Ground Water Monitoring&Remediation

Microcosm Assessment of a DNA Probe Applied to Aerobic Degradation of *cis*-1,2-Dichloroethene by *Polaromonas* sp. Strain JS666

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Abstract

A molecular biological tool based on an organism-specific DNA sequence does not necessarily indicate in situ activity but serves important functions of evaluating the potential for biodegradation and mapping the distribution of an organism. Currently, DNA-based probes are accepted as evaluative tools for site assessment. However, these techniques are far from standardized, and information on precision is usually lacking. Here, we present the development and evaluation of a DNA probe for *Polaromonas* sp. strain JS666, a bacterium that couples growth to aerobic oxidation of *cis*-1,2-dichloroethene (cDCE), and is therefore a promising candidate for bioaugmentation at sites where cDCE has accumulated in aerobic zones. The DNA probe was used in conjunction with quantitative polymerase chain reaction to track the abundance of JS666 in microcosms. This series of studies has allowed explicit resolution of the accuracy and precision of the probe and its correlation with variations in microcosm performance. We determined that the method is sufficient to monitor distribution of JS666 at bioaugmented sites. We found within environmental, mixed cultures, that the DNA target does not persist long after cell death, demonstrating that positive result from the probe is a strong indicator that degradation can occur in suitable environmental conditions. Finally, in the absence of suspected predation, the probe accurately and precisely tracks growth. Collectively, the studies appear to validate the utility of the molecular probe for site assessment in a bioaugmentation context.

Background

Chlorinated ethenes are among the most prevalent groundwater pollutants in the United States and are on the U.S. Environmental Protection Agency's (EPA's) list of primary regulated drinking water contaminants (EPA 2000). The cleanup of these and other volatile organic compounds is estimated to cost "more than $45 billion dollars (1996 dollars) over the next several decades" (EPA 2000). Of the technologies used to remediate contaminated sites, in situ bioremediation is recognized as being a promising and cost-effective solution (Lovley et al. 2003; Ritalahti et al. 2005). However, bioremediation depends on suitable conditions, including the presence of proper organisms with the metabolic capacity to degrade the pollutants, and favorable geochemical parameters. Even if those conditions are met, a site may require amendments to stimulate degradation. In cases where microorganisms with the degradative capacity are absent, sites will require augmentation with microorganisms (Ellis et al. 2000; Major et al. 2002; Rahm et al. 2006a).

Site characterization is essential to successful bioremediation, which typically involves three lines of evidence: (1) demonstration of the reduction of contaminant mass; (2) the potential for biodegradation, which can be demonstrated through geochemical data such as dissolved oxygen levels or redox potential; and (3) demonstration that microbial activity is responsible for observed reduction in contaminant levels (EPA 2000). This final line of evidence is often demonstrated through microcosms and/or column studies and is especially important in aerobic systems where, unlike reductive dehalogenation, there are no easily distinguishable daughter products to demonstrate biodegradation (Freedman et al. 2001). However, microcosm and column studies are time consuming and expensive and do not necessarily reflect in situ conditions, as the act of sampling a site may change the condition of the system (Hurst et al. 2002). It has been recently shown that compound-specific isotopic analysis can be useful in demonstrating aerobic biooxidation of *cis*-1,2-dichloroethene (cDCE), though few laboratories currently have the capability to perform these analyses (Abe et al. 2009).

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Molecular biological tools (MBTs) that can quantify the presence of desirable microorganisms (or, better yet, their activities) in situ are useful at both the decision-making stage when evaluating alternative technologies as well as in the monitoring of an implemented bioremediation technology (Stroo et al. 2006). MBTs can conceivably be based on DNA (targeting either phylogeny or genes of a degradative pathway), messenger RNA (mRNA; targeting upregulation of degradative genes), or proteins (targeting the degradative enzymes themselves). At present, there have been a limited number of studies conducted exploring the potential of mRNA-based probes (Holmes et al. 2004; Rahm et al. 2006b; Rahm and Richardson 2008a, 2008b) and even fewer that have explored their use in soil and groundwater (Baelum et al. 2008; Holmes et al. 2005). Although mRNA-based probes would appear conceptually to have greater potential to correlate with activity than would DNA-based probes, mRNA is very short lived and difficult to recover (Baelum et al. 2008). Because of this, the technology for their use has not yet reached practical utility and will likely be most effective in tandem with other molecular techniques and/or other more traditional methods of detection.

