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Field Evidence for a Protistan Role in an Organically-Contaminated Aquifer

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The association between protists, bacteria, and dissolved organic carbon (DOC) in an oxygen-depleted, 6 km-long wastewater contaminant plume within a sandy aquifer (Cape Cod, MA) was investigated by comparing abundance patterns along longitudinal and vertical transects and at a control site. Strong linear correlations were observed between unattached bacterial abundance and DOC for much of the upgradient-half of the plume (0.1−2.5 km downgradient from the source) that is characterized by quasi-steady state chemistry. However, a logarithmic decrease was observed between the number of protists supported per mg of DOC and the estimated age of the DOC within the plume. The relatively labile dissolved organic contaminants that characterize the groundwater sampled from the plume ≤0.1 km downgradient from the contaminant source appeared to indirectly support 3−4 times as many protists (per mg of DOC) as the older, more recalcitrant DOC in the alkylbenzene sulfonate (ABS)-contaminated zone at 3 km downgradient (∼30 years travel time). Substantive numbers of protists ($>10^4$/cm^3) were recovered from suboxic zones of the plume. The higher than expected ratios of protists to unattached bacteria (10 to 100:1) observed in much of the plume suggest that protists may be grazing upon both surface-associated and unattached bacterial communities to meet their nutritional requirements. In closed bottle incubation experiments, the presence of protists caused an increase in bacterial growth rate, which became more apparent at higher amendments of labile DOC (3−20 mgC/L). The presence of protists resulted in an increase in the apparent substrate saturation level for the unattached bacterial community, suggesting an important role for protists in the fate of more-labile aquifer organic contaminants.

Introduction

Most ecosystem-level research on subsurface bioremediation has centered exclusively on the role of bacteria in contaminant degradation (*1, 2*), although the existence of subsurface protists has been documented since 1983 (*3*) and protists are known to be common inhabitants of both pristine (*4*−*6*) and contaminated aquifers (*7*−*11*). However, little attention has been given to the role of protists in these subsurface ecosystems until just recently (*12*−*14*). In surface water habitats and wastewater treatment systems, protists are known to enhance bacterial degradation of organics by decreasing bacterial biomass resulting in greater uptake of the organic substrate per bacterium (*15*) or by recycling nitrogen or phosphorus (*16*−*19*). Protists are also capable of direct ingestion of high molecular weight dissolved organic matter (e.g., colloids) (*20, 21*) and viruses (*22, 23*). However, some recent modeling (*24*) and laboratory microcosm (*25*) studies have indicated predation actually may decrease rates of contaminant bioremediation in situ, at least for refractory compounds, by decreasing bacterial abundance in aquifers. Therefore, the specific role of protists in degradation of subsurface contaminants may vary from site to site, depending upon the nature and abundance of the contaminants.

As part of a collaborative project among the University of New Hampshire (UNH), the U.S. Geological Survey (USGS) and the Natural History Museum (London, UK), we are studying the distribution, ecology, community structure, and potential remediative role of protists in contaminated aquifers. The work reported here involves an abundant (∼10^4−10^5 per gram dry weight (gdw^{-1})) protistan community within a plume of organically contaminated groundwater originating from the Massachusetts Military Reservation (MMR) on Cape Cod, MA (*26*). While laboratory microcosm data suggest that there should be strong interactions between unattached bacteria and protists in the contaminant plume (*12, 13*), this has never been documented in situ.

The objective of the current study was to assess the likelihood of substantive bacterial-protistan interactions in the MMR plume by examining the association between protists, unattached bacteria, and dissolved organic carbon (DOC) on a spatial and temporal scale. Specifically, we developed and evaluated three questions: (1) Does the abundance of aquifer protists in the carbon-limited MMR plume correlate with the abundance of unattached bacteria and with concentrations of dissolved organic contaminants? (2) Does the magnitude of the protistan population that can be supported indirectly by each milligram of DOC in the plume decrease with increasing distance downgradient from the contaminant source and, therefore, with increasing age of the dissolved organic contaminants? (3) Does the presence of protists cause a substantive increase in the growth rate of the unattached bacteria in the MMR plume?

To evaluate these hypotheses, groundwater and aquifer sediments were collected along longitudinal and vertical transects of the contaminant plume and at a pristine (control) site and analyzed for the abundances of unattached and surface (sediment)-associated bacteria and protists, total organic carbon (TOC), DOC, and the potential terminal electron acceptors (TEAs) O_2, NO_3^-, and SO_4^{2-}. We used Fe^{2+} data collected by Savoie and LeBlanc (*27*). In addition, closed bottle incubation experiments were conducted to determine the effect of protists on bacterial growth rates at different concentrations of amended labile DOC.

Methods and Materials

Study Site. The MMR site in west, central Cape Cod is located in a sand and gravel outwash plain formed during the glacial retreat (*28*). The unconfined aquifer has been contaminated by the 60-year discharge of ∼1900 m^3/d of wastewater onto rapid sand infiltration beds (*29*) that has created a plume

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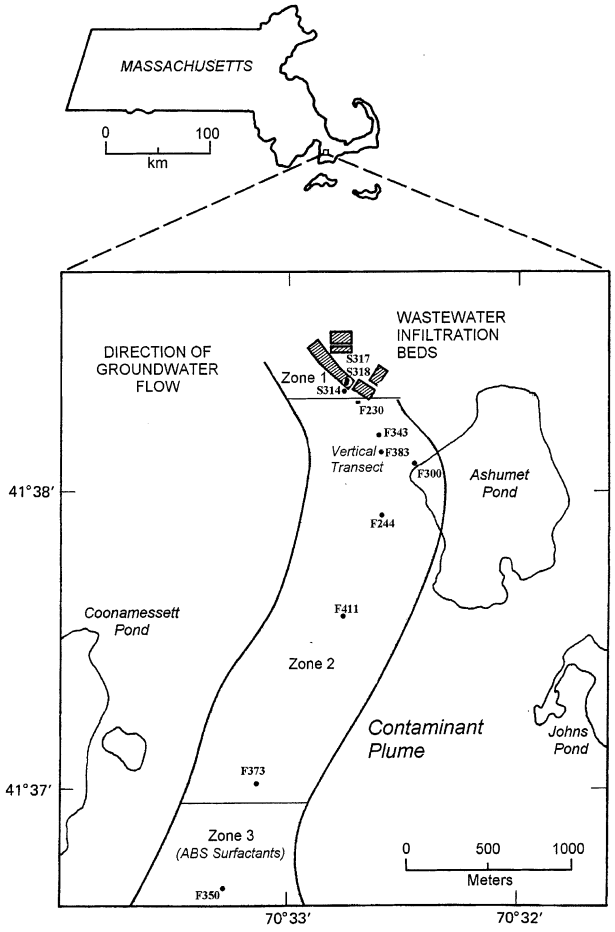

