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Protistan Predation Affects Trichloroethene Biodegradation in a Bedrock Aquifer▽

Joseph J. Cunningham III,[1]* Nancy E. Kinner,[1] and Maureen Lewis[2]

Bedrock Bioremediation Center, University of New Hampshire, Durham, New Hampshire,[1] and Air Force Institute of Technology, Wright-Patterson Air Force Base, Dayton, Ohio[2]

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Despite extensive research on the bottom-up force of resource availability (e.g., electron donors and acceptors), slow biodegradation rates and stalling at *cis*-dichloroethene (cDCE) and vinyl chloride continue to be observed in aquifers contaminated with trichloroethene (TCE). The objective of this research was to gauge the impact of the top-down force of protistan predation on TCE biodegradation in laboratory microcosms. When indigenous bacteria from an electron donor-limited TCE-contaminated bedrock aquifer were present, the indigenous protists inhibited reductive dechlorination altogether. The presence of protists during organic carbon-amended conditions caused the bacteria to elongate (length:width, ≥10:1), but reductive dechlorination was still inhibited. When a commercially available dechlorinating bacterial culture and an organic carbon amendment were added in he presence of protists, the elongated bacteria predominated and reductive dechlorination stalled at cDCE. When protists were removed under organic carbon-amended conditions, reductive dechlorination stalled at cDCE, whereas in the presence organic carbon and bacterial amendments, the total chlorinated ethene concentration decreased, indicating TCE was converted to ethene and/or CO_2. The data suggested that indigenous protists grazed dechlorinators to extremely low levels, inhibiting dechlorination altogether. Hence, in situ bioremediation/bioaugmentation may not be successful in mineralizing TCE unless the top-down force of protistan predation is inhibited.

The bacterially mediated sequential dechlorination of trichloroethene (TCE) to *cis*-dichloroethene (cDCE), vinyl chloride (VC), ethene, and CO_2 by dehalorespiration is often proposed as the most cost-effective in situ treatment to remediate chlorinated solvent-contaminated aquifers (35, 42). TCE mineralization to CO_2 requires specific electron donors (i.e., acetate and H_2) typically produced from readily fermentable organic carbon, the presence of specific bacterial species, and sulfate-reducing or methanogenic conditions (1, 4, 8, 15, 22, 25, 33, 35, 46). When the rate of mineralization is slow or stalled at one of the progeny (cDCE and VC), the problem is usually attributed to the bottom-up force of resource availability (e.g., the absence of a necessary condition such as suitable electron donors or bacterial species) (1, 4, 10, 22, 26, 43, 46). For example, whereas many bacterial species are capable of degrading TCE to cDCE and VC by dehalorespiration (33), only *Dehalococcoides ethenogenes* is known to convert VC to ethene (25). Hence, if an indigenous population of *D. ethenogenes* is not present in situ, the system will likely stall at cDCE or VC even if sufficient electron donor is added. Stalling is problematic because VC is more toxic than TCE (18). In this case, bioaugmentation with *D. ethenogenes* may trigger complete mineralization.

An established link exists for the top-down force of predator-prey relationships between protists and bacteria in a range of surface water systems (13, 19–21, 29). Our previous work with groundwater protists in a wastewater contaminated sandy aquifer demonstrated that size selective predation by protists affects biodegradation of the organic carbon. Our subsequent work in a TCE-contaminated bedrock aquifer at the Bedrock Bioremediation Center (BBC) research site (Portsmouth, NH) suggested that bottom-up resource availability could not totally explain stalls at cDCE. This led us to hypothesize that the top-down force of selective predation by protists on dehalorespiring bacteria inhibited the required compositional shift in the bacterial community to one dominated by *D. ethenogenes*, thus preventing the conversion of cDCE and VC to ethene and CO_2 (2, 15). This, coupled with bacterial studies by Travis and Rosenberg (41), Lewis (32) and Snyder et al. (40), led us to postulate that protistan predation could have a negative impact on TCE biodegradation. Continuously stirred reactors were used to examine how the presence of protists influenced the rate of bacterially mediated reductive dechlorination. Experiments were conducted using ambient (≤0.8 mM as C) and amended (10 mM as C) organic carbon concentrations with protists present and absent. TCE biodegradation was also assessed when the indigenous community was amended with a commercially available bacterial culture containing *D. ethenogenes* (34).

