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In Situ Bioremediation of Trichloroethylene Using *Burkholderia cepacia* G4 PR1: Analysis of Microbial Ecology Parameters for Risk Assessment

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Abstract

The introduction of bacteria into aquifers for bioremediation purposes requires monitoring of the persistence and activity of microbial populations for efficacy and risk assessment purposes. Burkholderia cepacia G4 PR1 constitutively expresses a toluene ortho- monooxygenase (tom) that aerobically mineralizes TCE. Groundwater and sediment from a potential release site have been used in laboratory microcosms to develop predictive models for the response of this organism. In sterile systems, PR1 maintains stable populations for extended periods. In non-sterile systems, the bacterium is eliminated concomitant with an increase in bacterivores. The half life for the organism in non-sterile systems increases logarithmically with increasing initial inoculation density above 1 x 10^6 PR1 ml^{-1}. Below this level of inoculation, the half life of PR1 increases with decreasing inoculation density. The inflection point corresponds to a numerical response threshold for bacterivores. In column systems designed to mimic aquifer flow, repeated pulses of PR1 build up bacterivore populations reducing the half life of the bacterium for subsequent additions. Addition of 0.5 µmM TCE in the elution stream results in prolonged survival of PR1. The results suggest that abiotic factors are not limiting to the bacterium in the target aquifer, but rapid losses from native bacterivores will occur.

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Introduction

The application of biotechnology to solve biological and ecological problems is becoming widespread in medicine and agriculture. The potential for use of genetically engineered or altered microorganisms (GEMS) for bioremediation of aquifers contaminated with toxic materials is high, despite the problems of physical access to this environment to define the ecological parameters for optimal microorganism function and to determine the extend of any risk involved in releasing non-native microorganisms into unconfined groundwaters. In natural communities, bacteria are subject to starvation, competition, predation and viral infection pressures in addition to limits imposed by physical and chemical conditions. It is these selective forces that maintain bacterial populations at relatively stable numbers in the environment. For successful bioremediation with introduced microorganisms (bioaugmentation), selective pressures must be altered within a target zone to maintain the high densities and activity required for treatment. If the goal of the introduction is activity through growth of the GEM rather than using GEM biomass as a static reagent, then the carrying capacity and potential niche space within the target environment for the GEM must be considered. Beyond a treatment zone where niche space may be altered for GEM survival, persistence and spread of GEMs and their genes require integration of the foreign bacterium (or genes) and displacement of species from existing microbial communities that have been derived by the selective pressures unique to that environment.

Analyses of population dynamics, trophic interactions and microorganism transport are critical for the application of successful bioaugmentation and require means to monitor both organisms and activity. Risk assessment associated with the introduction of non-native bacteria also requires a careful monitoring of the survival and dispersal of released microorganisms and altered genes. Although releases of non-native or recombinant bacteria have not been reported to result in adverse environmental effects to date, there is a responsibility to ensure that released microorganisms will be constrained by the selective pressures of the target environment. Monitoring of released populations of microorganisms thus aids in the final evaluation of both success and safety.

The use of chloroethenes including trichloroethylene (TCE) has led to an extensive contamination of groundwater resources in the United States. *In situ* bioremediation of this contaminant may be possible by the aerobic microorganism *Burkholderia cepacia* G4. *B. cepacia* G4 $PR1_{23}$ and $PR1_{31}$ constitutively express a toluene *ortho*-monooxygenase (*tom*) due to a secondary transposition of *Tn*5 sequence into a TCE degradative plasmid (TOM) (Nelson *et al.*, 1986; Shields *et al.*, 1992). Tn*5* mutagenesis also confers kanamycin (km) resistance to the organisms. $PR1_{23}$ contains an IS50R in the TOM_{23C} plasmid. $PR1_{31}$ contains a single Tn*5* in the chromosome and an IS50R in the TOM_{31C} plasmid. The IS50R elements in the plasmid of both strains are at nearly the same locations and are thought to be responsible for the constitutive expression of the tom gene.

This paper presents laboratory analysis of the behavior of *Burkholderia cepacia* G4 PR1 (PR1) in simulated aquifer conditions to address both function and fate questions for use of this microorganism in remediating TCE contaminated ground water. The laboratory analysis was targeted for a release at the "Borden Aquifer," a shallow sandy aquifer located on the Canadian Forces Base Borden, Alliston, Ontario.