FIGURE 1. Sampling points (USGS well locations) for bacteria, protists, and chemical constituents in the plume of contaminated groundwater that originated from the Massachusetts Military Reservation (MMR). Horizontal lines delineate the three geochemically distinct zones in the plume (*43*).

approximately 6 km long, 1.1 km wide, and 23 m deep (Figure 1). Although levels of DOC are ≤4 mg/L, several contaminants, including chlorinated solvents, nitrate, and ABS surfactants, are at concentrations that are of concern. For the purposes of this study, three distinct zones of the plume were identified, based upon groundwater chemistry. Zone 1 (0−0.1 km downgradient from the contaminant source) is characterized by highly labile DOC that is subject to rapid changes in concentration in response to changes in loading conditions. Zone 2 (0.1−2.5 km downgradient) is characterized by quasi-steady-state chemistry and decreasing DOC concentrations with increasing distance from the source. Zone 3 (2.5−5 km downgradient) is characterized by the presence of ABS surfactants and their breakdown products. The horizontal groundwater velocity, hydraulic conductivity, and average porosity at the site are 0.2−0.6 m/d, 60−90 m/d, and 30−40%, respectively. Flagellates (2−3 μm) in the plume include members of the genera *Bodo, Cercomonas, Cryptaulux, Cyanthomonas, Goniomonas,* and *Spumella*, along with some previously undescribed species (*10*). Few amoebae (*30*) and no ciliates (*9*) have been found.

Sampling. Aquifer sediments and groundwater samples were collected at 12 locations along a 3 km long longitudinal transect of the contaminant plume during the period from October 23 to November 7, 1990 (Figure 1). Samples were also collected along a vertical transect at 0.37 km downgradient from the plume source. Controls were collected at an uncontaminated (pristine) site 2.06 km southwest of the wastewater infiltration beds. After purging at least three well volumes, groundwater samples were collected from screened (250 μm slot width, 0.6 m interval) PVC monitoring wells (5.0 cm i.d.) using a stainless steel submersible pump equipped with Teflon tubing.

DOC and methylene blue activated substances (MBAS) samples were filtered in the field using 0.45 μm silver membranes, whereas samples for NO_3^- and SO_4^{2-} were filtered using 0.22 μm polycarbonate membranes. Samples for MBAS and unattached bacteria were preserved with formalin (1% and 2% v/v, respectively). Anion samples were frozen. TOC, DOC, MBAS, and unattached bacteria were chilled until analysis.

Aquifer sediments, analyzed for protistan and total bacterial abundance, were collected adjacent to the monitoring well, a few days after groundwater samples had been collected, using a wireline piston core barrel (*31*) assembled with a new 1.5 m aluminum sleeve for each sample (*32*). Approximately 1 m of aquifer sediments was recovered during coring. The core was cut into 0.3 m sections as outlined in Bunn (*32*). The upper and lower 0.15 m of the core were discarded because of the potential for contamination during collection/sampling. The upper and lower 0.3 m sections remaining were used to estimate bacterial and protistan abundance, respectively. Cores sections were transported on ice to UNH for protistan enumeration and shipped overnight to the USGS laboratory in Menlo Park, CA, for analysis of total bacterial abundance. All cores were stored at 4 °C for a maximum of 15 days before processing.

Analyses. Groundwater samples were analyzed in the field for specific conductance, dissolved oxygen (DO), temperature, and pH using an ICOM M90 multimeter (Corning, Inc.; Corning, NY). TOC and DOC were analyzed in duplicate using a heated persulfate oxidation (*33*) and 6 point calibration curves. Total anionic surfactants were measured as MBAS as outlined by Wershaw et al. (*34*) using 6 point calibration curves. Analyses for SO_4^{2-} and NO_3^- were performed using ion chromatography (*35*) and 5 point calibration curves.

Unattached bacterial abundance was determined by an acridine orange (AO) direct counting procedure (*36, 37*), using an aseptic wet-sieving procedure (*38*) to separate out coarser (>100 μm) grains, which do not harbor significant numbers of surfaced-associated bacteria and interfere with epifluorescence counting. Abundances of surface-associated bacteria were calculated by subtracting the numbers of unattached bacteria (determined for groundwater collected from a similar depth at a nearby well) from the total bacteria abundance obtained from the sediment analysis and correcting for porosity.

Protists were separated from sediment particles using a shaking technique (*32, 39*) and fixed with glutaraldehyde (1% v/v final sample concentration) and 143 μM DAPI (4′,6-diamino-6-phenylindole) (*32, 40, 41*). Stained samples were filtered through 0.8 μm (pore size) black, polycarbonate filters, using a vacuum ≤13 mmHg to prevent lysing the protists. The slides were counted by a scanning method developed by Bunn (*32*) using a brace attached to the microscope that controlled the area observed.

