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ORIGINAL ARTICLE

Enrichment of specific protozoan populations during *in situ* bioremediation of uranium-contaminated groundwater

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The importance of bacteria in the anaerobic bioremediation of groundwater polluted with organic and/or metal contaminants is well recognized and in some instances so well understood that modeling of the *in situ* metabolic activity of the relevant subsurface microorganisms in response to changes in subsurface geochemistry is feasible. However, a potentially significant factor influencing bacterial growth and activity in the subsurface that has not been adequately addressed is protozoan predation of the microorganisms responsible for bioremediation. In field experiments at a uranium-contaminated aquifer located in Rifle, CO, USA, acetate amendments initially promoted the growth of metal-reducing *Geobacter* species, followed by the growth of sulfate reducers, as observed previously. Analysis of 18S rRNA gene sequences revealed a broad diversity of sequences closely related to known bacteriovorous protozoa in the groundwater before the addition of acetate. The bloom of *Geobacter* species was accompanied by a specific enrichment of sequences most closely related to the ameboid flagellate, *Breviata anathema*, which at their peak accounted for over 80% of the sequences recovered. The abundance of *Geobacter* species declined following the rapid emergence of *B. anathema*. The subsequent growth of sulfate-reducing *Peptococcaceae* was accompanied by another specific enrichment of protozoa, but with sequences most similar to diplomonadid flagellates from the family *Hexamitidae*, which accounted for up to 100% of the sequences recovered during this phase of the bioremediation. These results suggest a prey–predator response with specific protozoa responding to increased availability of preferred prey bacteria. Thus, quantifying the influence of protozoan predation on the growth, activity and composition of the subsurface bacterial community is essential for predictive modeling of *in situ* uranium bioremediation strategies.

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Introduction

Anaerobic protozoa are important members of microbial communities inhabiting the anoxic zone of most aquatic environments. They have been found in a variety of pristine and contaminated freshwater subsurface habitats (Sinclair *et al.*, 1993; Novarino *et al.*, 1997; Ellis *et al.*, 1998; Zarda *et al.*, 1998; Kinner *et al.*, 2002; Luo *et al.*, 2005; Brad *et al.*, 2008; Yagi *et al.*, 2010; Lin *et al.*, 2012) as deep as 200 m below the surface (Sinclair *et al.*, 1993; Nagaosa *et al.*, 2008). Many of these protozoa are bacteriovorous, actively grazing on bacteria in the subsurface (Fenchel, 1982; Linley *et al.*, 1983; Fenchel, 1986; Fenchel and Ramsing, 1992; Kinner *et al.*, 1998; Decamp *et al.*, 1999; Kinner *et al.*, 2002) and having a major role in top-down biological control of bacteria and the regeneration of nutrients, such as nitrogen, phosphorus, trace metals and organics, in these ecosystems (Caron *et al.*, 1988; Caron, 1994; Eccleston-Parry and Leadbeater, 1995).

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Although protozoan grazing removes bacterial cells, the release of growth-limiting nutrients secreted by grazing protozoa can stimulate bacterial metabolism. For example, nitrification, methane production and sulfate reduction all increased in the subsurface in the presence of protozoan grazing, and the specific growth rate of bacteria was twofold higher in the presence of protozoa (Bloem *et al.*, 1988; Verhagen *et al.*, 1995; Strauss and Dodds, 1997; Biagini *et al.*, 1998). This enhanced metabolism can improve the efficiency of biodegradation of organic compounds, such as anaerobic sludge from dairy wastewater treatment plants (Priya *et al.*, 2008); hydrocarbons (Rogerson and Berger, 1983; Mattison and Harayama, 2001; Mattison *et al.*, 2005); and plant material (Biagini *et al.*, 1998; Ribblett *et al.*, 2005). Grazing by protozoa can also help prevent reduction in hydrological conductivity that might otherwise result from microbial biomass plugging aquifer pore spaces (Sinclair *et al.*, 1993; DeLeo and Baveye, 1997; Kinner *et al.*, 2002; Mattison *et al.*, 2002).

Alternatively, in some instances protozoan grazing can have a negative impact on groundwater bioremediation by critically reducing the number of contaminant-degrading microorganisms (Kota *et al.*, 1999). For example, protozoa inhibited trichloroethylene and BTEX (benzene, toluene, ethylbenzene, and *o*-, *m*- and *p*-xylenes) degradation in laboratory microcosms constructed with contaminated aquifer sediments (Kota *et al.*, 1999; Cunningham *et al.*, 2009) and when protozoan predation was not considered in a computer model that simulated bioremediation of trichloroethylene by a methanotrophic community, rates of trichloroethylene degradation were overestimated by 25% (Travis and Rosenberg, 1997).

Efforts to model the *in situ* bioremediation of uranium-contaminated water have become increasingly sophisticated with the introduction of genome-scale metabolic models to predict the growth and metabolic activity of the microorganisms thought to influence the bioremediation process (Scheibe *et al.*, 2009; Fang *et al.*, 2011; Lovley *et al.*, 2011; Mahadevan *et al.*, 2011; Zhuang *et al.*, 2011; Barlett *et al.*, 2012). However, these modeling efforts have not considered the potential role of protozoa in influencing microbial community dynamics. Here we report that stimulating the growth of the bacterial community with acetate to promote U(VI) reduction results in specific enrichment of protozoa with different protozoan genera responding to the growth of *Geobacter* or sulfate-reducing bacteria.

Materials and methods

Site and description of field site

In 2010, a small-scale *in situ* bioremediation experiment was conducted on the grounds of a former uranium ore-processing facility in Rifle, CO, USA, during the months of August–October as described previously (Miletto *et al.*, 2011; Giloteaux *et al.*, 2012). This same plot was biostimulated by acetate additions during the months of August–October in 2011. This research was part of the Uranium Mill Tailings Remedial Action (UMTRA) program of the US Department of Energy. The plot used in both field experiments was adjacent to a previously studied larger experimental plot at the site (Anderson *et al.*, 2003; Vrionis *et al.*, 2005). The monitoring array consisted of an injection gallery with six injection wells, nine downgradient wells and one background monitoring well-located upstream from the injection gallery (see Supplementary Material and Supplementary Figure S1). Groundwater for the experiment was collected from well CD-04 in 2010 and CD-01 in 2011.