(A portion of this research was originally submitted to the University of New Hampshire, Durham, by J. Cunningham as an M.S. thesis [12].)

MATERIALS AND METHODS

In experiment 1 (protists absent), protists were excluded from duplicate reactors using 0.8-μm-pore-size filtration, whereas in the second experiment protists were allowed to inhabit the reactors. (Note that preliminary experiments indicated 0.8-μm-pore-size cellulose nitrate membrane filtration removed the indigenous protist population without significantly affecting the abundance of indigenous bacteria.) Due to a leak developing in one of the reactors, as well as

* Corresponding author. Mailing address: University of New Hampshire, 237 Gregg Hall, 35 Colovos Road, Durham, NH 03824. Phone: (603) 862-0832. Fax: (603) 862-3957. E-mail: joseph.cunningham@unh.edu.

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difficulty in controlling the TCE concentration, experiment 2 (protists present) was repeated (i.e., runs 1 and 2). In experiment 3 (protists present with bacterial amendment), a bioaugmentation culture (KB-1; SiREM Laboratories, Guelph, Ontario, Canada) was added. In all of the experiments, the TCE degradation rate was determined for ambient (≤0.8 mM C) and amended (10 mM C) organic carbon conditions. Details can be found in a previous publication by Cunningham (12).

Two-liter reactors with Teflon-lined lids and dual sampling ports were created by using glass canning jars. Rocks obtained from an outcropping similar to the BBC site (fractured metasandstone and metashale) were split into fragments (2 to 6 cm long). Next, 500 g of the rock was suspended from the lids using nylon fishing line to mimic the fracture surface area to the porewater volume in the BBC fractured bedrock system.

Water for the reactors was obtained from BBC well 6 at a depth of ~36 m below ground surface and contained water that is typical for fracture rock aquifers in the region (pH ~8.5, < 1 mg/liter dissolved oxygen, <5 mM total organic carbon [TOC], abundant dissolved minerals). (Detailed water chemistry can be found in references 14, 16, and 17.) Well 6 is in a plume created by a leaking underground storage tank and contained ≤1.9 μM TCE, ≤1.5 μM cDCE, and <0.25 μM VC. The groundwater also contained an indigenous community of bacteria (size range, 0.3 to 0.8 μm) and nanoflagellates (1 to 5 μm) acclimated to the presence of the chlorinated ethenes. The reactors were housed in a nitrogen-filled glovebox at 10°C to maintain ambient subsurface conditions. Assembled reactors were autoclaved at 121°C at 15 lb/in^2 gauge for 60 min prior to the start of the experiment. Once the reactors were filled with ~1.5 liters of groundwater, ~0.62 ml of a 9.1 mM TCE solution was injected to achieve a final concentration of ~4 μM. The reactors were placed on magnetic stir plates (~100 rpm). For organic-carbon-amended conditions, a sodium lactate solution was injected into the reactors to yield a final concentration of ~10 mM C. For the bioaugmented experiment, 3 ml of KB-1 with an average density of 10^8 cells/ml was injected into the reactors as recommended by SiREM Laboratories.

During sampling, ~100 ml of water was withdrawn from the reactor by using a gastight syringe, and an equal amount of groundwater was injected to minimize headspace. TCE and sodium lactate, when appropriate, were added to the refill water to maintain the concentrations in the reactors at 4 μM and 10 mM, respectively (i.e., a draw-and-fill batch reactor).

The TOC samples were preserved with 2 ml of 1 N H_2SO_4 with a maximum hold time of 21 days prior to analysis by using the nonpurgeable organic carbon combustion method on a TOC-V CSH TOC analyzer (Shimadzu, Kyoto, Japan). The chlorinated ethene samples were analyzed by RLI Laboratories (Portsmouth, NH) using purge and trap gas chromatography with an electron capture detector in accordance with U.S. Environmental Protection Agency (EPA) guidelines (SWA-846, method 5030B/8260B; maximum hold time, 14 days).