An MBT that relies on an organism-specific DNA sequence also has benefits and drawbacks. DNA is relatively stable in the environment, which makes it easier to work with. However, it is not directly a measure of activity and might remain after cell death (Chen et al. 2007; Nocker et al. 2006), though one might expect DNA from dead cells to be degraded relatively rapidly (i.e., over days rather than weeks) within environmental, mixed cultures. DNA probes can serve the important purposes of showing the capability for biodegradation and also the spatial/temporal distribution of an organism following enhancement and/or bioaugmentation (Scheutz et al. 2008).

Currently, DNA probes, even those based on phylogeny and not function, are accepted as an indication of potential for degradation at bioaugmentation sites and are both qualitative (indicating presence) and quantitative (demonstrating abundance) (Ritalahti et al. 2005). The three requisite lines of evidence, including evidence from DNA-based probes, have been presented in successful bioaugmentation of sites affected by chlorinated ethenes through the process of reductive dechlorination (Major et al. 2002; Rahm et al. 2006a; Scheutz et al. 2008). In aerobic remediation where the end products are difficult to measure, the ability to monitor organisms could contribute to evidence that the process is microbially mediated (Dominguez et al. 2008). However, these techniques are far from standardized, and each probe requires an amount of examination to determine its utility. Work conducted with DNA-based probes in the context of aerobic remediation of contaminants such as chlorobenzenes and aromatic hydrocarbons include probes based on specific catabolic genes (Beller et al. 2002) and also on entire suites of oxygenases used by a number of different organisms (Baldwin et al. 2003). Studies involving MBT application for the aerobic remediation of chloroethenes have been limited to cometabolic systems probed for the genes of enzymes with broad substrate specificity such as nonspecific monooxygenases and are not quantitative (Hazen et al. 2009).

Coleman et al. isolated a beta-proteobacterium, *Polaromonas* sp. JS666, which is able aerobically to oxidize cDCE as carbon and energy source (2002). The organism is the first of its kind to be isolated. Because JS666 apparently requires no exotic growth factors, it is considered a promising bioaugmentation agent for aerobic sites where cDCE has accumulated. The genome of JS666 has been sequenced, and the results are discussed by Mattes et al. (2008). Initial work exploring the potential of JS666 as a bioaugmentation agent has been completed, namely, successful cDCE oxidation in microcosm studies constructed with various subsurface materials and inoculated with the organism (Giddings et al. 2010). Additionally, a pilot study using JS666 as a bioaugmentation agent began in October 2008 at St. Julien's Creek Annex (SJCA), Chesapeake, Virginia, USA.

Here, we present the development and application of a DNA-based probe based on the isocitrate lyase gene of JS666. This probe was used in conjunction with real-time, quantitative polymerase chain reaction (qPCR) to track the abundance of JS666 in microcosms (and is currently being applied in the ongoing SJCA field study). The microcosm studies allowed explicit resolution of the accuracy and precision of the probe and determination of the extent to which probe results correlate with variations in microcosm performance.

Materials and Methods

Culturing Technique

JS666 cultures were grown on neat cDCE (99%; TCI America, Portland, Oregon) in carbon-free minimal salts medium (MSM) at a pH of approximately 7.1 to 7.2, as described elsewhere (Giddings et al. 2010). Pure cultures were maintained through a series of 5% v/v culture transfers into 100 mL MSM in 160-mL serum bottles and fed a nominal concentration of cDCE of 51 mg/L. Additionally, purity checks by streak plating were routinely carried out.