The Old Rifle site is located on a flood plain of the Colorado River. Groundwater moves primarily in the topmost hydrostratigraphic unit of the unconfined aquifer, a sandy gravel, gravelly sand alluvium. The upper permeable layer (hydraulic conductivity ca. $37\,m\,d^{-1}$) is underlain by a relatively impermeable silty shale layer (conductivity ca. $0.005\,m\,d^{-1}$) from the weathered Wasatch formation (DOE, 1999; Anderson *et al.*, 2003; Yabusaki *et al.*, 2007).

Porosity of the Rifle sediments is ca. 25%, and only 38% of the aquifer material is contained in the <2 mm size fraction and the majority is gravel size (Fox *et al.*, 2012).

During the field experiment, a concentrated acetate/bromide solution (50/20 mM) mixed with native groundwater was injected into the subsurface to provide approximately 5 mM acetate to the groundwater over the course of 30 days in 2010 and 68 days in 2011as described previously (Anderson *et al.*, 2003; Williams *et al.*, 2011). Bromide was utilized as a non-reactive tracer.

Analytical techniques.

Samples for geochemical analyses were collected after purging 12 l of groundwater from the wells with a peristaltic pump. Ferrous iron was measured spectrophotometrically immediately after sampling using the phenanthroline method (AccuVac ampules; Hach Company, Loveland, CO, USA) for ferrous iron. After filtration through a 0.2 μm pore size polytetrafluoroethylene (Teflon) filter (Alltech Associates Inc., Deerfield, IL, USA), acetate concentrations were measured with a Dionex ICS-1000 ion chromatograph equipped with an IonPac AS22 column, an ASRS 300 suppressor and 4.5 mM carbonate/1.4 mM bicarbonate eluent (Dionex Corporation, Sunnyvale, CA, USA).

Extraction of nucleic acids from samples

DNA and RNA were extracted from groundwater collected from the U(VI)-contaminated aquifer

during the bioremediation field experiments. To obtain sufficient biomass from the groundwater, it was necessary to concentrate 50 l of groundwater by impact filtration on 293 mm diameter Supor membrane disc filters (Pall Life Sciences, Port Washington, NY, USA), which took about 3 min. All filters were placed into whirl-pack bags, flash frozen in a dry ice/ethanol bath and shipped back to the laboratory where they were stored at −80 °C. RNA was extracted from filters as described previously (Holmes *et al.*, 2005) and DNA was extracted with the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA).

Absorbance readings with the NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and agarose gel electrophoresis showed that high-quality DNA and RNA were extracted from the groundwater samples. To ensure that RNA samples were not contaminated with DNA, polymerase chain reaction (PCR) amplification with primers targeting the 16S rRNA gene was conducted on RNA samples that had not undergone reverse transcription.

A DuraScript enhanced avian RT single-strand synthesis kit (Sigma, Sigma-Aldrich, St Louis, MO, USA) was used to generate cDNA as described previously (Giloteaux *et al.*, 2012).

PCR amplification parameters and clone library construction

Several previously described primer pairs were used for the amplification of 16S rRNA, 18S rRNA and β-tubulin gene fragments from genomic DNA and cDNA constructed from mRNA extracted from groundwater. Gene fragments from the 16S rRNA and 18S rRNA genes were amplified with 8F (Eden *et al.*, 1991) and 519R (Lane *et al.*, 1985), and 515F (Giovannoni *et al.*, 1988) and 1209R (Reysenbach *et al.*, 1992); respectively; BT107F and BT261R (Baker *et al.*, 2004) were used to amplify the *β-tubulin* gene. The 18S rRNA and *β-tubulin* primer sets were both nonspecific and amplified both protozoan and non-protozoan gene sequences. Some of the non-protozoan gene sequences detected at this site came from plant, fungal and animal species, which accounted for ca. 5% and 25% of the 18S rRNA and *β-tubulin* clone libraries (see Supplementary Material, Supplementary Figure S2 and Supplementary Table S1). These studies focused exclusively on the protozoan sequences detected in these eukaryotic libraries.

Degenerate primers targeting the gene coding for the α-subunit of the dissimilatory sulfite reductase protein (*dsrA*) from *Peptococcaceae* species (dsrPept_380F and dsrPept_740R) (Supplementary Table S2) were designed from various *Desulfitobacteria*, *Desulfosporosinus* and *Desulfotomaculum dsrA* nucleotide sequences obtained from the NCBI GenBank website (http://www.ncbi.nlm.nih.gov).

A 50 μl PCR reaction consisted of the following solutions: 10 μl Q buffer (Qiagen, Valencia, CA, USA), 0.4 mM of each dNTP, 1.5 mM $MgCl_2$, 0.2 μM of each primer, 5 μg bovine serum albumin, 2.5 U *Taq* DNA polymerase (Qiagen) and 10 ng of PCR template. Amplification was performed with a minicycler PTC 200 (MJ Research, Waltham, MA, USA) starting with 5 min at 94 °C, followed by 35 cycles consisting of denaturation (45 s at 94 °C), annealing (see Supplementary Table S1), extension (90 s at 72 °C) and a final extension at 72 °C for 10 min.

After PCR amplification of these gene fragments, PCR products were purified with the Gel Extraction Kit (Qiagen), and cloned into the TOPO TA cloning vector, version M (Invitrogen, Carlsbad, CA, USA). In all, 100 plasmid inserts from each of these clone libraries were sequenced with the M13F primer at the University of Massachusetts Sequencing Facility.

Calculation of diversity indices

The Shannon–Wiener and Simpson indices of diversity were used to determine the diversity of taxa present in groundwater collected from the site. The Shannon–Wiener diversity index (H') was calculated as follows (Margalef, 1958; Dunbar *et al.,* 1999):

$$H' = -\sum_{i=1}^{s} p_i \ln(p_i)$$

Simpson's diversity index (D) was calculated with the following equation (Simpson, 1949):

$$D = \sum_{i=1}^{s} p_i^2,$$

where p_i in both of these equations represented the proportion of the ith phylotype.

Species evenness was represented by Pielou's evenness index (J'), which was calculated from H'/H'_{max}, where H'_{max} is equal to $\ln(s)$ and s is the total number of phylotypes (Pielou, 1966).