Redox and pH were measured with a platinum redox probe filled with AgCl solution and an Orion pH probe, respectively. Then, 50 ml of sample was placed in a 60-ml sterile centrifuge tube, and redox and pH measurements were taken. Next, 3 ml of formalin was injected as a preservative, and the samples were stored for ≤21 days at 10 ± 2°C prior to bacterial and protistan analysis. For bacterial abundance, 5 ml of sample was passed through a 25-mm sterile membrane filter with a 0.22-μm pore size (catalog no. 110656; Whatman International, Kent, United Kingdom) under ~5 mm Hg of vacuum and then stained with 0.01% acridine orange (30). The stained slides were examined using ×1,000 magnification on a Nikon Optiphot-2 microscope equipped with a B2H filter cube and an external high-pressure mercury vapor lamp. The bacteria were enumerated by randomly selecting seven fields and counting the number of fluorescing green or orange. For protistan abundance, ≤35 ml of sample was passed through a 25-mm sterile filter with a 0.8-μm pore size (E08BP02500; Osmonics, Inc., Trevose, PA) and stained with primulin (6, 30). The stained slides were examined at ×400 magnification using a UV-2A filter cube. The entire filter was scanned because of the relatively low abundance of protists. For protists and bacteria, one slide was evaluated per sample taken from each reactor.

Quantitative PCR and terminal restriction fragment length polymorphism (TRFLP) analyses were conducted on four samples by Mary Voytek's laboratory (U.S. Geological Survey [USGS], Reston, VA). (Details of the procedure can be found in reference 27.)

The TCE degradation rates were calculated by graphing the chlorinated ethene concentrations (TCE, cDCE, VC, and total chlorinated ethenes) and visually identifying areas of active reductive dechlorination (exclusive of lag time). These data were plotted (ln concentration versus time), and the degradation rate constant and the contaminant half-life ($t_{1/2}$, ln 2/k) were calculated.

RESULTS

Protists absent. Protists were successfully removed by filtration in the protists absent phase of the experiment (days 0 to 82), during which biodegradation without protistan predation was examined. For the first 26 days, the reactors simulated in situ conditions (<0.8 mM TOC). No significant changes in chlorinated ethene concentrations, bacterial abundance or morphology were observed (Fig. 1a, 2a). From day 27 to 80, the reactors were amended with organic carbon. cDCE increased above ambient levels on day 40 and reached its first peak on day 67, coinciding with a proportional decrease in TCE, whereas the total molar chlorinated ethene concentration remained stable, indicating that reductive dechlorination was occurring. No further changes in TCE or cDCE were observed, nor did VC increase. The half-life of TCE under carbon-amended conditions was 5.7 days (Table 1). There was no significant change in bacterial abundance when TOC was amended (Student *t* test, $P = 0.05$).

On day 83, the 0.8-μm-pore-size filtration of the refill water ceased, and indigenous protists returned to the reactors. On day 85, TCE was injected into the reactors to raise the concentration to ~4 μM; it was rapidly converted to cDCE and eliminated by day 90. Concurrently, the protistan abundance increased and a morphological change was observed, with >90% of the bacteria becoming elongated (length:width [L:W], ≥10:1) by day 95 (Fig. 2a).

Protists present. The protists present experiment was repeated twice. Runs 1 and 2 lasted 51 and 100 days, respectively, and were used to examine bacterial biodegradation with protists present. Organic carbon was added on day 19 during run 1. Within 5 days, 90% of the bacteria were elongated (L:W, ≥10:1). This morphology remained predominant until the termination of the experiment. There was no statistically significant increase (Student *t* test, $P = 0.05$) in bacterial or protistan abundance. No changes occurred in any of the chlorinated ethene concentrations, (i.e., no biodegradation occurred).

The first 55 days of run 2 were under in situ conditions (<0.8 mM TOC). There were problems controlling the TCE concentration in the reactors because of a faulty injection syringe, and the planned concentration of 4.8 μM was exceeded. In spite of these problems, relevant data were obtained. Although the TCE concentration increased from ~2.7 to ~7 μM, the cDCE, VC, bacterial, and protistan concentrations remained stable (Fig. 1b and 2b). A total of 100% of the bacteria were coccoidal.