Sediment and Groundwater Types

Subsurface sediment or groundwater samples were obtained from aerobic, cDCE-contaminated sites at Savannah River (SRS), South Carolina, or SJCA, Virginia, respectively. Moisture content of the SRS sediment was determined gravimetrically from heat-dried sediment samples, and pH of both the sediment and the groundwater was measured using an Accumet microelectrode with a calomel reference. Additionally, pH measurements were taken from individual microcosms at the conclusion of each experiment to assure that pH never became prohibitively low. All sediment and groundwater samples were shipped on ice to the laboratory and stored at 4 °C in the dark for later use.

Microcosms

All microcosms were prepared aseptically under a laminar flow hood, with PCR-clean spatulas in autoclaved 160-mL serum bottles and contained either 50 g (dry weight) sediment or 50 mL groundwater. For each sediment treatment, MSM was aseptically added to yield a total of 50 mL of liquid (including the moisture contributed from

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soil). Microcosms amended with MSM required no other neutralization beyond that provided by the phosphate-buffer component of the MSM. cDCE-fed microcosms were administered 2.3 μL of cDCE (59 mg/L nominal concentration) via syringe through ethanol-swabbed, flamed septa. All experiments were conducted at 22 °C in the dark, agitated at 60 rpm. The experimental treatments summarized in Table 1 were performed to achieve the following objectives:

1. To determine whether the probe could usefully detect growth.
2. To gain a measure of the precision of the probe in soil, as well as to provide variation in cDCE-degradation performance to attempt correlation with probe results.
3. To investigate JS666 die-off (including endogenous decay and cell death due to predation or toxicity) and microbiostasis.

Analytical Methods

Total quantities of cDCE in bottles were measured from 100-μL headspace samples by gas chromatography with a flame-ionization detector and a packed column, as described in detail elsewhere (Giddings et al. 2010). Standards to quantify cDCE were created in either dH_2O or sediment and MSM.

Optical density at 600 nm (OD600) in a Biophotometer was used to estimate the initial target inoculations for each experimental subculture culture. This was performed with an established correlation between OD600 and cell counts from real-time qPCR using primers targeting the isocitrate lyase gene of JS666.

Sampling Procedure and Nucleic Acid Extraction

DNA was extracted from pure cultures or from microcosms using the UltraClean Soil DNA Kit (Mo Bio, Carlsbad, California). Microcosms were sampled in a manner so that the analyzed sample had the same proportions of supernatant and sediment as in the microcosms. Moist sediment was sampled with a PCR-clean spatula and weighed. Based on the previously measured moisture content of the sediment, the weights of dry solids and water in this sediment sample were calculated. Next, with a disposable, sterile syringe, an appropriate mass of supernatant was withdrawn from the microcosm and added to this sediment sample to achieve a supernatant/solids ratio identical to that of the microcosm as a whole. Total sample size was approximately 0.5 g (but precise mass was noted for later calculations). Liquid cultures (without soil) were sampled more simply: 0.5 mL of liquid was withdrawn by sterile disposable syringe. This small amount was used to avoid having to pelletize the cells to discard the supernatant, as the culture does not cohere easily. Instead, we could extract DNA from the entire sample to prevent inadvertently disposing of cells. Pure-culture DNA was used to create standards for each of the primer probes. All DNA extracts were stored at −20°C until later use.

The total concentration of DNA in each sample was quantified with fluorometry using the intercalating reagent PicoGreen (Invitrogen). A Fluoroskan Ascent spectrophotometer (Thermo Scientific, Waltham, Massachusetts) measured fluorescence of PicoGreen bonded to double-stranded DNA at an excitation wavelength of 485 nm and emission wavelength of 538 nm. Lambda DNA (Invitrogen) was used as a primary standard. Because DNA in extracts from pure JS666 cultures was assumed to be entirely comprised of JS666 DNA, the DNA concentrations of JS666-DNA stocks were fluorometrically determined by applying the lambda DNA standard curve. Standard curves for JS666 were generated using serial dilutions of DNA of known concentration extracted from pure cultures and applied in the standardization of qPCR analyses.