Growth Rate Experiment. For the closed bottle incubation experiments, 0.2 L of groundwater collected from the MMR control site (well F393) was dispensed into each of two series of sterile glass bottles. Groundwater added to the first series of bottles was filtered through a 3 μm (porosity) polycarbonate membrane filter to remove protists. The filtrate was examined microscopically to ensure that protists had indeed been removed. The bottles were amended with various volumes of a sterile 18 mM acetate solution such that the concentrations in the bottles at time = 0 were 0, 0.2, 0.5, 1.0, 3.0, 10.0, and 20.0 mg/L (expressed as amended DOC). For the filtered and unfiltered series, two to three replicates were prepared of each final concentration of amended DOC. Samples were

TABLE 1. Depth, Location, Chemistry, and Attached Bacteria at Sampling Points along Vertical and Horizontal Transects through the MMR Contaminant Plume

USGS well no.	depth (m)	dist (km)	specific conductance (uS/cm)	pH	MBAS (mg/L)	DOC (mg/L)	potential electron acceptors: DO (mg/L)	NO_3^- (mg/L as N)	SO_4^{2-} (mg/L as S)	Fe^{2+} [d] (mg/L)	surface-associated bacteria ($\times 10^6$ cm^{-3})
S317[a]	8.2	0.01	454	5.7	0.2	3.5	3.60	13.9	10.3		7.6 ± 0.5
S318[a]	11.0	0.05	409	5.8	0.2	4.4	0.07	6.9	10.7		4.4 ± 0.9
S314[a]	10.7	0.08	435	5.5	0.1	3.4	0.40	14.4	11.0		6.6 ± 0.5
F230[a]	14.9	0.12	414	6.0	0.2	3.6	0.03	12.3	10.6		8.7 ± 0.9
F343[a]	24.1	0.20	316	6.1	0.2	2.5	<0.01[c]	0.2	4.5	22	n/a[e]
F383[a]	7.0	0.37	119	5.8	0.1	1.6	9.10	2.8	5.3	<0.1	2.3 ± 0.1
F383[a,b]	9.8	0.37	356	5.7	≤0.02[c]	1.4	<0.01[c]	8.3	11.3	<0.1	2.3 ± 0.2
F383[b]	12.2	0.37	284	5.5	0.2	2.9	<0.01	≤0.06[c]	8.9	<0.1	7.0 ± 0.5
F383[b]	18.6	0.37	306	5.9	0.1	2.8	<0.01[c]	≤0.06[c]	8.9	3.3	1.4 ± 0.1
F383[b]	25.0	0.37	248	5.8	0.2	3.1	<0.01[c]	≤0.06[c]	8.2	3.5	8.3 ± 1.7
F300[a]	9.1	0.69	204	6.4	0.2	2.9	0.03	≤0.06[c]	3.5		13 ± 5.0
F244[a]	27.4	0.87	247	6.2	0.1	2.1	0.04	≤0.06[c]	8.6	<0.1	13 ± 1.6
F411[a]	24.7	1.6	204	5.8	0.1	1.7	0.02	1.9	6.4	<0.1	7.3 ± 0.2
F373[a]	18.3	2.4	138	5.5	≤0.02[c]	0.9	1.30	4.4	2.2	<0.1	7.6 ± 1.7
F350[a]	23.5	3.0	212	5.8	0.8	4.1	0.02	0.2	7.8	<0.1	5.3 ± 0.4
F393	11.3	control	59	5.5	<0.02[c]	0.8	4.10	0.1	2.2		0.2 ± 0.1

[a] Longitudinal transect. [b] Vertical transect. [c] Method detection limit. [d] Data from ref *27*. [e] n/a = not available.

taken from each bottle at 0, 3, 5, 10, 21, and 46 h into the incubations. Bacterial abundance and frequency of dividing cells (FDC) were determined as described in ref *42*. Bacterial growth rates were calculated from changes in bacterial abundance over the 46 h incubation at the various amended DOC concentrations. When protists were absent, the observed growth rate was equivalent to the true bacterial growth rate because bacterial die-off was negligible during the course of the experiment. When protists were present, the apparent bacterial growth rate represented the net effect of bacterial growth and protistan predation. Again, bacterial die-off was negligible during the course of the experiment. The true bacterial growth rate when protists were present was estimated as the net bacterial growth rate plus the loss due to protistan predation.

Results

Microbial and Chemical Distributions. Groundwater chemistry, including concentrations of DOC and potential TEAs, for the uncontaminated control site (F393) and longitudinal and vertical transects through the MMR plume are listed in Table 1. DOC concentrations along the two transects were modest (<5 mg/L), but elevated relative to uncontaminated zones of the aquifer (0.8 mg/L at control well F393). As reported by Harvey and Barber (*43*), there was a general decreasing trend in DOC with increasing distance downgradient and a second DOC peak 3 km downgradient, which corresponded to a zone contaminated with highly recalcitrant alkylbenzene sulfonate (ABS) surfactants (zone 3, Figure 1) used at MMR prior to 1965. DO concentrations along the transects were generally very low (<0.05 mg/L) with exception of the groundwater sampled immediately downgradient (within 0.1 km; S317, S314) of the contaminant source and near the water table (depth = 7 m; S317, F383). The presence of 1.30 mg/L of DO at F373 appeared to be an anomaly, possibly due to a sampling or analytical error. Levels of other potential TEAs (NO_3^- and (or) SO_4^{2-} and occasionally Fe^{2+}) were typically high in the oxygen-depleted (suboxic) zones of the plume (Table 1).

Decreasing trends in the abundances of protists and unattached bacteria with increasing distance (0.1−3.0 km, zones 2 and 3) downgradient along the longitudinal transect of the MMR plume are depicted in Figure 2. The spatial distribution of unattached bacteria along the longitudinal transect downgradient from zone 1 was similar to the pattern observed earlier (e.g., refs *42* and *43*), although in the present

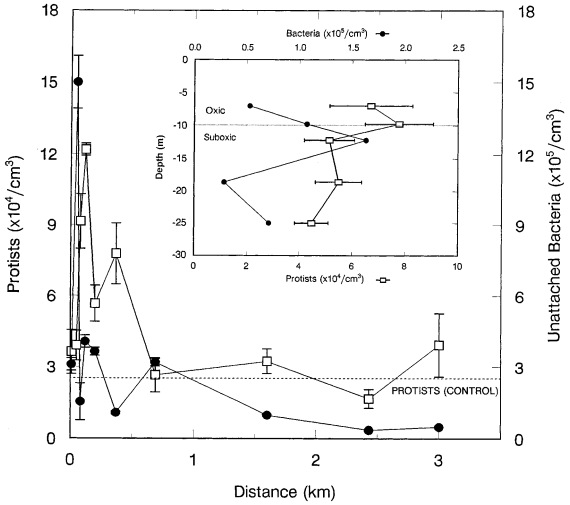

FIGURE 2. Variations in abundance of aquifer protists and unattached bacteria along the longitudinal MMR plume transect. Dashed line indicates protistan abundance in uncontaminated aquifer sediments (USGS well F393). Inset: Variations in protistan and unattached bacterial abundances along a vertical transect through the plume at 0.37 km downgradient from the source. Dashed line delineates oxic and suboxic zones. Note: zones 1, 2, and 3 = 0−0.1, 0.1−2.5, and 2.5−5 km downgradient, respectively.