On day 55 organic carbon was added. The TCE concentration rapidly increased to ~6.8 μM but rapidly declined after the amount of TCE in the refill water was reduced in an effort to reestablish the desired concentration in the reactors. The TCE concentration reached the target range (4 to 5 μM) on day 80; however, it rapidly increased to ~7 μM and then ~10 μM on day 90. The problem was traced to the syringe and solved. Throughout this period, the cDCE and VC concentrations remained stable, whereas the total molar chlorinated ethenes closely tracked the TCE concentration, indicating reductive dechlorination was not occurring. Although the mean bacterial abundance was not significantly different (Student *t* test, $P = 0.05$) from that observed at ambient conditions,

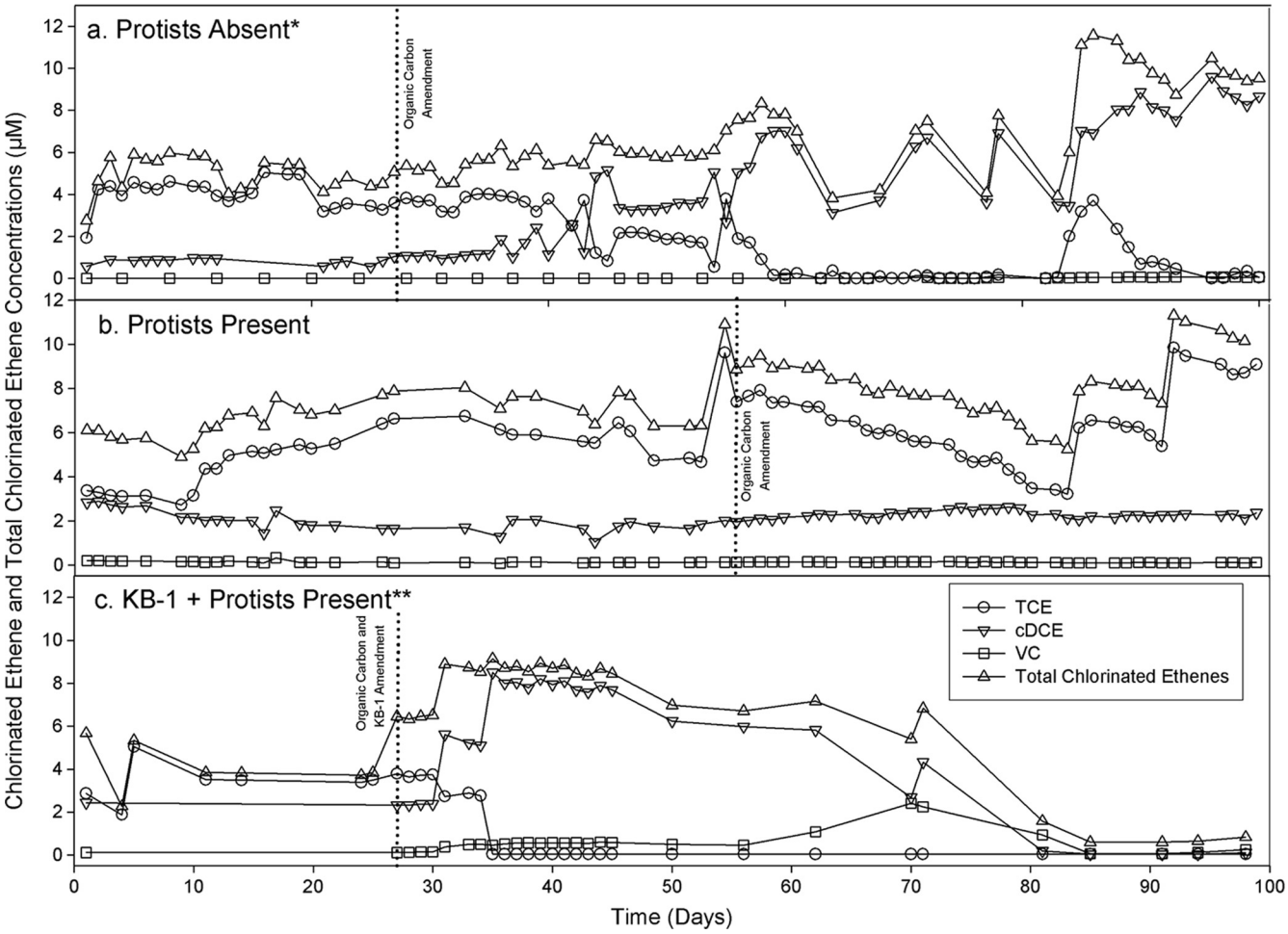

FIG. 1. TCE, cDCE, VC, and total chlorinated ethene concentrations (in μM) versus time with protists absent (a), protists present (b), and KB-1 plus protists (c). *, protists allowed to return on day 83; **, protists absent from days 70 to 100.

>90% of the bacteria became elongated (L:W, ≥10:1). The measured Eh was approximately −212 mV, indicating sulfate reducing conditions predominated. The protistan abundance was highly variable but not significantly different (Student *t* test, $P = 0.05$) from that observed under ambient conditions.

Overall, when protists were present, TCE was not degraded even when organic carbon was added. The organic carbon did stimulate the elongation of bacteria when protists were present.