Testing and design of qPCR primers

The following primer sets were used to quantify 16S rRNA and citrate synthase (*gltA*) gene and mRNA transcript copies found in groundwater collected during both field experiments by quantitative (q)PCR: 16S rRNA was amplified with 338F (Weisburg *et al.*, 1991) and 518R (Muyzer *et al.*, 1993), and *gltA* was amplified with CS375F and CS598R (Holmes *et al.*, 2005). New qPCR primer sets targeting *dsrA* and *β-tubulin* genes were designed according to the manufacturer's specifications (Applied Biosystems, Carlsbad, CA, USA) and had amplicon sizes ranging from100 to 200 bp. qPCR primers targeting all *β-tubulin* protozoan genes found in the groundwater (qbetGen_260F/qbetGen_340R) were designed from sequences found in our clone libraries and from representative protozoan *β-tubulin* sequences obtained from the GenBank database.

Primers for qPCR were also designed to target specifically *Breviata* and *Hexamita β-tubulin* genes, and *Desulfosporosinus dsrA* genes in the groundwater (Supplementary Table S2). The *Breviata β-tubulin* primer pair (qbetBrev_521F/qbetBrev_610R) was designed from a *β-tubulin* clone (clone RB) that had 86% nucleotide identity to β-tubulin from *Breviata anathema* and accounted for 65% of the *β-tubulin* clone library assembled from groundwater collected on day 14 during the 2010 field experiment. The *Geobacter gltA* primer pair (CS375F/CS598R) was previously designed from a *Geobacter* sequence most similar to *Geobacter* sp. M18 (Holmes *et al.*, 2005) and accounted for 79% of the *Geobacter gltA* clone library assembled with groundwater collected at the peak of Fe(III) reduction during the 2010 field experiment. The *Hexamita* primer pair (qbetHex_309F/qbetHex_542R) was designed from a *β-tubulin* clone (clone RH) that was 84% identical to the nucleotide sequence of *β-tubulin* from *Hexamita inflata* and accounted for 86% of the *β-tubulin* sequences detected on day 46 in 2011. The *Desulfosporosinus dsrA* primer pair (qdsrA_56F/qdsrA_217R) was designed from a *dsrA* clone (clone RD) that was 98% identical to *Desulfosporosinus youngiae* and accounted for 95% of the clone library in groundwater collected on day 39 in 2011.

Quantification of gene and transcript abundance by qPCR

qPCR amplification and detection were performed with the 7500 Real-Time PCR System (Applied Biosystems) using genomic DNA and cDNA made by reverse transcription from mRNA extracted from groundwater collected during the bioremediation experiment.

All qPCR assays were run in triplicate. Each reaction mixture consisted of a total volume of 25 µl and contained 1.5 µl of the appropriate primers (stock concentrations, 1.5 µM), 5 ng cDNA and 12.5 µl Power SYBR Green PCR Master Mix (Applied Biosystems). Standard curves covering eight orders of magnitude were constructed with serial dilutions of known amounts of purified cDNA quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) at an absorbance of 260 nm. Transcript abundances and qPCR efficiencies (95–99%) were calculated from appropriate standard curves.

Optimal thermal cycling parameters consisted of an activation step at 50 °C for 2 min, an initial 10 min denaturation step at 95 °C, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. After 50 cycles of PCR amplification, dissociation curves were made for all qPCR products by increasing the temperature from 60–95 °C at a ramp rate of 2%. The curves all yielded a single predominant peak, further supporting the specificity of the PCR primer pairs.

Cell number estimates from qPCR results

Bacterial and protozoan cell numbers were estimated from 16S rRNA and *β-tubulin* gene copies determined by qPCR with 338F/518R and qbetGen_2260f/340r. Before estimates could be made, the average number of 16S rRNA and *β-tubulin* gene copies found within bacterial and protozoan genomes were determined. Analysis of all available bacterial genomes indicated that bacteria have an average of 4.3 copies of the 16S rRNA gene per cell (Lee *et al.*, 2009) (http://rrndb.mmg.msu.edu/search.php), while the number of 18S rRNA gene copies can vary considerably among protists and individuals can have anywhere from 1to 1000 copies of this gene in their genome (Saito *et al.*, 2002; Galluzzi *et al.*, 2004; Zhu *et al.*, 2005; Terrado *et al.*, 2011). This enormous variability among 18S rRNA gene copy number would make it difficult to extrapolate cell numbers based on qPCR results. Therefore, the *β-tubulin* gene was selected for qPCR analysis. Analysis of 74 different protozoan genomes showed that protozoa have an average of 1.94 *β-tubulin* gene copies in their genome (Supplementary Table S3).

Cell numbers were estimated from the following equations:

(1) Weight of the gene for X copies:

$$\underbrace{\frac{S \times MW_{\mathrm{bp}}}{N_{\mathrm{A}}}}_{W = \text{Weight of 1 copy of gene}} \times C_{\mathrm{cell}}$$

where S is the average size of the gene (bp); MW_{bp} is the average weight of a basepair: $660\,\mathrm{g\,mol^{-1}}$; N_{A} is the Avagadro constant: $6.023 \times 10^{23}\,\mathrm{mol^{-1}}$; and C_{cell} is the average number of gene copies per cell. (In our study $C_{\mathrm{cell}} = 4.3$ for 16S *rRNA* and 1.94 for *β-tubulin*).

(2) Quantity of the gene from sample collected:

$$\frac{\mathrm{qPCR_C} \times W \times q_{\mathrm{DNA}}}{V}$$

where $qPCR_{\mathrm{C}}$ is the number of qPCR gene copies; W is the weight of one copy of the gene; q_{DNA} is the quantity of DNA extracted from sample; and V the volume of groundwater collected on filter.

(3) Number of cells per volume of sample:

$$\frac{(2)}{(1)}$$

Phylogenetic analysis

16S and 18S rRNA and functional gene sequences were assembled with Geneious 5.6 and compared with GenBank nucleotide and protein databases with the blastn and blastx algorithms (Altschul *et al.*, 1998). Alignments were made in ClustalX (Thompson *et al.*, 1997) and corrected with PROSEQ v.2.9 (Filatov, 2002) before phylogenetic trees were constructed with MEGA v.4 (Tamura *et al.*, 2007). The neighbor-joining algorithm was used to construct all phylogenetic trees (Saitou and Nei, 1987). All evolutionary distances were computed with the Poisson correction method with 1000 bootstrap

replicates. Homologous coverage calculations and rarefaction analyses of each library were performed as described previously (Giloteaux *et al*., 2010).