study bacterial abundances in the most distal part of the transect were not much different than those observed at the uncontaminated control site. A corresponding, decreasing trend in abundance with increasing distance downgradient along the longitudinal transect was not observed for the surface-associated bacterial community (Table 1). However, their numbers were still at least an order of magnitude higher than that observed for uncontaminated aquifer sediments sampled from the F393 control site. Abundances of unattached bacteria and protists varied along the vertical transect at 0.37 km downgradient from the contaminant source (Figure 2 inset) and did not correlate with levels of DO. Large protistan populations (>10^4/cm^3) were observed in suboxic zones.

Associations of Protists with Bacteria and DOC. Strong relationships were observed between protistan abundance and DOC and between unattached bacterial abundance and DOC for aquifer samples collected along the longitudinal

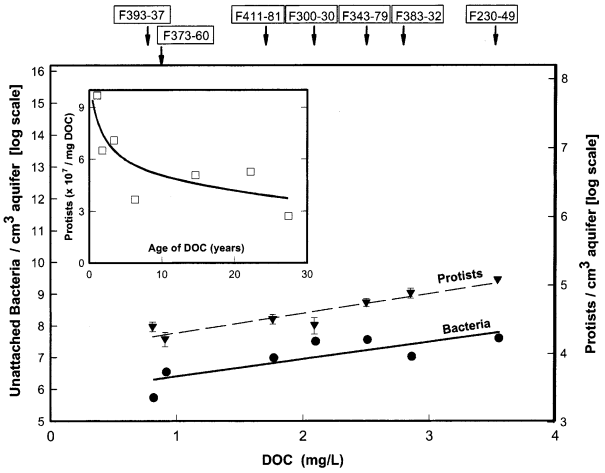

FIGURE 3. Logarithmic relationships for protistan and unattached bacterial abundances as a function of DOC for samples collected along the longitudinal transect through zone 2. Bacteria vs DOC linear $r^2 = 0.61$, $p = 0.0043$; logarithm (shown) $r^2 = 0.66$, $p = 0.026$. Protists vs DOC linear $r^2 = 0.83$, $p = 0.005$; logarithmic (shown) $r^2 = 0.88$, $p = 0.002$. Error bars represent standard error of the mean of 5−10 replicate fields. Protists = filled triangles. Unattached bacteria = filled circles. Slopes of unattached bacteria vs DOC and protists vs DOC (shown) are significantly different at $p = 0.20$. Inset depicts the logarithmic regression ($r^2 = 0.67$; $p = 0.02$) between the abundance of aquifer protists as a function of the distance downgradient from the source and the estimated age of the DOC.

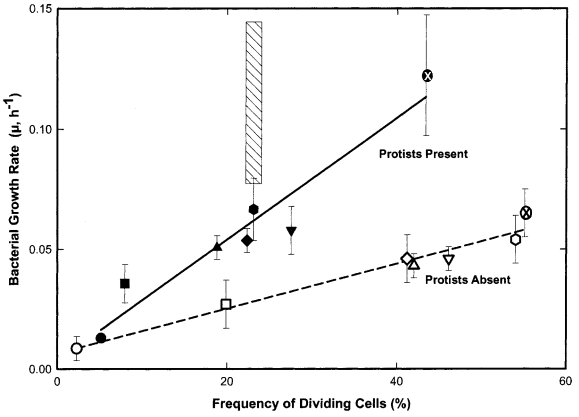

FIGURE 4. Apparent (measured) and corrected (for protistan grazing) growth rates and frequency of dividing cells (FDC) of groundwater bacteria (collected at USGS well F393; background (refractory) DOC = 0.9 mgC/L) for different concentrations of amended DOC (0−20 mgC/L) at $t = 46$ h during closed-bottle incubation experiments with acetate. Filled and empty symbols indicate protists present and protists absent, respectively. Amended DOC concentrations (mg/L): ○ = 0.0; □ = 0.2; △ = 0.5; ▽ = 1.0; ◇ = 3.0; ⬡ = 10.0; ⊗ = 20.0. Error bars represent standard error of the mean of 5−10 replicate fields. Linear relationships for protists absent and present are as follows: $0.094x + 0.0067$, $r^2 = 0.96$, $p = 0.0001$; and $0.253x + 0.0039$, $r^2 = 0.91$, $p = 0.0006$, respectively. The slopes of the lines are significantly different ($p < 0.002$). The large, vertical bar indicates the range of true bacterial growth rates when protists are present and the amended DOC = 10 mgC/L. This rate was calculated using a grazing rate of 0.77 bacteria/protist·h measured on MMR samples at 10 mgC/L (*13*) where the true bacterial growth rate = (apparent bacterial growth rate + loss of bacteria due to predation).

transect in zone 2 (Figure 3). The linear correlation between abundances of protists and unattached bacteria for this sample set was rather weak ($r^2 = 0.36$, $p = 0.15$, not shown). In general, the protistan abundance supported by a mg of DOC decreased with increasing distance downgradient (Figure 3 inset). The relatively young and, therefore, more-labile dissolved organic contaminants that characterize the groundwater sampled from the plume at ≤0.1 km (zone 1) downgradient from the contaminant source appeared to support 3−4 times as many protists (per mg of DOC) as the older, more recalcitrant DOC in the ABS-contaminated zone at 3 km (~30 years travel time). A declining logarithmic function yielded the best fit for the regression of the relationship between specific protistan abundance and the estimated age of the DOC (Figure 3 inset).