Protists present with bacterial amendment. For the first 30 days during ambient conditions, no significant changes in TCE, cDCE, VC, or total chlorinated ethenes were observed; bacterial and protistan abundances were similar to those found in situ, and the bacteria were 100% coccoidal.

KB-1 and organic carbon were added on day 27, and an E_h (reduction potential) of –220 mV (sulfate reducing conditions) prevailed by day 37. TCE declined significantly by day 35 and approached the detection limit (0.07 μM), whereas the cDCE concentrations increased from ~2 μM to a peak of 7 μM. The concentration of cDCE remained constant from day 35 to day 45 and then began to steadily decrease, approaching the detection limit (0.10 μM) on day 80. There was an increase in VC from ~0.5 μM starting on day 55, peaking at ~2.2 μM on day 71. VC decreased to the detection limit (0.16 μM) on day 82

TABLE 1. First-order biodegradation constants and half lives

Treatment group	Mean TCE and cDCE reduction and production biodegradation constants (day^{-}) ± SEM[a]			
	TCE reduction	cDCE production	cDCE reduction	VC production
Protists absent	−0.120 ± 0.018 (5.7)	+0.184 ± 0.024	ND	ND
Protists present	ND	ND	ND	ND
KB-1 + protists	−0.589 ± 0.190 (1.2)	+0.817 ± 0.263	−0.184 ± 0.018 (3.8)	+0.059 ± 0.013

[a] ND, not detected. Half-life values are indicated in parentheses in days.

