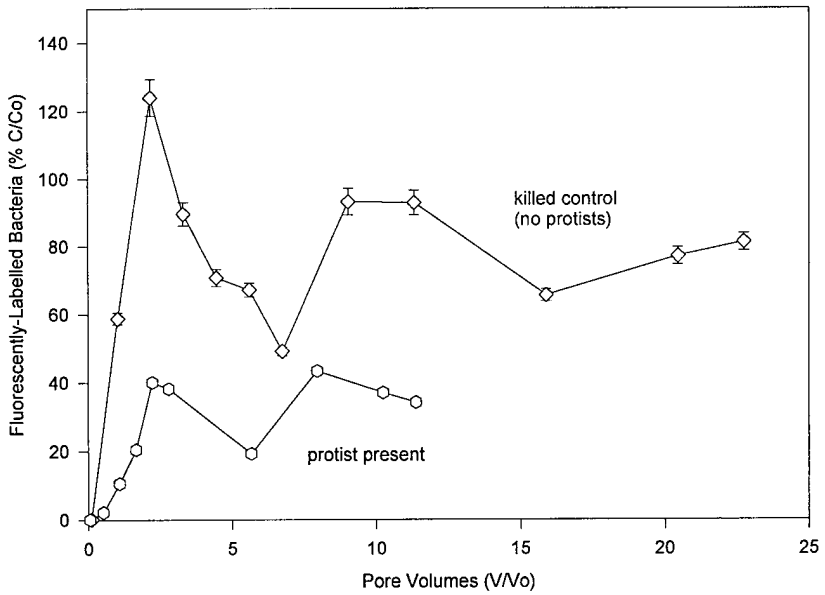

Fig. 3. Breakthrough of fluorescently labelled FLB in Column I when protists present and in killed control. Error bars, which are too small to be seen in some cases, represent the standard error of the mean of the number of fields counted or scans made.

earlier and in much greater abundance when flagellates were not present (i.e., in the killed controls). These data strongly suggest that the flagellates were grazing on the fluorescently labelled FLB and were most likely direct interception feeders [43]. It is also possible that autoclaving affected bacterial transport in the killed controls. It has been observed in at least one study that autoclave sterilization can decrease bacterial transport through subsurface material, in part, because of aggregation and surface charge changes in the clays [441. However, what little clay is present in the aquifer sediments from Cape Cod [29] was largely removed during sieving, so this effect would be expected to be minor in our experiments.

An estimate of the clearance rate of the flagellates was made using the data from the killed controls and aquifer microcosms following a mass balance approach once the effluent FLB concentration achieved steady state. This could be done because it was assumed that the DAPI-labelled FLB did not grow in the S318 groundwater during the short (< 1.9 h) contact time.

$$QC_o = QC_e + QC_{ad} + QC_{prot}$$

where Q = flowrate (ml min^{-1}), C_o = influent concentration of fluorescently labelled FLB (# ml^{-1}), C_e = effluent fluorescently labelled FLB concentration (# ml^{-1}), C_{ad} = (fraction of FLB adsorbed in killed control)·C_o = 0.2 C_o, C_{prot} = concentration decrease due to protistan ingestion of fluorescently labelled FLB (# ml^{-1}).

The grazing and clearance rates estimated from our study have the same problem as many others; they are based on protists ingesting bacteria that may have been somewhat altered by the presence of the fluorescent stain. Still, the aquifer microcosm grazing and clearance rates (Table 4) are in the range of those reported in the literature for flagellates in other aqueous environments [1,2,4,45,46]. It is likely that the estimates derived from our aquifer microcosms are high because the concentration of flagellates in the effluent was probably lower than the total protistan population present in the columns. This has not been a concern in most other studies because they have examined the effect of protistan grazing in non-porous media (i.e., lakes, rivers, marine and estuarine environments). The total flagellate population in the aquifer microcosms could not be measured without removing soil from the columns. This was not possible because the columns had to be used

by releasing ammonia in nitrogen-limited environments [12-16]. However, it is unlikely this was the case in our microcosms or at the Cape Cod site where DOC is limiting and nitrogen is in abundance [47].

We are presently conducting feeding experiments with fluorescently labelled FLB of different size classes to determine if the aquifer flagellates are selectively grazing. In addition, we are conducting laboratory column and in situ studies to monitor the population dynamics of FLB and flagellates under various DOC loading conditions. These experiments should further elucidate the role of flagellates in organically contaminated aquifers and improve estimates of their grazing and clearance rates. Further studies must also investigate the role of aquifer flagellates upon the population dynamics of surface-associated bacteria.

Acknowledgments

This research was funded by the U.S. National Science Foundation (Grants BSC 9012183 and BSC 9312235) awarded to the University of New Hampshire. The assistance of the Massachusetts/Rhode Island District of the USGS is gratefully acknowledged. We also thank Fabio Roldan-Garcia (UNH) for preparing the graphics and Naleen Mayberry and Cyndee Gruden for reviewing the manuscript. Funding for Ms. Kazmierkiewicz-Tabaka was provided through a fellowship from the Central European University.

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