The nucleotide sequences of 18S rRNA, *dsrA*, *β-tubulin* and 16S rRNA genes amplified from the uranium-contaminated aquifer have been deposited in the GenBank database under accession numbers HF568845–HF568867 and HF569144–HF569146.

Results and discussion

Specific enrichment of Breviata species in response to Geobacter growth

Addition of acetate to the groundwater during the 2010 field experiment resulted in a significant increase in bacteria, followed by an increase in protozoa (Figure 1a). Bacterial cell numbers estimated from qPCR of the 16S rRNA gene with the equation described in the Materials and methods section were similar to those estimated by fluorescence *in situ* hybridization analysis (Holmes *et al*., 2013). In 2010, bacterial cell numbers estimated by qPCR ranged from 2.31×10^4 to 2.02×10^6 cells per ml and the number of cells estimated by fluorescence *in situ* hybridization ranged from 3.89×10^4 to 2.19×10^6 (Holmes *et al*., 2013). Protozoan cell numbers estimated from qPCR of the protozoan *β-tubulin* gene indicated that the number of protozoan cells ranged from ca. 1.0×10^2 to 3.75×10^3 cells per ml.

These protozoan abundances were similar to those observed in other pristine and contaminated subsurface aquifers where 10^2–10^4 individuals per ml have been detected (Sinclair and Alexander, 1989; Sinclair *et al*., 1993; Ellis *et al*., 1998; Zarda *et al*., 1998; Ekelund *et al*., 2001). The ratio of protozoan to bacterial cell numbers observed in the groundwater was also consistent with previously reported ratios from other freshwater aquifers (Sinclair and Alexander 1989; Sinclair *et al*., 1993; Zarda *et al*., 1998); ca. $1{:}10^2$ in 2010 and ca. $1{:}10^3$ in 2011.

As noted in previous studies (Anderson *et al*., 2003; Holmes *et al*., 2005; Vrionis *et al*., 2005; Williams *et al*., 2011), the increase in bacteria could be attributed to enhanced growth of *Geobacter* species, as evidenced by an increase in *Geobacter gltA* gene copies (Figure 1b). Analysis of 16S rRNA gene sequences demonstrated that *Geobacter* species became the predominant bacterial species in the groundwater, accounting for as much as 89% of the bacterial community (Figure 1c) before a decline in numbers that was coincident with the increased abundance of the protozoa (Figure 1a).

Before acetate injections, the protozoan community was highly diverse with as many as 42 different protozoan 18S rRNA gene sequences detected in the groundwater. Shannon–Wiener (H') and Simpson diversity (D) indices calculated with formulas described in the Materials and methods section were 2.81 and 0.9 (Supplementary Table S4B). The protozoan community was dominated by species from the classes *Spirotrichea* (34.2% of the 18S rRNA gene sequences) and *Chrysophyceae* (20.4% of the 18S rRNA gene sequences).

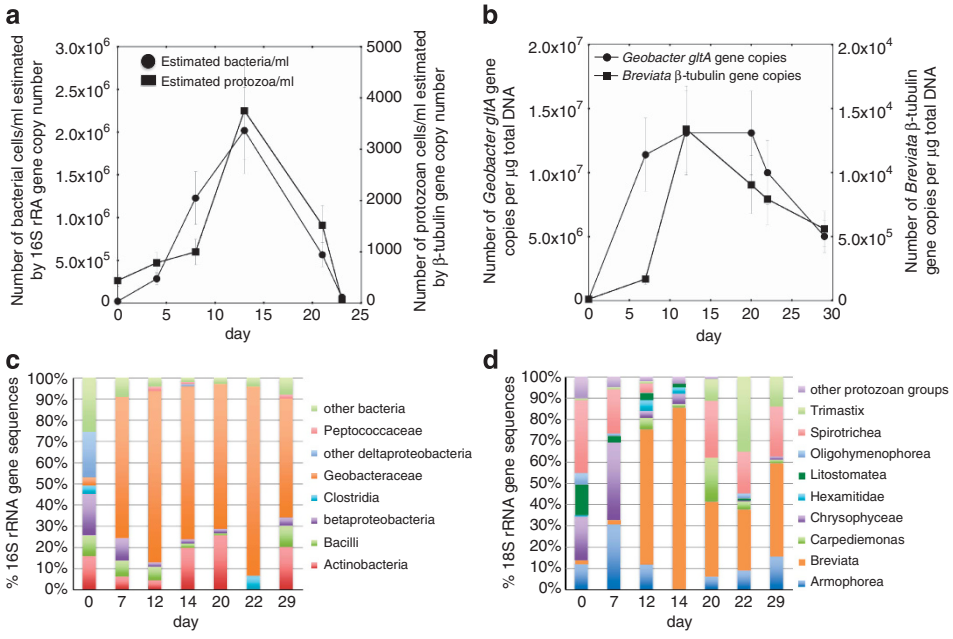

Figure 1 Microbial community analyses of groundwater samples collected during the Fe(III)-reducing phase of the 2010 field experiment. (**a**) Estimates of bacterial and protozoan cell numbers calculated from 16S rRNA and protozoan *β-tubulin* (*b2t*) gene copy numbers estimated by qPCR (Pearson's correlation, $r = 0.90$, $P = 0.015$). (**b**) The number of *Breviata β-tubulin* and *Geobacter* citrate synthase (*gltA*) gene copies per µg total DNA ($r = 0.51$, $P = 0.02$). (**c**) Percentage breakdown of bacterial 16S rRNA gene sequences detected in clone libraries constructed from genomic DNA extracted from groundwater. (**d**) Percentage breakdown of protozoan 18S rRNA gene sequences detected in clone libraries constructed from genomic DNA extracted from groundwater.

The majority (63.6%) of *Spirotrichea* 18S rRNA gene sequences were most similar to the ciliated protozoan species *Euplotes aediculatus* (96% similar) represented by clone RDL11 in Figure 2. The majority of *Chrysophyceae* sequences (84.8%) were most similar to the *Spumella*-like flagellate JBM/S11 (98% similar) represented by clone RDL4 as in Figure 2.