Probe Development and Real-Time qPCR

The genome of *Polaromonas* sp. JS666 was sequenced by the Joint Genome Institute Microbial Sequencing Program (GenBank accession numbers CP000316-CP000318), which facilitated the selection of target genes for the molecular probe. The chromosomal gene, isocitrate lyase, was selected as the target for our molecular probe. Although a cDCE-specific degradative gene would perhaps have been a preferred target, the cDCE-degradative pathway has not been elucidated in this organism. Mattes et al. (2008) and Jennings et al. (2009) have suggested the involvement of monooxygenases and glutathione-*S*-transferases, respectively, but JS666 has several genes of each type and the role of any specific one in cDCE degradation remains unproven. Its isocitrate lyase gene is a functional gene in the glyoxylate cycle and has ample variability and sequence stability for the design of strain-specific primers. Additionally, it has more sequence variability than the 16S rRNA gene. The Beacon Designer 4 software program aided in the design of JS666 primers and optimized efficiency for real-time PCR assays. The isocitrate lyase primer set AceA 276F (TGCCGCTGACAACAACAC) and AceA 414R (ATCAATGCCTTTGGAGTGC) has an amplicon length of 139 bp.

Table 1
Experimental Studies

Objective	Material	Inoculation Level			cDCE Level	MSM Buffer
Growth and probe correlation	MSM-only (in duplicate)	1X	0.1X	0.01X	1C	√
Probe accuracy and precision in soil	SRS sediment (in quadruplicate)	1X			1C	√
Starvation & die-off	SRS sediment (in triplicate)	10X			0	√
	SJCA GW (in duplicate)	1X	0.05X		0	

Note: For reference, "1X" = 7×10^6 cells/mL (3.5×10^8 copies/bottle); "1C" = 59 mg/L cDCE (nominal concentration).

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The specificity of the primers was confirmed with database searches in GenBank. It was also tested with conventional PCR with both the isocitrate lyase and a 16S rRNA primer set (8f/1492r) as a positive control on DNA extracted from a number of sources. The DNA came from pure JS666 culture, SRS sediment and groundwater, two topsoil samples with high organic carbon content from Ithaca, New York, collected 4 inches bgs, *Escherichia coli*, primary effluent collected from the Ithaca Area Wastewater Treatment Plant, and a mixed dehalogenating culture that contained *Dehalococcoides ethenogenes*. Additionally, a negative control containing DNA-clean water was carried through the experiment. Gel electrophoresis with ethidium bromide staining was used to determine the presence of any nonspecific amplification from the primer sets. Amplification was only observed in the reactions carried out with JS666 and the isocitrate lyase primer sets and all DNA with the 16S rRNA primer sets, and no amplification was observed in the negative controls, demonstrating that the primer set was specific to JS666 for our purposes.

The DNA, and therefore the number of target genes in each sample, was quantified by qPCR with a thermocycler (iCycler Detection System; BIO-RAD, Hercules, California) with the intercalation agent iQ SYBR Green (BIO-RAD). The reactions were carried out under the following conditions: 2 min at 50°C followed by 3 min at 95°C; next 40 cycles (denaturation at 95°C for 15 s, annealing and extension at 63°C for 1 min), where fluorescence was measured after every cycle. Each reaction was performed in triplicate, and a melt curve was completed following the amplification reactions to confirm the specificity of the primers and the reactions.