Procedures

Media, Growth, and Cell Counts. PR1 was maintained as a frozen stock and on a basal salts medium (BSM) with 20 mM lactate and 1.5% agar in petri plates for working cultures. For experimentation, cells were grown in batch cultures in BSM 20 mM lactate, glucose, phenol, m-cresol, or phthalic acid. A commercial spring water, Georgia Mountain Water (GMW) was used in place of a limited supply of Borden aquifer water where bulk use was required. Cell cultures were routinely checked for TOM activity using the TFMP assay (described below). Enumeration of total CFU in aquifer samples was done using Difco R2A agar.

Direct counts of total bacteria and protists in formalin or Lugol's fixed samples were performed using the DNA fluorochrome DAPI (Porter & Feig, 1980; Pomeroy, 1984). Minimum numbers for direct counts of bacteria were 10 microscope fields and 200 cells. Minimum numbers for protist DAPI counts were 10 fields and 100 cells. Plate counts of culturable bacterial cells were performed by spread plates (50-100 μl) of serial dilutions. Where possible, plate counts within 10-100 CFU/plate were used.

Tracking Methods. Four methods of tracking and confirming PR1 have been developed.

1) Selective plating was based on phthalic acid, phenol, and cresol utilization and the presence of kanamycin (km) resistance associated with *Tn5*.

2) A monoclonal antibody specific to PR1 LPS has been used for direct fluorescent counts of PR1 from suspensions and for identifying colonies on filter blots of agar plates as previously described (Winkler et al., 1995).

3) A colorimetric reaction (triflouromethyl phenol, TFMP to triflouroheptadienoic acid, TFHA) was used to assay for the toluene monooxygenase enzyme that degrades TCE (Shields *et al.*, 1991). This reaction was used for both activity determinations in cell suspensions and for identifying colonies on plate blots. For cell suspensions, absorbance at 600 nm (A_{600}) was determined for 1 ml. Cells are then pelleted and resuspended in 1 ml of 1.0 mM TFMP in 10 mM Tris-Cl pH 8.5. The suspension was incubated in a 25ml Erlenmeyer flask at 30°C for 20 min. Cells were repelleted in a 1.5 ml Eppendorf tube to transfer the cell-free supernatant to a microcuvette and read absorbance at 386 and 600 nm. To base the reaction on protein, a conversion factor of A_{600} x 0.290 provided an estimate of mg/ml protein. To convert to μM TFHA, absorbance at 385 nm was multiplied by 0.0269. For distinguishing between colonies on agar plates, filter paper with colony blots was soaked with 1.0 mM TFMP in 10mM Tris-Cl pH 8.5, and any color change recorded.

4) A nucleic acid thermocyler amplification assay using primers unique to the *Tn5* insertion site has been developed and tested. The primers were designed based on the assumption that the insertion points would be unique for PR1. This assay was performed for both extracted DNA from sediment samples and as an assay for whole cells in ground water suspension.

Protist MPN. PR1 was grown on BSM lactate km medium to stationary growth phase, pelleted, and washed free of metabolites and resuspended to an optical density (OD) of 0.2 @ 480 nm on an HP spectrophotometer.

Using Corning 24-well culture plates, 1 ml of the suspension of bacteria was placed into each, with 8 wells per dilution of sample and six dilutions (10^{-1} to 10^{-6}). Approximately 1 ml subsamples of Borden aquifer material were removed from core material of the saturated zone and placed in pre-weighed 15 ml disposable Falcon centrifuge tubes. Each sample tube then received 10 ml of filter sterilized Borden aquifer water (BAW). Dilution tubes (5 each) for each subsample received 9 ml of BAW. Tubes with the soil/water sample were vortexed well for approximately 1 min and allowed to stand for approximately 20 sec to allow larger sand grains to sediment. Eight 1 ml portions of the diluted sediment were distributed to 8 wells in the 24-well plate to establish a 10^{-1} dilution with a final prey density of 0.1 OD. This was repeated for serial dilutions made from the initial 10^{-1} suspension. Initial dilution tubes containing sediment were dried and weighed to obtain the gram dry weight (gdw) of each sample.

Plates were examined every two or three days starting on day 4 and extending through day 28. Plates were viewed on an inverted microscope (Zeiss) and scored positive or negative for flagellates, ciliates, naked and testate amoebae.