Both of these dominant protozoan species have been detected in other contaminated environments. For example, *Spumella* species are frequently found in freshwater aquifers contaminated with sewage (Novarino *et al*., 1994; Kinner *et al*., 1998); coal-tar waste (Yagi *et al*., 2010) and polyaromatic hydrocarbons (Lara *et al*., 2007), and freshwater *Euplotes* species have been isolated from activated sewage sludge (Salvado *et al*., 1997) and industrial effluents (Rehman *et al*., 2008). Studies have also shown that *Euplotes* species are resistant to high concentrations of heavy metals and can use bioaccumulation to remove up to 90% of these metals from the environment (Schlenk and Moore, 1994; Madoni *et al*., 1996; Shakoori *et al*., 2004; Martin-Gonzalez *et al*., 2006; Chaudhry and Shakoori, 2011). It is possible that the continuous U(VI) removal observed at the Rifle site after acetate additions have stopped (N'Guessan *et al*., 2008) might be due to bioaccumulation of U(VI) by metal-resistant *Euplotes* species in the subsurface.

With the addition of acetate, protozoan species richness dropped down to 7 and H' and D were as low as 0.78 and 0.31 (Figure 1d and Supplementary Table S4B). This decrease in protozoan diversity could be attributed to a specific enrichment of 18S rRNA gene sequences most similar to the bacteriovorous ameboid flagellate, *B. anathema* (95% similar; Figure 2), which accounted for as much as 86% of the protozoan population (Figure 1d). As acetate and Fe(II) concentrations started to decline after day 23 (Figures 1b and c and Supplementary Figure 3A), the number of *Breviata* 18S rRNA sequences decreased and overall protozoan diversity increased ($H' = 1.56$, $D = 0.71$) (Figures 1b and d and Supplementary Table S4B). This increase and subsequent decline in numbers of *Breviata* species tracked with changes in the abundance of *Geobacter* species (Figure 1b), and the number of *Geobacter gltA* and *Breviata β-tubulin* gene copies were correlated (Pearson's correlation, $r = 0.51$, $P = 0.02$).

In the 2010 field experiment, sampling was ended as soon as sulfide began accumulating in the water, indicative of the start of sulfate reduction (Supplementary Figure 3A). In 2011, acetate amendments were made in the same well and additions and monitoring were extended through sulfate reduction (Supplementary Figure 3B). Similar to the 2010 field experiment, when acetate was added to the groundwater, a correlation was observed

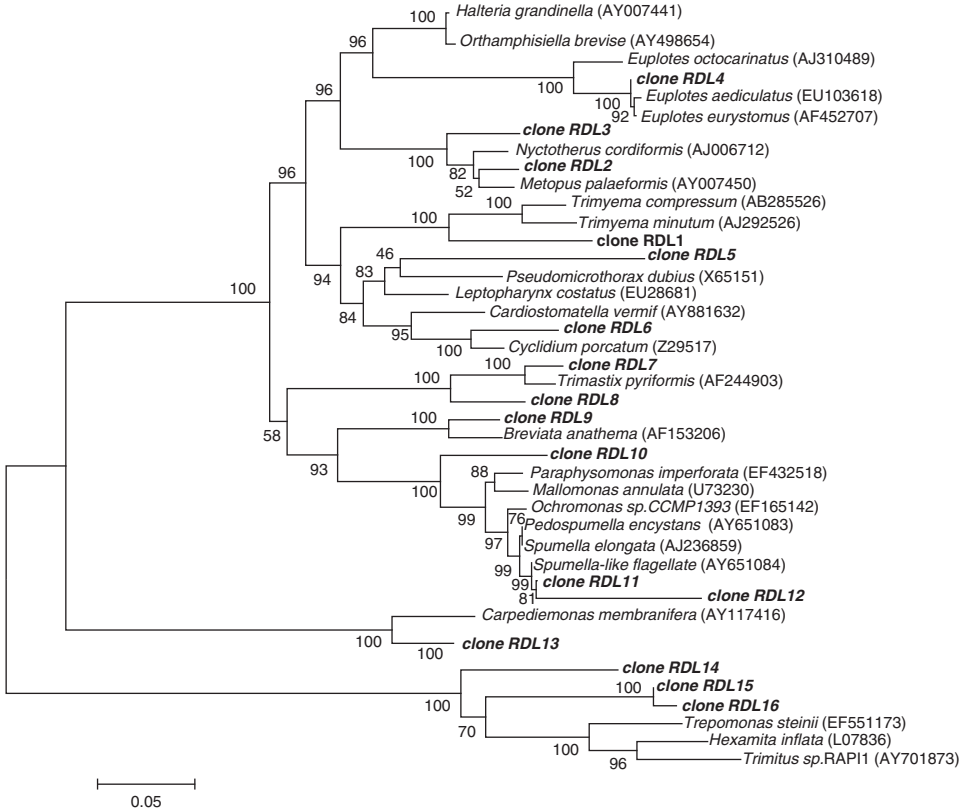

Figure 2 Phylogenetic tree comparing 18S rRNA gene sequences detected in the groundwater to sequences from previously characterized protozoa.

between bacterial and protozoan abundance ($r = 0.59$, $P = 0.02$) (Figure 3a), and bacterial and protozoan diversity decreased significantly during the Fe(III)-reducing phase of the experiment; H' for the bacterial community decreased from 2.59 on day 0 to 1.82 on day 18 and H' for the protozoan community decreased from 1.97 to 0.70 on these same days (Supplementary Tables 4C and D). Bacterial cell numbers estimated by qPCR ranged from 2.30×10^4 to 1.09×10^7 cells per ml and protozoan cell numbers ranged from 3.32×10^3 to 5.06×10^4.

The initial increase in bacterial and protozoan cell numbers could again be attributed to an enrichment of *Geobacter* and *Breviata* species (Figure 3b), with *Geobacter* and *Breviata* species accounting for as much as 79% and 67% of the bacterial and protozoan communities detected during this time (Figure 3c).

Quantitative reverse transcription PCR of *Breviata β-tubulin* gene transcripts demonstrated that relative expression of this gene, and thus presumably *Breviata* metabolic activity, was high during the increase in *Breviata* numbers (Figure 3d). *Breviata β-tubulin* gene transcript abundance correlated well ($r = 0.75$, $P = 0.007$), with relative expression of the *Geobacter* citrate synthase gene, *gltA*, which is known to be an indicator of *Geobacter* metabolic activity (Holmes *et al*., 2005).