To provide a normalization parameter for eventual field studies, we designed a technique for quantitatively measuring both JS666 and total eubacterial 16S rRNA targets. The 16S rRNA primers used, 799F (GGTAGTCYAYGCMSTAAACG) and 1044R (GACARCCATGCASCACCTG), have a similar annealing temperature to that of the isocitrate lyase primers and were therefore run with the same protocol (Bach et al. 2002).

qPCR Applied to Sediment Systems

To overcome soil-matrix inhibition to the PCR reaction, DNA extracts from sediment were diluted. To determine the minimum level of dilution required, the following procedure was performed for sediment or groundwater. A known amount of JS666 was used to inoculate a sediment sample. Next, a DNA extraction was performed. This DNA was diluted 1-, 5-, 10-, 20-, 50-, 100-, and 200-fold and the copy number was measured using qPCR. These were compared against the expected amount of DNA as determined by a liquid extraction performed on the same inoculum. We determined that DNA extracted from SRS and SJCA materials required a 50-fold and a 5-fold dilution, respectively. All microcosms were sampled for DNA at the completion of each experiement.

Preliminary work on the recovery of JS666 from sediment by qPCR was performed by comparing the qPCR copy numbers to the numbers of cells in the inoculating cultures as determined by heterotrophic plate counts and viable microscopic counts. Serial dilutions of JS666 pure cultures were prepared by making 10-fold dilutions of a source culture. This resulted in four JS666 cultures labeled A-D (most concentrated to least concentrated) and one blank. The concentration of cells in the diluted cultures was determined by heterotrophic plate counts and LIVE/DEAD microscopic counts. Heterotrophic plate counts were carried out on 1/4-strength trypticase soy agar plates. LIVE/DEAD staining was used to obtain direct microscopic counts of the live cells and apparently dead cells with damaged membranes.

To test for differences in extraction efficiencies, a Mo Bio DNA Soil Isolation Kit was used to extract DNA from 0.5-mL aliquots of the JS666 cultures and approximately 0.2 mL of the same JS666 cultures applied to approximately 0.3 g of SRS sediment (0.41 g dry weight, exact measurements noted for analysis).

Expression Data Analysis

To damp-out errors associated with plate-to-plate variation in standard curves, fluorescence data generated by the iCycler were analyzed using the DART-PCR technique as outlined and developed by Peirson et al. (2003). The DART-PCR tool uses linear regression to calculate an initial fluorescence level, R_0, in each well (Schefe et al. 2006). The JS666 standard conversion factor between initial fluorescence (R_0) and ng of DNA per reaction was created for qPCR. This number, in units of R_0/ng DNA, was found by averaging pure JS666-DNA samples of known concentrations from standard curves. Measured concentration of DNA was converted to copies of target gene per microliter of sample (Equations 1 and 2), where the size of the JS666 genome is 5.9 Mb as reported by the Joint Genome Institute Microbial Sequencing Program. The total mass of DNA (grams) per mole of JS666 cells is thus given by

$$\left(\frac{\text{gDNA}}{\text{mol}}\right) = \left(5.9 \times 10^6\,\text{bp}\right) \times 660\left(\frac{\text{Daltons}}{\text{bp}}\right) \tag{1}$$

and therefore the number of copies per μL is found by

$$\left(\frac{\text{Copies}}{\mu\text{L}}\right) = \frac{6.02 \times 10^{23}\left(\frac{\text{Copies}}{\text{mol}}\right) \times C_{\text{DNA}}\left(\frac{\text{g}}{\mu\text{L}}\right)}{\left(\frac{\text{gDNA}}{\text{mol}}\right)} \tag{2}$$

where C_{DNA} is the concentration of DNA as measured by fluoroskan.

This number was found for the isocitrate lyase gene and the 16S rRNA by averaging 13 pure JS666-DNA samples of known concentrations (as determined with fluoroskan) and was $1.97 \times 10^{-9} \pm 4.43 \times 10^{-10}$ and $4.65 \times 10^{-10} \pm 8.79 \times 10^{-11}$ R_0/gene copy, respectively.