Viruses. An attempt was made to isolate viruses capable of infecting PR1 from Borden aquifer sediment. Sediment was sterilized with chloroform vapor under vacuum for 1 week to eliminate native microorganisms, then allowed to air dry for 2 days. PR1 was grown on BSM 20 mM lactate medium, washed and resuspended in sterile spring water to an OD of 1.0. The PR1 suspension (10 ml) was placed into a Falcon 15 ml centrifuge tube with 10 mM lactate and approximately 2 ml of the air-dried sterile sediment. This sediment/PR1 suspension was mixed on a rotatory wheel for 2 hours, followed by a 1 hour settling time. The supernatant was then mixed 50:50 with BSM 10 mM lactate with 0.75% agar and poured over BSM 10 mM 1.5% agar in petri plates. These pour plates were monitored for 13 days for clearing zones (plaques) indicating lysis of PR1.

Groundwater Survival. Survival in sterile and non-sterilized Borden ground water was determined by plate counts (Phthalate/Km), 4',6-diamidino-2-phenylindole (DAPI) counts and direct immunofluorescence counts (fmab) and was previously reported for a 30-day incubation (Winkler *et al.*, 1994). Mab blots of plate counts and DAPI counts of this presterilized treatment were subsequently obtained after a 7 month duration.

Anaerobic Effects. *Survival of PR1 deprived of* oxygen was monitored in suspensions of cells in sterile ground water bubbled with 0.2 μm filtered N_2 gas. Incubations were maintained in a constant temperature bath and continuously agitated with magnetic stir bars. Temperature and oxygen concentrations were continuously monitored using Nessler oxygen probes and meters connected to a 386 MS-DOS computer fitted with a data acquisition card. DAPI direct counts and CFU response were determined over time.

Extinction Rates in Sediment Slurries. Sediment slurries were set up with approximately 10 g wet weight of Borden aquifer sediment and 30 mls of Borden aquifer water in 125 ml flasks or 100 ml serum bottles. Repeated experiments were conducted at various initial densities of PR1 ranging from 9×10^3 to 9×10^8 cells ml^{-1} of slurry. A typical experiment compared uninoculated controls, flasks receiving nutrient addition only, PR1 only, and PR1 plus a nutrient. Other experiments compared different levels of inoculated PR1 only. Slurries were kept at constant temperature (18 and 10°C) and mixed at each sampling point. Numbers of PR1 were monitored over time by plate counts, confirmed by monoclonal antibody or TFMP blots. DAPI counts of both total bacteria and protists were recorded.

Extinction rate constants were determined by regression analysis as the slope of natural log-transformed loss data. Extinction rate constants (k) were determined by regression analysis as the slope of linear portions of natural log-transformed cell loss data using the formula.

$$k = \frac{\ln N_{to} - N_{tf}}{t_f - t_o}$$

These estimates included both growth and predation losses of the bacterium. Dividing these extinction rate constants by 0.693 (natural log of 2) provides a time interval for reduction of the population by half (half life). These half life estimates were then plotted as an inoculation-density dependent function and fitted to a predictive regression model, providing an estimator for survival times of PR1 in the aquifer.

Growth responses of the bacterivorous protist assemblage in Borden sediment were also determined from cell count data and growth rate constants calculated similarly as the extinction rates. Growth rate constants were plotted as a function of inoculated PR1 density.

PR1 Response in Flow Columns. Sediment columns were established to mimic the groundwater flow at the Borden site. A chromatography column was fitted with cut GC vials closed with Teflon septa to provide sampling ports. Teflon tubing and fittings were used for all flow lines containing TCE. Tygon tubing connected to a constant temperature recirculating bath was used to jacket the column and maintain it at 15°C. Flow was controlled at the column outflow and set to the flow rate in the target environment (2 cm day^{-1}). GMW was used as the eluant. Cells were added by syringe pump. An overflow ensured constant supply of eluant, or cell suspension, to the top of the sediment. Pore water samples were taken by syringe (200-0 μl each) and used for plate counts and direct counts of bacteria and protists at each sampling port.

Typically, an inoculum of two void volumes at 10^8 cells ml^{-1} was added to the column, followed by continued flow of GMW or GMW + 0.5 μM TCE. Samples (200 μl) from each port were taken by syringe from the center of the sediment to avoid edge effects. Samples were split for plate counts and preserved for direct counts. Samples for TCE analysis where taken on alternate days to avoid large disturbances of the flow field by removal of large volumes.