Closed-bottle incubation experiments with acetate indicated that the presence of protists corresponded to increases in the net growth rate of unattached groundwater bacteria across the entire range of amended DOC concentrations (Figure 4). However, at concentrations of amended DOC ≤3 mgC/L, this increase was very modest. In contrast, at the highest concentration of amended DOC (equivalent to 20 mgC/L), the presence of protists appeared to cause a 2-fold increase in net growth rate. In the absence of protists, increasing the amended DOC above 0.5 mgC/L had only a nominal effect upon the net growth rate. However, in the presence of protists, increasing the amended DOC concentration from 10 to 20 mgC/L resulted in increases in net growth rate from 0.067 to 0.122 h^{-1}.

Discussion

Protistan abundances (Table 2) clearly demonstrate that these organisms are major constituents of the aquifer's microbial community and are present in appreciable numbers throughout the plume. Their abundance in some core samples is similar to that reported by Sinclair et al. (*7*) for a jet fuel plume (44 mg C/L) undergoing H_2O_2 addition to enhance bioremediation (Table 2), but considerably greater than that reported for petroleum-contaminated sites where bioremediation is very slow. Our data also suggest that protists in contaminated groundwater habitats are not limited to aerobic environments (Figure 2 inset) as previously thought (*44*). The greater protistan abundances observed, compared to those reported in the literature, may be related, in part, to the concentration and nature of the sewage-derived organic carbon in the MMR plume. The concentration of readily degradable DOC, especially in zone 1, is considerably higher than in many hazardous waste plumes. In addition, previous studies of protistan abundances in aquifers have used the Singh most probable number (MPN) enumeration technique with cultured bacteria (e.g., *Enterobacter aerogenes*) as the food source (*5*−*8*) instead of a direct count method. Direct count methods, such as the one we employed, typically result in higher abundances than culturing techniques. This is especially true in the case of aquifer protists because their food sources are unknown, and it is well documented (*12*, *13*) that MMR and other protists are highly selective with respect to prey size, species, and growth condition. Subsequent enumerations of aquifer sediments with primulin (a eukaryotic stain) and DAPI yielded similar abundances suggesting that our DAPI results were accurate. The direct count method does not definitively indicate whether the protists are trophic or encysted, although the flagellates enumerated generally exhibited typical trophic characteristics (e.g., flagella).

The strong relationships between protistan abundance and DOC (Figure 3) support the hypothesis that the protists in the MMR plume are actively involved in the decrease in organic contamination. Sinclair et al. (*7*) suggested that protists may enhance in situ bioremediation by maintaining the hydraulic conductivity of the aquifer. This has led to speculation that an important role of protists in a highly contaminated aquifer undergoing engineered bioremediation may be their ability to reduce the degree of pore clogging by

TABLE 2. Ratios of Bacterial to Protistan Abundances in the MMR Plume and Other Saturated/Aquatic Environments

environment	abundance (#/gdw)	ratio bacterial to protistan abundance
	MMR Plume[a]	
2−3 μm protists	2.6×10^4−1.9×10^5	
unattached bacteria	8.4×10^3−2.3×10^6	(<1 to 38:1)
attached bacteria	3.5×10^5−2.1×10^7	(9 to 500:1)
total bacteria	3.6×10^5−2.2×10^7	(9.3 to 512:1)
	Abandoned Hydrocarbon Refinery (Aquifer) (*11*)	
protists[b]	1−1.2×10^3	
total bacteria[d]	4×10^8−8×10^8	10^3:1
	Jet Fuel Plume (Aquifer) (*7*)	
protists[b]	$<2.1 \times 10^0$−2.4×10^4	
total bacteria[c]	1.4×10^7−3.2×10^7	(1×10^3−6.7×10^6:1)
	Jet Fuel H_2O_2 (Aquifer) (*7*) Bioremediation Site	
protists[b]	5.8×10^4−6.5×10^5	
total bacteria[c]	2.2×10^7−3.2×10^7	(49 to 379:1)
	Coal Tar Site (Aquifer) (*8*)	
protists[b]	<50−1.9×10^4	
total bacteria[d]	3×10^6−10^8	(5.3×10^3−6.0×10^4:1)
	Pristine Aquifers[e] (*4*−*6*)	
protists[b]	10^0−10^1	
total bacteria[d]	1−5.6×10^7	(2.5×10^6−1.8×10^7:1)

[a] #/ccaq converted to #/gdw (#/ccaq ÷ 0.65 = #/gdw). [b] Protists = MPN/gdw. [c] Bacteria = CFU/gdw using PTYG media. [d] Bacteria = # as direct count/gdw. [e] Gravelly, sandy loam and sand, silt grains.

preying upon bacteria. However, DeLeo and Baveye (*45*) have concluded that several factors allow bacterial prey to persist and clog pore spaces in aquifer microcosms despite protistan predation.

The MMR plume is carbon-limited (*46*) and has bacterial abundances (*38*) too low to reduce hydraulic conductivity. However, our research has demonstrated that the protists in the MMR plume are preying upon bacteria. For example, laboratory studies with 2−3 μm flagellates isolated from the MMR plume sediments have shown the protists can consume 12−74% of the standing crop of 0.8−1.5 μm unattached bacteria/day (*13*). This size class of bacteria is the fastest growing within the pore fluids of the MMR sediments. Protistan predation in flow-through columns of aquifer sediments and grazing experiments with fluorescently labeled bacteria indicate the protists have clearance rates (1.4−12 nL/protist·d) comparable to similarly sized nanoflagellates in surface waters (*12, 13*). Protistan predation has also provided an answer for the greater than expected losses that result as unattached bacteria are advected downgradient within the MMR plume, considering only bacterial growth, advection, and sorption.