Results and Discussion

Accuracy and Precision

The accuracy of the DNA probe and recovery of JS666 DNA were first assessed by inoculating sediment samples with aliquots of serially diluted, pure-culture JS666, and

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then comparing the copy number of isocitrate lyase genes (copies/mL) determined from qPCR to the cell counts obtained from heterotrophic plate counts (colony forming unit [CFU]/mL) and direct microscopic counts (cells/mL). Given that there is a single copy of the isocitrate lyase gene per cell, the cell counts should directly correlate with the copy numbers calculated from qPCR. Results are presented in Figure 1. Although there is some discrepancy in the most concentrated of the serial dilutions, there is generally satisfactory agreement among enumeration methods.

The test for differences in extraction efficiencies (between sediment and pure liquid cultures) using a Mo Bio DNA Soil Isolation Kit showed that the efficiency in sediment was actually elevated more than 10-fold relative to that in pure liquid culture ($1.6 \times 10^7 \pm 7.5 \times 10^6$ vs. $3.3 \times 10^5 \pm 1.2 \times 10^4$ copies/mL). This might be due to soil's protection of the DNA, preventing excessive DNA damage during the bead-beating portion of the extraction procedure, or alternately the sediment-aided lysis of cells. Given adequate dilution of the DNA sample, though counter to what might be expected, the presence of sediment did not appear to impede DNA extraction.

A microcosm study prepared with SRS sediment and amended with MSM was constructed in quadruplicate to assess the overall precision of the qPCR method (including the soil-extraction step). Initially, each of the four bottles was extracted in triplicate immediately following inoculation, yielding a sample size of 12 extractions. The calculated copy numbers per bottle were not found to differ statistically for any of the extractions or bottles ($\alpha = 0.01$). The average copy number in each bottle was 3.0×10^8 ($\pm 1.3 \times 10^8$ std dev) per bottle, which corresponds to a coefficient of variation (CV) of 43%. This also confirmed that our calculated inoculation levels (target level of 3.5×10^8 copies/bottle, as per Ramadan et al. [1990]) were achieved. When using a fluoroskan following PicoGreen staining of double-stranded DNA to calculate the total DNA (ng) in each bottle, the CV was much lower, only 20%. The total DNA in the bottle was $1.1 \times 10^6 \pm 2.2 \times 10^5$ ng. The large

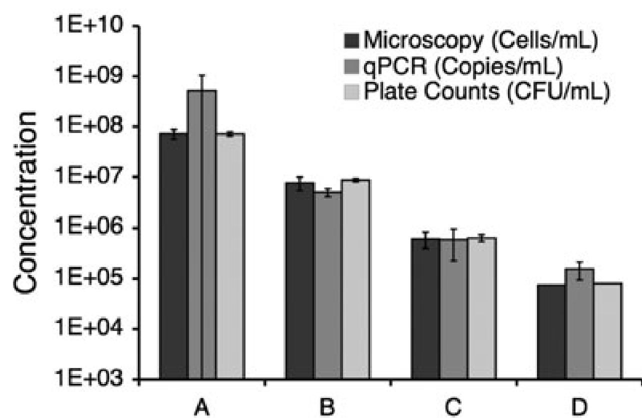

Figure 1. Comparison of copy number calculated from qPCR of JS666/sediment samples to cell counts from microscopy and heterotrophic plating. JS666 cultures in sediment were extracted in duplicate from duplicate sediment samples. Error bars represent standard deviations.

difference between these CVs is most likely due to error propagation in the qPCR process. Because of the mathematics involved (i.e., extrapolation backward many cycles from the threshold cycle number, CT), small variations in CT will translate into large variations in the estimate of initial copy number. Additionally, the CV of 43% includes not only the imprecision of the qPCR procedure itself but also the DNA extraction and comparison across plates. The R_0/rxn of replicate samples of pure JS666 DNA had intrarun (within plate) CVs of 0.6% to 1.5%, whereas the interrun (across plates) CV was 21%. This suggests that simply comparing samples across qPCR plates accounts for approximately half of the variance seen in the overall process.