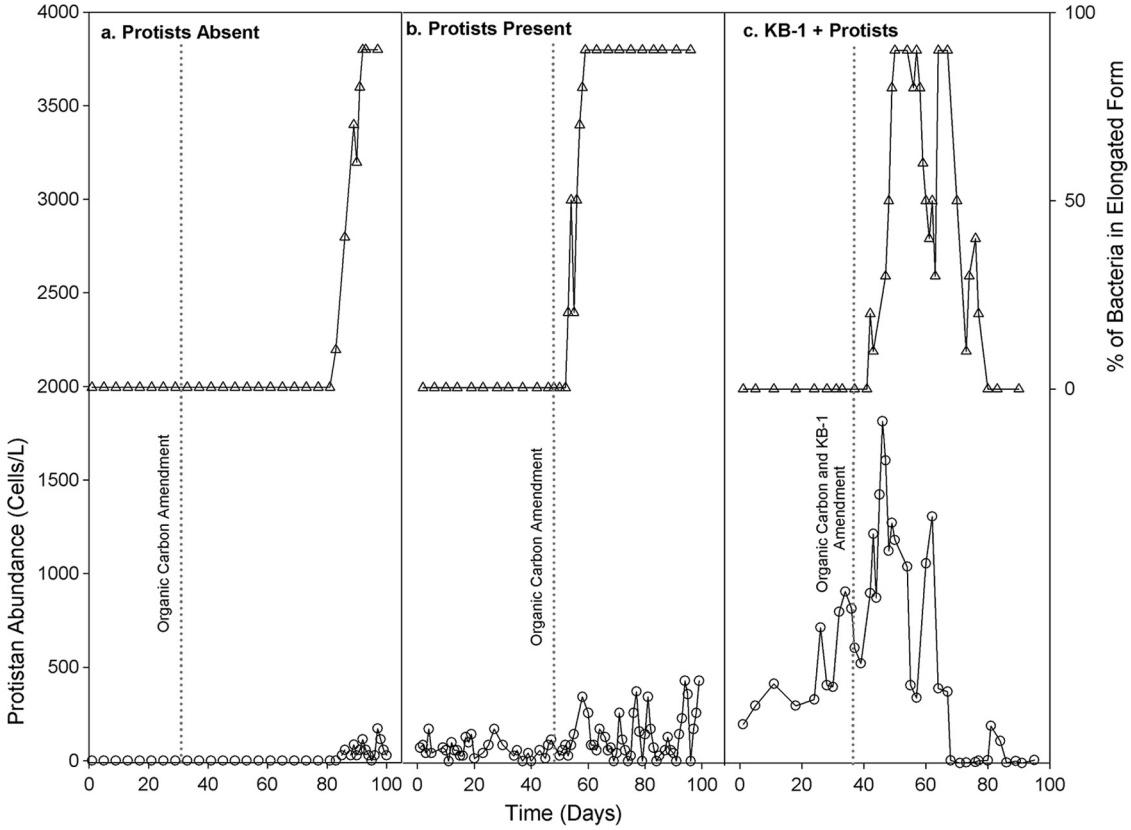

FIG. 2. Bacterial morphology as a function of protistan abundance versus time.

(Fig. 1c). Total chlorinated ethenes were stable at 8.6 ± 1.08 μM until day 71, when they rapidly decreased, suggesting VC was being converted to ethene and thus complete reductive dechlorination was occurring. We were unable to calculate the VC degradation rate because degradation was so rapid that insufficient data were collected. The half-lives for TCE and cDCE degradation were ~1.2 days and ~3.8 days, respectively (Table 1).

DISCUSSION

TCE and its progeny were not degraded when protists were absent and there were no amendments. Once organic carbon, which is limiting at the BBC site, was added, TCE was degraded to cDCE at rates similar to those observed in the literature (5, 7). The transformation of TCE to cDCE in the presence of sodium lactate indicated that bacteria capable of producing H_2 or acetate (1, 4) and removing the first chlorine atom from TCE were present in the BBC groundwater. The degradation "stalled" at cDCE even though adequate amounts of organic carbon were present, suggesting that the species of bacteria required for further dechlorination were not present. Overall, when protists were absent and only bottom-up forces prevailed, partial TCE degradation occurred.

TCE and its progeny were not degraded under in situ or amended conditions when protists were present, indicating top-down forces controlled. When sodium lactate was added, the redox potential of the reactors decreased to a level consistent with fermentation and possibly sulfate reduction, indicating that bacteria were using the sodium lactate and producing H_2 and acetate. Hence, protistan grazing was negatively impacting the bacteria that dechlorinate TCE. This corroborates experiments conducted by Lewis (32) and computer modeling by Travis and Rosenberg (41). The bacteria capable of dechlorination (e.g., *D. ethenogenes* and *Geobacter* spp.) are typically between 0.2 and 0.8 μm and within the size range ingested by subsurface nanoflagellates (31).