The protists in the MMR plume are likely to affect the rate at which the bacteria degrade organic contaminants. In other carbon-limited ecosystems, protistan predation increases bacterial productivity and the uptake rate of carbon per unit bacterial biomass (*15, 47, 48*). Results of our closed bottle incubation experiments with acetate begin to elucidate how growth rates of unattached bacteria in the plume may be affected by the protists at different DOC concentrations. At low concentrations of amended DOC (0−1 mgC/L) and, in the absence of protists, the bacteria exhibit productivities comparable to those observed for in situ unattached bacterial communities in zones 1 and 2 (*42*). Because the bacteria used in the closed bottle experiments were collected from the pristine region of the aquifer (F393), the data suggest that unattached bacteria may be able to respond quickly to modest increases in DOC (e.g., up to 0.5 mgC/L) of a readily degradable organic compound. At amended DOC concentrations above 0.5 mgC/L, little increase occurred in the net growth rate, indicating that the oligotrophic bacteria may have been near substrate saturation and, therefore, have a relatively low K_s for acetate.

Travis and Rosenberg (*24*) in their model of in situ TCE bioremediation (>2 mg/L) and Kota et al. (*25*) in their BTEX (0.4−0.5 mg/L each compound) aquifer microcosms observed that predation decreased bacterial abundance. When protists were present during our closed bottle incubations, they had only a modest impact on the net bacterial growth rate when the amended DOC concentration was ≤3 mgC/L. At higher amended DOC concentrations (10−20 mgC/L), however, the 2-fold increase in net growth rate suggests that protistan predation causes an increase in the apparent K_s. The DOC is probably going into production of new bacterial biomass. The true growth rates of the bacteria in the presence of protists could not be estimated because we did not quantify protistan predation rates during the experiments. In addition, it is likely that the composition of the bacterial community in the filtered and unfiltered bottles became progressively different over the course of the incubations. Despite these factors, these data allow us to conclude that protists probably have a bigger effect on bacterial productivity in more contaminated aquifers. It appears that their predation upon groundwater bacteria enables the bacteria to degrade organic contaminants more effectively, at least in the case of readily degradable organic compounds. This has important ramifications for remediation of contaminated aquifers and is worthy of further study.

In zone 1 of the MMR plume, the unattached bacterial population is large enough to be the major carbon source for the protists. The lack of correlation between protistan abundance and DOC or bacterial abundance in zone 1 is not surprising considering the dynamic nature of the plume in this region. DOC concentrations in zone 1 are constantly fluctuating (*49*) reflecting changes in loading and discharge patterns at the MMR wastewater treatment plant. Studies conducted by Murphy et al. (*50*) and Bengtsson (*51*) with aquifer sediments have shown that the unattached fraction of a bacterial community responds more readily than the attached fraction to changes in DOC loading. In laboratory column studies with acetate meant to simulate conditions at the head of the MMR plume, the unattached bacterial and protistan populations responded quickly and exhibited growth rates of 0.050 ± 0.021 h^{-1} (true bacterial growth, not including the effect of predation) and 0.024 ± 0.018 h^{-1}, respectively (*52*). These data suggest that microbial popula-

tion responses to change in DOC loading in zone 1 are rapid. Therefore, to detect the relationships between DOC, and unattached bacterial and protistan abundances, sediment and groundwater samples would need to be taken on a daily basis for several consecutive days. In the future, bacterial abundances should also be differentiated based upon size class, due to the size selective nature of protistan predation (*13*).

In zone 2, where short-term fluctuations have much less of an effect upon plume chemistry than in zone 1, the relationships between protists, DOC, and bacteria are more easily determined. It is clear that as the DOC ages and becomes more recalcitrant, due to preferential microbial use of the more-labile compounds during transport down-gradient, the number of protists that can be supported by each mg of DOC sharply diminishes (Figure 3 inset). While there is some correlation between the unattached bacteria and protistan abundances in zone 2, predation on unattached bacteria alone would not appear adequate to sustain the protists (Figure 2, Table 2). Typically, much higher ratios of bacterial prey to protists (up to 1000:1) are observed in other environments (Table 2). Therefore, we must consider the possibility that the protistan community in zone 2 may also be preying upon at least some of the surface-associated bacteria. A modest community of "creeping" protists, likely to feed on loosely attached bacteria, has been found in the plume (*10*). Hydrodynamic perturbation studies have shown bacteria loosely (electrostatically) associated, at least initially, with the grains in so-called "secondary minima" are also present (Harvey et al., unpublished). It is not known whether bacteria located in secondary minima can serve as food sources for the protists. This possibility may have important ramifications concerning microbial degradation of subsurface contaminants, particularly in some carbon-limited systems where abundances of unattached bacteria are relatively low ($\sim 10^5$/mL).

The lack of correlation between bacteria and heterotrophic protists observed in some aquatic environments has been attributed to bacterial losses due to other predators (*53*) or phage (*54*). The impact of phage in the MMR plume has not been investigated and is beyond the scope of this study. Losses of bacteria to other predators (e.g., ciliates and microinvertebrates) will not be a factor; however, because these larger organisms are not present in the MMR plume, as their size inhibits their transport and distribution in the aquifer. The lack of large predators also eliminates the possibility of their predation on the protists.

The possibility that the protists in the MMR plume are consuming high molecular weight DOC, as shown in the laboratory by Sherr (*20*) and Tranvik et al. (*21*), is unlikely because the DOC consists almost entirely of lower molecular weight compounds (<30 000 MW; L. B. Barber II, USGS, unpublished) (*49*). It is unlikely that the protists could compete with bacteria by direct uptake of these small dissolved organics molecules. Ledin (*55*) has shown that colloidal organic matter, which could be a protistan food source, can be created from dissolved organic matter (DOM) in surface waters under certain geochemical conditions. Protists could also egest submicron and colloidal organic matter, especially during their lag phase of growth, and eventually reingest it as shown by Isao et al. (*56*) and Pelegri et al. (*57*). The extent of such processes in the MMR plume is unknown, but unlikely in zones 2 and 3 where chemical conditions are more stable. Gonzalez et al. (*22*) have shown that flagellates can graze phage-size particles, but judging from the molecular weight distribution of DOM passing through a 0.45 μm filter, phage do not constitute a significant fraction of the available organic carbon in the plume.