Currently, there is no standard procedure for nucleic acid extraction from sediment systems, and comparing across studies is difficult. qPCR has been demonstrated to be sensitive and accurate, but interrun variability (i.e., across plates) tends to be high (Dionisi et al. 2003; Powell et al. 2006; Smith et al. 2006). Moreover, most researchers report only variations in their interrun CT values, which when converted to absolute numbers would have significantly higher variation (Smith et al. 2006). For example, Powell et al. found in their intrarun assays that the CVs calculated on the CT values was between 1.2% and 1.4% for the differing gene assay, but much higher when calculated on the number of copies per microliter (2006). These CVs were 16% and 18%, respectively, and even higher when calculated for interrun values: 25% to 38% (2006).

Tracking Growth and Degradation Performance

To determine whether the probe could usefully track cell growth within microcosms (in the absence of complicating factors such as predation), MSM-only microcosms were constructed in duplicate and inoculated at three different levels—1X (7×10^6 cells/mL or 3.5×10^8 per bottle), 0.1X, and 0.01X—with an initial, nominal cDCE concentration of 59 mg/L.

Coleman et al. (2002) report a yield coefficient for JS666 of 6.1 g protein/mol cDCE. If we assume a protein mass per cell of 1.55×10^{-13} g, as for *E. coli* (Neidhardt et al. 1990), then a rough estimate of expected cellular yield is 4×10^7 cells formed per μmol cDCE degraded. In Figure 2A, the 1X, 0.1X, and 0.01X microcosms each completely degraded 30 μmol, for an expected synthesis of 1.2×10^9 cells per bottle. Measured qPCR-based JS666 levels in the various microcosm types at conclusion of the study agree reasonably well with quantities of cDCE degraded. The two 1X replicates showed an average increase of 4.8×10^8 cells above inoculum level, whereas the 0.1X and 0.01X microcosms showed increases of 1.6×10^9 and 1.0×10^9 cells per bottle, respectively (Figure 2B). The average of all six bottles provides a yield of $4.9 \times 10^7 \pm 2 \times 10^7$ cells/μmol cDCE degraded, which is in accord with protein-based yield estimates.

Precision is, of course, only one issue of importance in the utility of an MBT. Another question is whether the MBT can adequately track the performance of the targeted organism. The SRS sediment with MSM microcosms prepared in quadruplicate that had been used to assess MBT precision at time zero were subsequently monitored over time,

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with expected variations in cDCE-degradation performance observed among them that could be tested against results from DNA probing.

Figures 3A and 3B present the time-course results for the quadruplicates. The microcosms were sampled in triplicate at inoculation and then at days 39, 45, and 59. These times correspond to when the microcosms had degraded all or most of the present cDCE, and then approximately 7 and 15 days after degradation had completely ceased in all bottles. Given an analytical precision of ±43% (as determined earlier), the isocitrate lyase gene copy numbers in bottles A and B at the day-39 sampling time (~4×10^8 copies/bottle) were not significantly different, and they had performed similarly in cDCE degradation. However, bottle D had the lowest (1.4×10^8 copies/bottle) and bottle C had the highest (8.4×10^8 copies/bottle) concentrations. This partially confirms what was observed in the cDCE-degradation patterns. At day 45, bottle D had significantly lower DNA levels than the others, though bottle D had performed the best in cDCE degradation, and bottle C had performed the worst.

It is possible that the levels of JS666 DNA that were observed are the result of sediment microbiostasis, which is a balance among growth, decay, and other pressures, such as predation or unfavorable abiotic effects (van Veen et al. 1997). Some suppression mechanisms (e.g., predation and phage infection) would be expected to elevate in response to JS666 population levels, tending to equalize resulting observed populations so that any growth is not seen, especially in aerobic environments (Madsen et al. 1991). However, protozoan grazing may become less of an issue in deeper aquifers as their presence decreases as oxygen becomes limiting (Madsen et al. 1991). In our experience, SRS material was not a favorable environment for JS666 growth. Because of this, there was a prolonged incubation required to achieve cDCE degradation in SRS sediment that could lead to this stasis, which we were most likely observing.