Enrichment of Hexamitidae in the sulfate reduction phase

Similar to previous field experiments (Dar *et al*., submitted for publication; Vrionis *et al*., 2005; Miletto *et al*., 2011), continued addition of acetate after the Fe(III)-reducing phase of the experiment promoted sulfate reduction. Sulfate reduction during the 2011 experiment started as early as day 7 when sulfide began to accumulate in the groundwater (Supplementary Figure 3B). A dramatic increase in the number of *dsrA* gene copies associated with sulfate-reducing bacteria was observed after day 25 (Figure 4a).

In previous field experiments, it was found that sulfate reducers phylogenetically affiliated with either the family *Desulfobacteraceae* or *Peptococcaceae* were significant members of the bacterial community (Anderson *et al*., 2003; Vrionis *et al*., 2005; Miletto *et al*., 2011; Dar *et al*., submitted for publication). In the 2011 field experiment, *Peptococcaceae* were predominant, accounting for 89% of the bacterial 16S rRNA gene sequences on day 39 when H_2S concentrations were at their peak (Figure 4b). The majority of *Peptococcaceae* clones were most similar to *Desulfotomaculum acetoxidans* (95% similar) and *Desulfosporosinus* sp. A10 (96% similar) (Figure 5). There was a strong correlation between sulfide concentrations in the groundwater and both the proportion of

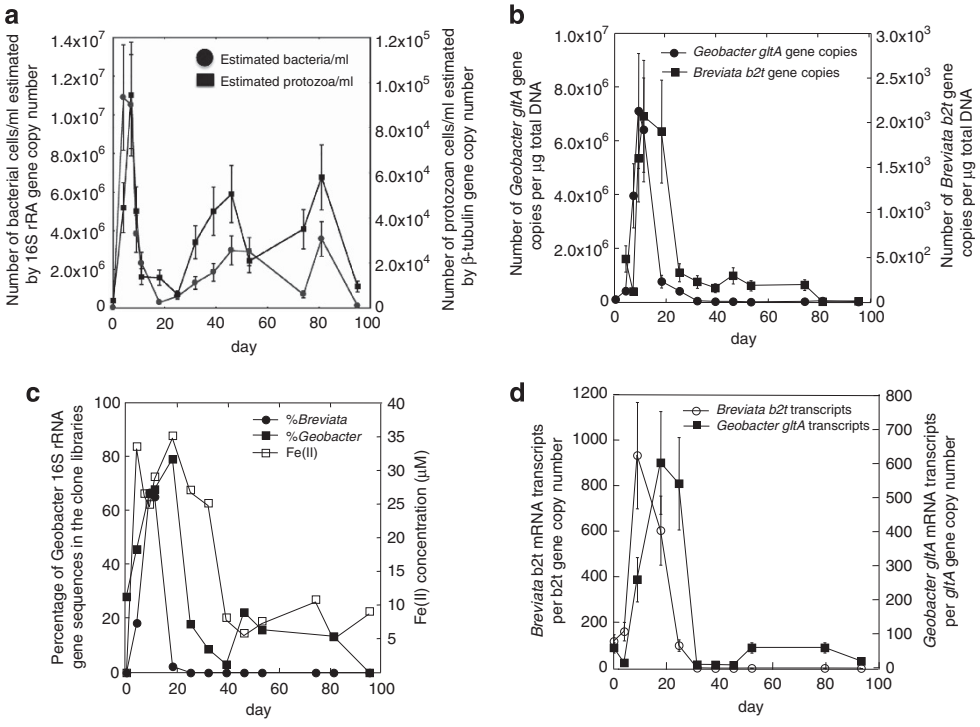

Figure 3 Analysis of bacterial and protozoan communities in the groundwater during the Fe(III)-reducing phase of the 2011 field experiment. (**a**) Estimates of bacterial and protozoan cell numbers calculated from 16S rRNA and protozoan *β-tubulin* (*b2t*) gene copy numbers estimated by qPCR (Pearson's correlation, $r = 0.59$, $P = 0.02$ (**b**) The number of *Breviata β-tubulin* (*b2t*) and *Geobacter* citrate synthase (*gltA*) gene copies per µg total DNA ($r = 0.63$, $P = 0.01$). (**c**) Percentage of *Breviata* 18S and *Geobacter* 16S rRNA gene sequences in clone libraries compared to Fe(II) concentrations ($r = 0.68$, $P = 0.01$). (**d**) The number of *Geobacter gltA* and *Breviata β-tubulin* mRNA transcripts normalized against the number of *Geobacter gltA* and *Breviata β-tubulin* gene copies ($r = 0.75$, $P = 0.1$).