Potential Host Range for the TOM Plasmid. Twenty-four random isolates on R2A medium from Borden aquifer sediment were used to determine the potential spread of the plasmid from $PR1_{31}$. Transformations were attempted by standard direct filter matings. Antibiotic sensitivity of the isolates and PR1 were determined to provide for selective plating of donor, recipient and transconjugant. Overnight cultures of $PR1_{31}$ were grown in BSM-20 mM lac. Potential recipients were grown in R2A broth (Difco). Five mls of donor and recipient were pelleted separately. Each pellet was resuspended in R2A broth to A_{600} OD=1.0. Viability of each was checked by spread plate. One ml of each culture was transferred to a 5ml test tube and mixed. The mixed suspension was filtered onto 0.2 μm membrane filters with a presterilized swinex and sterile syringe. The filter was then placed on an R2A agar plate. The filter was incubated at 30°C overnight. The filter was then transferred to a tube containing 1ml R2A broth and vortexed. Resulting suspension was plated on selective media. Selectivity of media was checked by plating an aliquot of donor and recipient. Putative transconjugants were tested for positive TFMP reaction, indicating the presence of the TCE-degrading toluene monooxygenase, TCE mineralization and presence of the TOM plasmid by the nucleic acid amplification assays.

Transport. PR1 and transconjugants were tested for their relative transport potential in Borden aquifer sediment. Cells were grown on R2A broth medium and harvested at stationary growth phase. Cells were pelleted and washed free of medium and metabolites and adjusted to 0.1 OD @ 480 nm. Columns were set up using 2 ml glass syringes filled with sterilized Borden sediment packed under water saturation and vibration. Flow was controlled at the column outflows and set to the flow rate in the target environment (2 cm day^{-1}). One void volume of cell suspension was added to the columns and chased by sterilized Borden aquifer water. Fractions of 3 drops each were collected at the outflow in 96-well tissue culture plates. Fractions were serially diluted and plated on selective media. Output concentrations were plotted as a percentage of the inflow concentration to determine relative transport propensity for the different bacteria.

Results and Discussion

Thermocyler Amplification of TOM Sequences. This assay provided a definitive test for the presence of the PR1 degradative plasmid. Lower detection limits determined by dilution of cells in Borden water and sediment slurries yielded approximately 1×10^2 and 1×10^3 cells ml^{-1}, respectively.

MPN for PR1 Predators. MPN data for PR1-consuming bacterivores from four separate subsamples of Borden aquifer material indicated numerous protists in the aquifer system. Ciliates were rare, but recorded at a mean (± standard deviation) MPN value of 0.678 ± 0.255 gdw^{-1}. The ciliate observed is apparently an undescribed species of Hymenostome and is the first report of a ciliate from aquifer sediments. Means and standard deviations for other protists were: flagellates, $1.71 \times 10^4 \pm 2.13 \times 10^4$ gdw^{-1}; naked amoebae, $2.20 \times 10^3 \pm 1.49 \times 10^3$ gdw^{-1}; and testate amoebae $3.22 \times 10^2 \pm 1.77 \times 10^2$ gdw^{-1}, indicating a prevalence of potential PR1 predators in the target environment. Attempts were made to isolate PR1 viruses, but none were recovered.

Groundwater Survival. PR1 was recoverable after 7 months incubation in sterilized aquifer water, but was quickly eliminated in non-sterile water. Response of PR1 to anaerobic conditions indicated little effect of oxygen deprivation on $PR1_{31}$ culturability through 2 days at 20°C and through 25 days at 15°C, although at reduced numbers. Direct counts with the DNA fluorochrome DAPI remained constant through the incubation period indicating the cells and DNA of PR1 remained intact despite a loss of culturability. This finding is significant in that most aquifers are anaerobic, and prolonged

persistence of the bacterium beyond an aerobic treatment zone will not be limited by anaerobiasis. These preliminary experiments suggested that abiotic factors of the target environment would not be limiting to PR1 survival, but biological interactions were potentially important.

Potential Host Range of TOM Plasmid. Out of 24 random isolates, 10 (42%) were positive for TOM_{31C} transfer by detection of TOM target sequences. *Tom* enzyme activity was present in 80% (8 of 10) of the transconjugants as indicated by a positive transformation of trifluoromethyl phenol (TFMP) to trifluoroheptadianoic acid (yellow product) and mineralization of TCE. These data indicate a wide potential host range within the target environment for TOM, highlighting the need to ensure tracking capability for both the organism and its associated genetic elements.

Transport of PR1 and Transconjugants. Five out of seven positive transconjugants had greater relative transport through Borden sediment material than did PR1. These data suggest that plasmid transfer within the aquifer may be a major factor in determining the spread of the novel DNA within the system. Detailed analyses of transport phenomena associated with PR1 and Borden aquifer material have been presented elsewhere (Lawrence & Hendry, 1996, 1998; Hendry et al., 1997).