Sherr and Sherr (*58*) developed a model of carbon fixation and organic carbon cycling in surface waters showing the

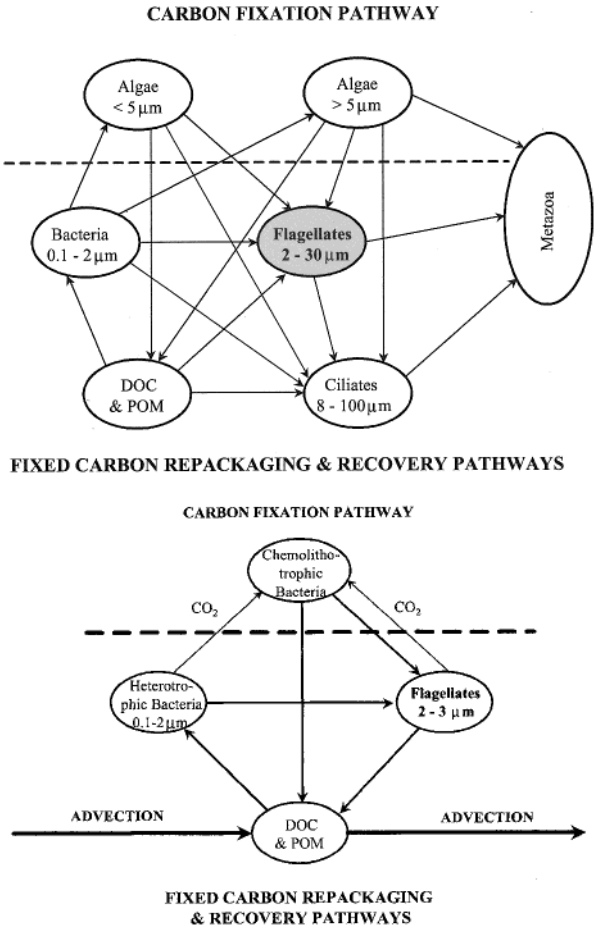

FIGURE 5. (a) Classical carbon flow diagram for the microbial community in surface waters (redrawn from (*58*) with permission from the publisher). (b) Analogous carbon flow diagram for the microbial community inhabiting the MMR contaminant plume.

interactions between protists and other constituents of the microbial community (Figure 5a). Our data suggest that a less complex model of carbon cycling exists in the MMR plume linking DOC, heterotrophic and chemolithotrophic bacteria, and protists (Figure 5b). This simplified model would be typical of most aquifers where larger eukaryotes are excluded. Hence, direct relationships between protists and bacteria in the contaminated subsurface may be easier to elucidate than in other environments. The field data from the MMR plume, coupled with this information and our previous results indicating that protists may consume a substantial fraction of the unattached bacterial standing crop per day (*12, 13*), suggest that the degradation rate of at least the more labile organic contaminants in aquifers is enhanced by protists. Further studies must be conducted to document the extent of protistan predation on surface-associated bacteria, their role in carbon flux including Azam's (*59*) organic matter field concept, and the inhibition of protists by certain toxic contaminants.

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Literature Cited

(1) Norris, R. D.; Hinchee, R. E.; Brown, R.; McCarty, P. L.; Semprini, L.; Wilson, J. T.; Kampbell, D. H.; Reinhard, M.; Bouwer, E. J.; Borden, R. C.; Vogel, T. M.; Thomas, J. M.; Ward, C. H. *In Situ Bioremediation of Groundwater and Geological Material: A Review of Technologies*; Kerr Environ. Research Laboratory; U.S. EPA: Ada, OK, 1993.

(2) Committee on In Situ Bioremediation. *In Situ Bioremediation When Does It Work*; National Research Council; National Academy Press: Washington, DC, 1993.

(3) Hirsch, P.; Rades-Rohkohl, E. *Dev. Ind. Microbiol.* **1983**, *24*, 183−200.

(4) Beloin, R. M.; Sinclair, J. L.; Ghiorse, W. C. *Microb. Ecol.* **1988**, *16*, 85−97.

(5) Sinclair, J. L.; Ghiorse, W. C. *Appl. Environ. Microbiol.* **1987**, *53*, 1157−163.

(6) Sinclair, J. L.; Randke, S. J.; Denne, J. E.; Hathaway, L. R.; Ghiorse, W. C. *Ground Water* **1990**, *28*, 369−377.

(7) Sinclair, J. L.; Kampbell, D. H.; Cook, M. L.; Wilson, J. T. *Appl. Environ. Microbiol.* **1993**, *59*, 467−472.

(8) Madsen, E. L.; Sinclair, J. L.; Ghiorse, W. C. *Science* **1991**, *252*, 830−833.

(9) Kinner, N. E.; Bunn, A. L.; Harvey, R. W.; Warren, A.; Meeker, L. D. In *U.S. Geological Survey Water Resources Investigations Report 91-4034*; Reston, VA, 1991; pp 141−143.

(10) Novarino, G.; Warren, A.; Kinner, N. E.; Harvey, R. W. *Geomicrobiol.* **1994**, *12*, 23−36.

(11) Zarda, B.; Mattison, G.; Hess, A.; Hahn, D.; Höhener, P.; Zeyer, J. *FEMS Microbiol. Ecol.* **1998**, *27*, 141−152.

(12) Kinner, N. E.; Harvey, R. W.; Kazmierkiewicz-Tabaka, M. *FEMS Microbiol. Rev.* **1997**, *20*, 249−259.

(13) Kinner, N. E.; Harvey, R. W.; Blakeslee, K.; Novarino, G.; Meeker, L. D. *Appl. Environ. Microbiol.* **1998**, *64*, 618−625.

(14) Novarino, G.; Warren, A.; Butler, H.; Lambourne, G.; Boxshall, A.; Bateman, J.; Kinner, N. E.; Harvey, R. W.; Mosse, R. A.; Teltsch, B. *FEMS Microbiol. Rev.* **1997**, *20*, 261−276.

(15) Hunt, H. W.; Cole, C. V.; Klein, D. A.; Coleman, D. C. *Microb. Ecol.* **1977**, *3*, 259−278.

(16) Johannes, R. E. *Limnol. Oceanogr.* **1965**, *10*, 434−442.