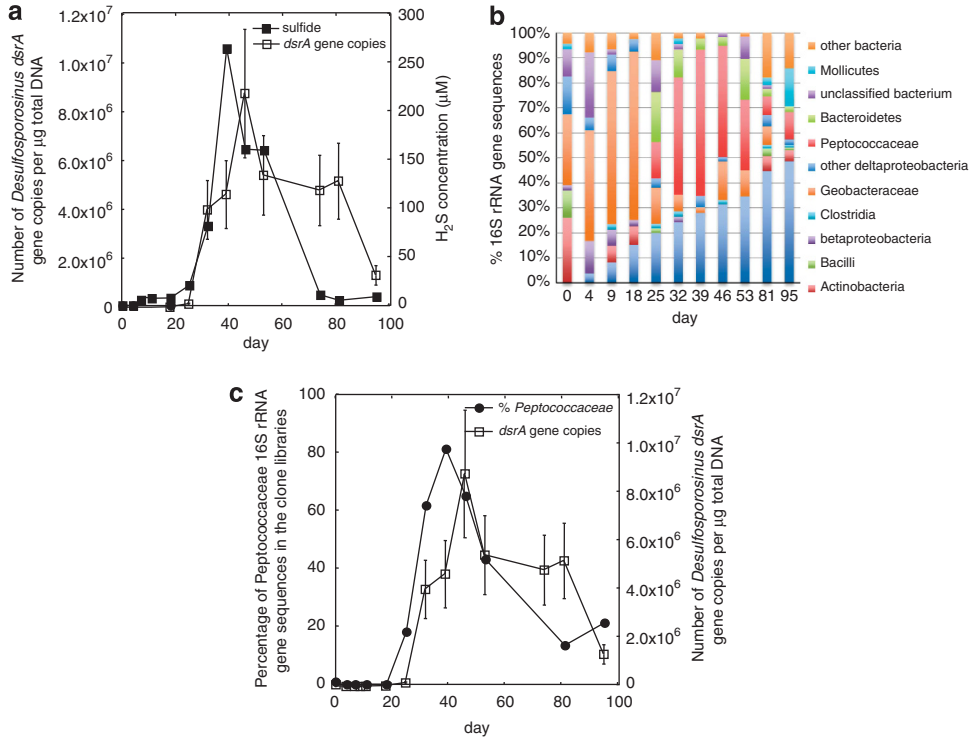

Figure 4 Analysis of the bacterial and protozoan communities found in the groundwater during the sulfate-reducing phase of the 2011 field experiment. (**a**) H_2S concentration (µM) and the number of *dsrA* gene copies per µg total DNA ($r = 0.67$, $P = 0.002$). (**b**) Percentage breakdown of bacterial 16S rRNA gene sequences detected in clone libraries. (**c**) Proportion of *Peptococcaceae* 16S rRNA gene sequences compared with the number of *dsrA* gene copies per µg total DNA ($r = 0.79$, $P = 0.002$).

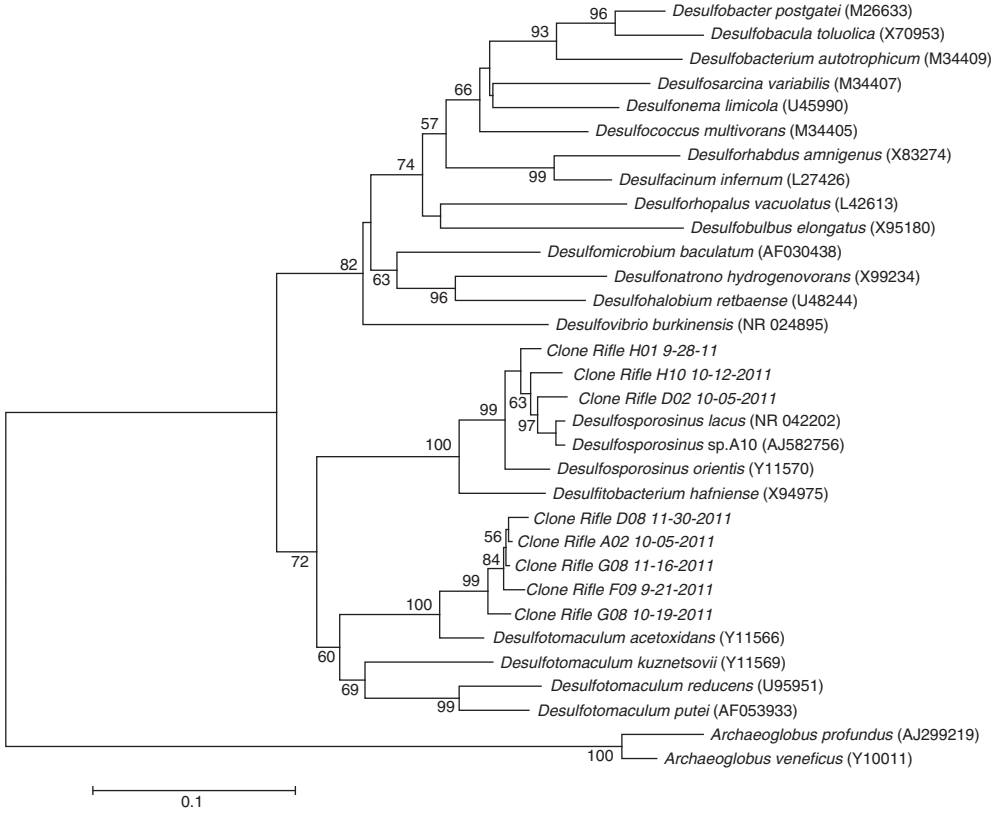

Figure 5 Phylogenetic tree comparing 16S rRNA gene sequences from sulfate-reducing *Peptococcaceae* species detected in the groundwater to sequences from previously described sulfate-reducing prokaryotes.

Peptococcaceae sequences in the clone libraries ($r=0.72$, $P=0.007$) and the number of *Desulfosporosinus dsrA* gene copies estimated by qPCR ($r=0.67$, $P=0.002$). In addition, there was a strong correlation between the proportion of *Peptococcaceae* sequences and *dsrA* gene copies ($r=0.79$, $P=0.002$) (Figure 4c).

The rise in sulfide concentrations and *Peptococcaceae* sequences was associated with a shift in the protozoan community. Species from the family *Hexamitidae* became predominant and accounted for up to 100% of the 18S rRNA gene sequences detected in the groundwater during this period (Figures 6a and b). All of the *Hexamitidae* 18S rRNA gene sequences were most similar to *H. inflata* (84% similar) or *Trepomonas* sp. *steinii* (83% similar), represented by clones RDL14-15-16 as in Figure 5. Clone library and qPCR studies showed a direct correlation between the percentage and abundance of sulfate-reducing *Peptococcaceae* and *Hexamitidae* species and between *Hexamitidae* and sulfide concentrations ($r=0.64$ and 0.67, $P=0.02$) (Figure 6b). qPCR analysis of *Hexamita β-tubulin* and *Desulfosporosinus dsrA* gene copies also demonstrated a strong correlation between the abundance of *Hexamitidae* and *Desulfosporosinus* cells in the groundwater ($r=0.57$, $P=0.03$) (Figure 6c). Furthermore, quantitative reverse transcription-PCR of *Hexamita β-tubulin* and *Desulfosporosinus dsrA* mRNA transcript abundance indicated that *Hexamitidae* species were metabolically active during the period when *Hexamitidae* were increasing in abundance and when *Desulfosporosinus* had high relative expression of *dsrA* ($r=0.53$, $P=0.05$) (Figure 6d).