Extinction Rates in Sediment Slurries. In aquifer sediment slurries, numbers of native bacteria isolated on ph/km medium varied below an upper limit of approximately 5×10^5 CFU ml^{-1}. Direct epifluorescence microscopy counts of bacterivorous protists were below 8×10^2 ml^{-1} in unamended incubations. Where PR1 was added, selective plate counts, as confirmed by monoclonal antibody blots, decreased to zero concomitant with a rise in protist numbers. CFU of phthalate-utilizing bacteria increased following protist mineralization of PR1, suggesting that general enrichment of the system will increase population levels of other bacteria. None of these isolates, however, were found to have acquired TOM plasmid from PR1 in any of these experiments. Addition of substrate with PR1 (phenol or phthalate) had no significant effect on PR1 survival.

Total protist population response increased with the decrease in PR1 at inoculation from 1×10^8 to 5×10^8 cells ml^{-1}. Maximum protist response did not increase when the inoculation density was raised from 5×10^8 to 1×10^9 cells ml^{-1}. However, maximum protist population response was sustained for a longer duration, and precipitous loss of PR1 does not occur at the 1×10^9 cells ml^{-1} inoculation density until after 22 days of incubation.

Extinction rates fit well to a logarithmic curve, providing an estimated life span for PR1 within a treatment zone, and indicate a threshold of 1×10^8 ml^{-1} to 2×10^8 ml^{-1}, above which further increases in survival rate with increased inoculation density is minimal. In addition, extinction to a lower threshold of 1×10^7 bacteria ml^{-1}, the level of PR1 considered necessary for significant TCE mineralization activity, is shown in Figure 1. This predictive model provides an estimated cycling time for maintaining high densities within a treatment zone.

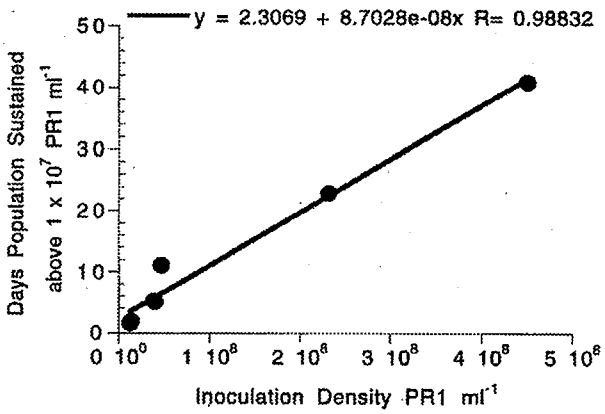

Figure 1. A predictive model for maintaining PR1 populations above a 1×10^7 cell ml^{-1} threshold.

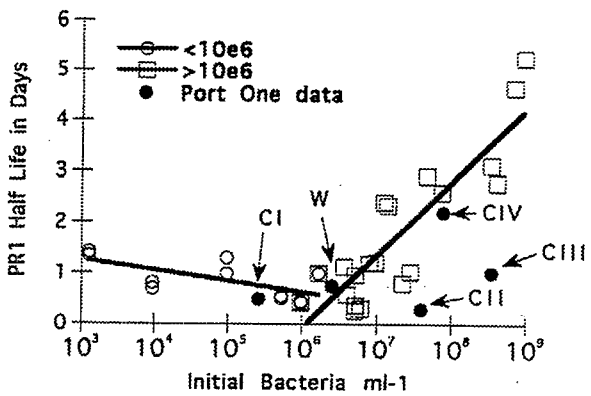

Figure 2. Half-life prediction models based on sediment slurry incubations and half-life estimation for PR1 in flow columns. CI through CIV refer to separate column experiments.

The lowest survival rates occurred at inoculation densities around 1 x 10^6 PR1 ml^{-1}. Inoculation densities above and below this level resulted in increased survival times. This phenomenon is shown in Figure 2. The inflection point of lowest PR1 survival coincides with a threshold of response for bacterivores, as indicated by the numerical response curve for Borden aquifer protists grown on PR1 shown as Figure 3. This growth response of the native bacterivore assemblage has its greatest range of increase at 10^6 PR1 ml^{-1} and above. Thus, inoculation at or above the inflection point stimulates bacterivore excystment and numerical response, accelerating bacterial losses. Inoculation below this point, as would occur as a plume of bacteria leaving a treatment zone enters the downstream aquifer, would result in bacterial cell losses being restricted to encounters with existing active bacterivores, and survival rates are extended. Given that the densities of native bacteria in the ground water are below the inflection point, most bacterivores in the unamended aquifer are probably either encysted or just meeting maintenance energy requirements. At low PR1 densities, predator-prey contact rates in the sediment slurries would have been conceivably higher than contact rates that would occur in the undisturbed aquifer, making these estimates an upper limit to removal rates in the natural system.