(17) Clarholm, M. *Soil Biol. Biochem.* **1985**, *17*, 181−187.

(18) Sherr, B. F.; Sherr, E. B.; Berman, T. *Appl. Environ. Microbiol.* **1983**, *45*, 1196−1201.

(19) Verhagen, F. J. M.; Duyts, H.; Laanbroek, H. J. *Appl. Environ. Microbiol.* **1993**, *59*, 2099−2106.

(20) Sherr, E. B. *Nature* **1988**, *335*, 348−351.

(21) Tranvik, L. J.; Sherr, E. B.; Sherr, B. F. *Mar. Ecol. Prog. Ser.* **1993**, *92*, 301−309.

(22) Gonzalez, J. M.; Suttle, C. A. *Mar. Ecol. Prog. Ser.* **1993**, *94*, 1−10.

(23) Pesan, B. F.; Weinbauer, M. G.; Peduzzi, P. *Mar. Ecol.* **1994**, *15*, 281−290.

(24) Travis, B. J.; Rosenberg, N. D. *Environ. Sci. Technol.* **1997**, *31*, 3093−3102.

(25) Kota, S.; Borden, R. C.; Barlaz, M. A. *FEMS Microbiol. Ecol.* **1999**, *29*, 179−189.

(26) Kinner, N. E.; Harvey, R. W. In *U.S. Geological Survey Water Resources Investigations Report 93-4015*; Reston, VA, 1993; pp 249−252.

(27) *USGS Water Resources Investigations Report 97-4269*; Savoie, J., LeBlanc, D. R., Eds.; Reston, VA, 1998.

(28) Garabedian, S. P.; LeBlanc, D. R. In *U.S. Geological Survey Water Resources Investigations Report 91-4034*; Reston, VA, 1991; pp 1−10.

(29) LeBlanc, D. R. In *U.S. Geological Survey Open File Report 84-475*; LeBlanc, D. R., Ed.; Boston, MA, 1984; pp 1−9.

(30) Warren, A.; Butler, H. *Natural History Museum Report SPC-94-4023*; London, UK, 1994.

(31) Zapico, M. M.; Vales, S.; Cherry, J. A. *Ground Water Monit. Rev.* **1987**, *7*, 74−87.

(32) Bunn, A. L. Ph.D. Dissertation, University of New Hampshire, Durham, NH, 1992.

(33) *Standard Methods for the Examination of Water and Wastewater*, 17th ed.; Greenberg, A. E., Clesceri, L. S., Eaton, A. D., Eds.; American Public Health Assoc.: Washington, DC, 1989.

(34) Wershaw, R. L.; Fishman, M. J.; Grabbe, R. R.; Lowe, L. E. In *U.S. Geological Survey Techniques of Water Resources Investigations*; 1983; Book 5, Chapter A3.

(35) Pfaff, J. D.; Brockhoff, C. A.; O'Dell, J. W. *Test Method 300.0*; U.S. EPA, Environmental Monitoring and Systems Laboratory: Cincinnati, OH, 1989.

(36) Hobbie, J. E.; Daley, R. J.; Jasper, S. *Appl. Environ. Microbiol.* **1977**, *33*, 1225−1228.

(37) Harvey, R. W. *Limnol. Oceanogr.* **1987**, *32*, 993−995.

(38) Harvey, R. W.; Smith, R. L.; George, L. *Appl. Environ. Microbiol.* **1984**, *48*, 1197−1202.

(39) Dye, A. H. *Estuar. Coast. Mar. Sci.* **1979**, *8*, 199−204.

(40) Rogerson, A. *Arch. Protistenkunde* **1988**, *135*, 289−298.

(41) Caron, D. A. *Appl. Environ. Microbiol.* **1983**, *46*, 491−498.

(42) Harvey, R. W.; George, L. H. *Appl. Environ. Microbiol.* **1987**, *53*, 2992−2996.

(43) Harvey, R. W.; Barber, L. B., II *J. Contam. Hydrol.* **1992**, *9*, 91−103.

(44) Harvey, R. W.; Garabedian, S. P. *Environ. Sci. Technol.* **1991**, *25*, 178−185.

(45) DeLeo, P. C.; Baveye, P. *Geomicrobiol J.* **1997**, *14*, 127−149.

(46) Smith, R. L.; Duff, J. H. *Appl. Environ. Microbiol.* **1988**, *54*, 1071−1078.

(47) Kuikman, P. J.; Van Elsas, J. D.; Burgers, S. L.; Van Veen, J. A. *Soil Biol. Biochem.* **1990**, *22*, 1063−1073.

(48) Mattison, R. G.; Harayama, S. *FEMS Microbiol. Lett.* **2001**, *194*, 39−46.

(49) Striker, L. K. Master's Project Paper, University New Hampshire, Durham, NH, 1998.

(50) Murphy, E. M.; Ginn, T. R.; Chilakapati, A.; Resch, C. T.; Phillips, J. L.; Wietsma, T. W.; Spadoni, C. M. *Water Resour. Res.* **1997**, *33*, 1087−1103.

(51) Bengtsson, G. *Microb. Ecol.* **1989**, *18*, 235−248.

(52) Hines, L. E. Masters Thesis, University New Hampshire, Durham, NH, 1998.

(53) Gasol, J. M.; Vaqúe, D. *Limnol. Oceanogr.* **1993**, *38*, 657−665.

(54) Bratbak, G.; Thingstad, F.; Heldal, M. *Microb. Ecol.* **1994**, *28*, 209−221.

(55) Ledin, A. Ph.D. Thesis, Linkoping University, Linkoping, Sweden, 1993.

(56) Isao, K.; Hara, S.; Terauchi, K.; Kogure, K. *Nature* **1990**, *345*, 242−244.

(57) Pelegri, S. P.; Christaki, U.; Dolan, J.; Rassoulzadegan, F. *Microb. Ecol.* **1999**, *37*, 276−284.

(58) Sherr, E. B.; Sherr, B. F. *Limnol. Oceanogr.* **1988**, *33*, 1225−1227.

(59) Azam, F. *Science* **1998**, *280*, 694−696.

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