Implications

These studies demonstrate that the specific enrichment of bacteria associated with the addition of acetate to the subsurface to promote U(VI) reduction in turn promotes the growth of specific bacteriovorous protozoa. Although it seems likely that the enrichment of different genera of protozoa in response to changes in the most abundant genera of bacteria is related to prey preferences, it is possible that other factors, such as changes in groundwater chemistry, might also be important.

Breviata species that become predominant during the *Geobacter* bloom are likely to have an impact on the growth and activity of the *Geobacter* species considered to be important in U(VI) reduction at this site (Lovley *et al.*, 2011; Williams *et al.*, 2011). *Breviata* have not been noted as dominant members of any previously reported protozoan communities. *Breviata* sequences were recovered from Dutch agricultural soils (Moon-van der Staay *et al.*, 2006) and closely related ameboid flagellates from the

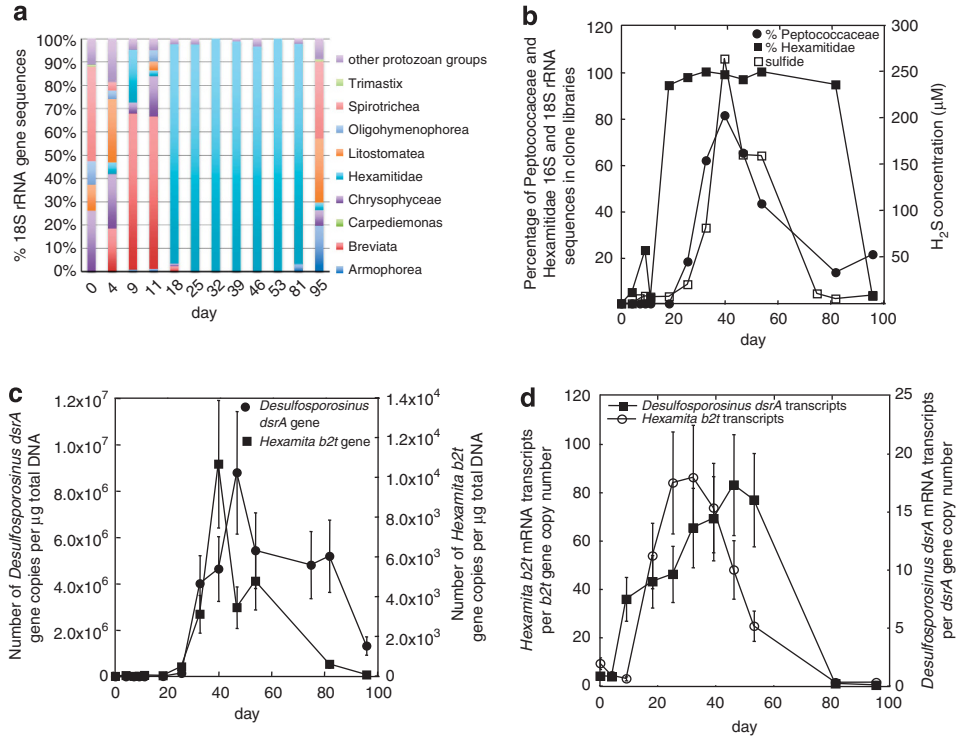

Figure 6 Analysis of the protozoan community associated with the sulfate-reducing phase of the 2011 field experiment. (**a**) Percentage breakdown of protozoan 18S rRNA gene sequences detected in clone libraries. (**b**) Percentage of *Hexamitidae* 18S and *Peptococcaceae* 16S rRNA gene sequences in clone libraries compared with sulfide concentrations. *Peptococcaceae* vs *Hexamitidae* rRNA sequences ($r=0.64$, $P=0.02$); *Hexamitidae* vs H_2S concentrations ($r=0.67$, $P=0.02$). (**c**) Comparison of *Hexamita β-tubulin* (*b2t*) and *Desulfosporosinus dsrA* gene copies per μg total DNA ($P=0.57$, $P=0.03$). (**d**) The number of *Desulfosporosinus dsrA* and *Hexamita β-tubulin* mRNA transcripts normalized against the number of *dsrA* and *β-tubulin* gene copies ($r=0.53$, $P=0.05$).

genus *Mastigamoeba* have been detected in anoxic sediments collected from a sewage-contaminated aquifer (Novarino *et al.*, 1997), a marine tidal flat (Dawson and Pace, 2002) and several freshwater ponds (Bernard *et al.*, 2000). Although previous descriptions of *B. anathema* have classified this organism as a microaerophile (Klebs, 1892; Walker *et al.*, 2006; Minge *et al.*, 2009), we were able to grow *Breviata* under strictly anaerobic conditions with *Geobacter uraniireducens* provided as a food source (data not shown). Protozoa related to the *Hexamitidae* species that arose with the growth of sulfate reducers following the addition of acetate have been found in other freshwater aquatic environments (Lee *et al.*, 2005; Luo *et al.*, 2005), some of which contain high sulfide concentrations (Horie, 1969; Luo *et al.*, 2005). In addition, *Trepomonas* species have been seen consuming bacteria in laboratory cultures, which provides support for *in situ* predation by *Hexamitidae* (Dujardin, 1841; Proceedings of Societies: Dublin Microscopical Club, 1879; Eyden and Vickerman, 1975).

At present, there is insufficient data to quantify the impact that *Breviata* and *Hexamitidae* species grazing on *Geobacter* and *Peptococcaceae* species might have on *in situ* U(VI) reduction. Previous studies have suggested that protozoan grazing can accelerate the metabolism of bacterial communities (Bloem *et al.*, 1988; Verhagen *et al.*, 1995; Strauss and Dodds, 1997; Biagini *et al.*, 1998) and may prevent the reduction of subsurface permeability by preventing the accumulation of bacterial biomass (Kinner *et al.*, 2002; Mattison *et al.*, 2002). However, when the concentrations of electron donor, that is, acetate, are high, as they were during the field experiments, the number of catalytic units capable of reducing U(VI) is likely to be a major factor controlling the rate of U(VI) reduction. Thus, grazing of *Geobacter*, and possibly other bacterial species, would be expected to lower the rate of U(VI) reduction. These considerations indicate that attempts to model predictively the dynamics of microbial growth and activity during *in situ* uranium bioremediation (Scheibe *et al.*, 2009; Lovley *et al.*, 2011; Mahadevan *et al.*, 2011) should include protozoan grazing.

Conflict of Interest

The authors declare no conflict of interest.

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