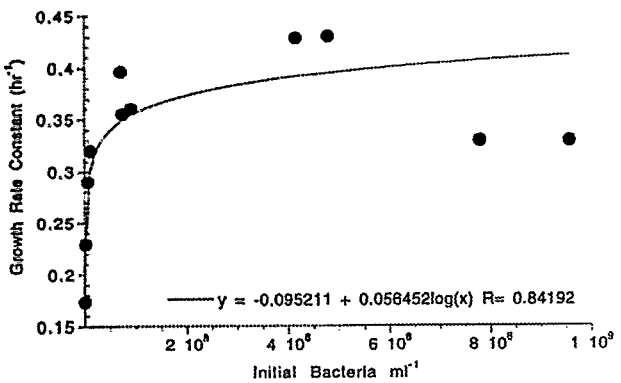

Figure 3. Bacterivore numerical response from sediment slurries.

PR1 Response in Flow Columns. With an inoculum of two void volumes at 1x 10^8 cells ml^{-1}, a population above 10^7 CFU ml^{-1} pore water was maintained for 5 days at the top of a column. Native bacterivores responded to the addition of PR1 to the column by increasing protist numbers in proportion to the decrease of PR1 cells. A linear decrease in PR1 numbers with distance through the column was observed prior to bacterivore impacts. By days 8 and 10, the combined effect of predation and elution decreased PR1 numbers in the upper portion of the column. By day 15, the pulse of PR1 was eliminated at the upper and lower portions of the column, leaving residual cells in the central portion. Most of the bacterivores form resistant cysts on sediment surfaces when food is not available, so a decrease in protist numbers over time is likely due to re-encystment of these organisms after depletion of PR1.

Data from subsequent runs demonstrated accelerated losses, presumably due to an increased reservoir of encysted bacterivores. A run with TCE added to the inflow had greater persistence of PR1, with a sustained population above 10^7 CFU ml^{-1} through 9 days of elution. TCE breakthough did not occur until after the sampling at 15 days of elution. Half-life estimates for data collected at the topmost sampling port indicate that survival rates are equal to or lower than the survival rates determined for the sediment slurries (Figure 2). The relatively slow transport of PR1 through Borden sediment limits the effects of elution on the disappearance of cells from the sediment, but repeated inoculation increases bacterivore populations and lowers survival rates (CII and CIII points). Addition of TCE to the column (CIV) appeared to have inhibitory effects on bacterivores and increased PR1 survival to similar rates as found in the slurries where bacterivore impact was affected by the limitation of growth response to the inoculated bacteria. The toxicity of TCE to bacterivores may restrict the effect of the built-up reservoir of encysted bacterivores. If this scenario is correct, then as TCE concentrations are reduced, bacterivore impact would increase, limiting the dispersal of PR1 in the non-contaminated parts of the system.

This study has shown that the abiotic conditions of the aquifer do not appear to be detrimental to survival of this organism, but that biological interactions may be of primary concern. Predation has often been demonstrated to affect the removal of allochthonous bacteria and certainly is a factor where inoculation is above the lower growth thresholds for bacterivores. Competition is also often cited as the cause of inoculated bacteria disappearance. Competitive ability for available growth substrates and resistance to starvation would be strong determinants of microorganism displacement and survival, although the ability to integrate cooperatively into existing microbial communities may be equally important. In this investigation, competition was not investigated directly, although the increased survival rate observed for inoculation below bacterivore excystment and growth thresholds suggests that competition may be less important than predation. Competition between bacteria may also be manifest in the ability to attach and colonize

surfaces where growth substrates are more readily obtained, especially in habitats with low organic content. Surface colonization and availability of micropore spaces may also provide a refuge from bacterivores.

This work has sought to apply ecological principles in defining the interactions that determine persistence and activity of GEMS in the environment. Growth substrate availability, competition, and predation are dominant forces that control bacterial densities and community structure in natural habitats. The reactions of introduced microorganisms to these selective forces will determine their persistence and activity, especially when the target concentrations of bacteria are above normal thresholds for natural systems.

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