When KB-1 was added in the presence of protists and sodium lactate, TCE was rapidly converted to cDCE. The extremely high initial abundance of KB-1 bacteria likely temporarily overwhelmed the effects of predation, allowing TCE to be converted to cDCE. Ultimately, the protistan community responded in typical predator-prey dynamics and reestablished top-down control on the bacterial community. Protists do not actively "hunt" for bacteria but rather create currents with their flagella that allow them to graze upon bacteria that are drawn toward them (9, 11, 28, 29). This method of feeding reduces the probability that individual species will be grazed to extinction. Quantitative PCR and TRFLP analyses (43) suggested that the protists grazed the dechlorinating bacteria in KB-1 to extremely low levels, and cDCE was slowly degraded over a period of 20 days, with a concurrent increase in VC (Fig. 1c). One caveat to this hypothesis is necessary because little is known about the propensity of dechlorinators, including *D. ethenogenes*, to attach. Only aqueous samples were collected from the reactors, so the data only reflect the unattached

bacterial community. Although it appears that KB-1 species (e.g., *D. ethenogenes* and *Geobacter* spp.) were removed by grazing, it is possible that they attached to the reactor surface to seek refuge and reduce the probability of being preyed upon or to improve mass transfer kinetics (23, 24).

Once the VC concentration was ≥0.16 μM, protistan abundance decreased dramatically (Fig. 2c). Chlorinated ethene toxicity to groundwater protists has been previously proposed by Snyder et al. (40) as an explanation for decreased protistan abundance during TCE mineralization. Snyder et al. (40) did not report concentrations of cDCE, VC, or ethene and therefore were not able to demonstrate which compounds were toxic to protists. The results of the present study suggest that VC, not TCE, may be responsible for decreased protistan abundance during TCE biodegradation; however, little is known about the toxicity of TCE and its progeny to groundwater protists. With the top-down force of predation inhibited, the system switched to a bottom-up control, likely allowing the compositional shift discussed by Becker (2) and Duhamel and Edwards (15) to occur. VC and the total chlorinated ethene concentrations decreased, suggesting that *D. ethenogenes* introduced in the KB-1 inoculation, whose abundance had been limited by protistan predation, were active and likely converting VC to ethene.

These results indicate that the traditional concept of TCE biodegradation as bottom-up controlled is only correct in aquifers where protists are absent because of size exclusion or chemical inhibition. In these aquifers, the addition of electron donor and/or bacterial augmentation can result in complete TCE mineralization. However, if protists are present, and selectively graze key bacterial species, these bioremediation methods may result in partial or negligible biodegradation (i.e., stalls). This may explain the "hit or miss" nature of bioremediation at many sites: the top-down force of predation may be inhibited at some sites but a major factor at others. Hence, it should be a standard protocol to determine whether protists are present at a site prior to instituting bioremediation.

The impact of protistan predation can be mitigated by prey avoidance strategies (e.g., attachment, elongation, or top-down control on protistan predation). Typically, predators on nanoflagellates are larger than 5 μm; hence, they are unlikely to exist in the subsurface due to size exclusion, with the exception of hyporheic zones (3). Subsurface protists are essentially unregulated by top-down forces and, in the absence of toxic chemicals, only bottom-up forces control their abundance. In our experiments, bacteria elongated within 5 days of the organic carbon amendment when protists were present (Fig. 2). Bacterial elongation appears to be a direct response to protistan predation in the presence of elevated organic carbon within the reactors (39). (Note that no elongated bacteria have been observed at the BBC site and were not present in the refill water.) Bacterial elongation, to a size the predator is unable to consume, has been previously reported (37–39). Unfortunately, except for the first few days when protists were allowed to return during the protists absent experiment (Fig. 1a and 2a) elongated bacteria were not associated with TCE biodegradation even when high concentrations of organic carbon were present. Quantitative PCR and TRFLP analyses indicated that the elongated bacteria were of the *Gammaproteobacterium* genus, which have been shown to elongate under heavy predation (36–39) but are not known to perform reductive dechlorination.

Perhaps the most detrimental impact of protistan predation upon TCE biodegradation was what appeared to be selective grazing on *D. ethenogenes*. These bacteria are the key to mineralization of TCE and are a major constituent of bioaugmentation amendments (e.g., KB-1). Our experiments suggest that further research needs to be performed to determine: (i) the specific grazing rates of protists on key dechlorinators and their influence on community structure, (ii) how predation can be controlled or eliminated, and (iii) what strategies can be developed to make key dechlorinators such as *D. ethenogenes* less susceptible to predation (e.g., elongation and attachment).

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