Another microcosm study was constructed in SRS sediment without any cDCE or additional carbon source to monitor decline in probe signal (both for the isocitrate lyase and for the universal eubacterial 16SrRNA gene) over an extended period of starvation. Over 4 weeks, with no added carbon source, there was an obvious downward trend in JS666 DNA levels (Figure 4A) but not of the universal eubacterial 16S rRNA gene (Figure 4B), suggesting that there was no loss of total DNA or problems with extraction. Within 1 week, the total amount of JS666 present was reduced by almost three orders of magnitude from 1.6×10^9 to 5.0×10^6 cells/bottle, which supports the idea that any growth of JS666 would be difficult to detect due to rather robust mechanisms apparently operating in the SRS sediment system causing relatively rapid reduction of JS666 DNA. This suggests explanation for the swift decline in DNA observed (Figure 3) between days 39 and 45 in bottle D of the previous experiment following cessation of cDCE degradation, as the growth expected from the amount of cDCE degraded was never observed. Bottle D was the bottle that completed cDCE degradation first among the quadruplicates.

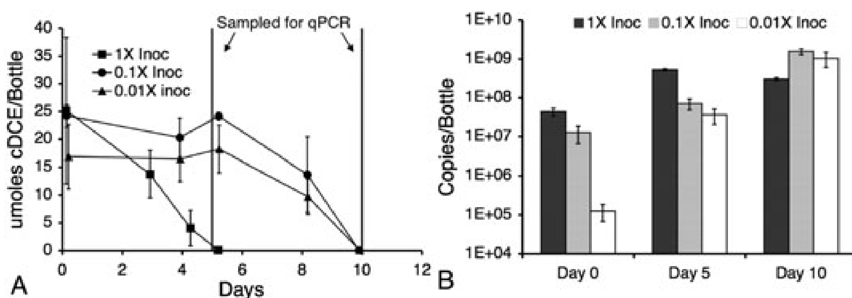

Figure 2. (A) cDCE degradation in no-soil, MSM-only microcosms at various inoculation levels. Microcosms were prepared with 59 mg/L cDCE and inoculated at target values of 1X (3.5×10^8 cells/bottle), 0.1X or 0.01X. (B) Corresponding qPCR data for the JS666-specific probe. DNA was extracted in duplicate. Error bars represent the standard deviations of duplicate microcosms and duplicate DNA extractions.

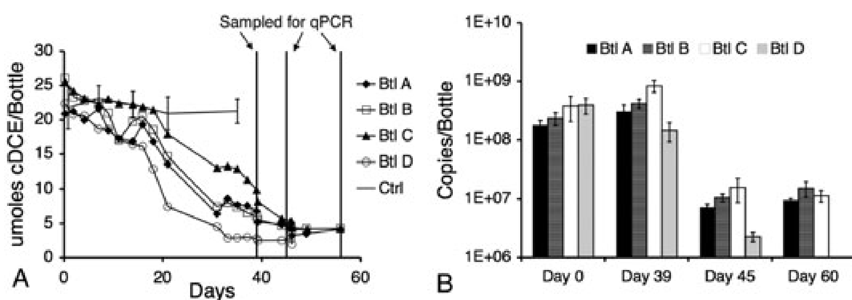

Figure 3. (A) cDCE degradation in SRS sediment inoculated with JS666, prepared in quadruplicate, and (B) corresponding average copies of JS666 per bottle from triplicate extraction samples, as enumerated via qPCR (data unavailable for bottle D for sample day 60). Error bars represent standard deviations.

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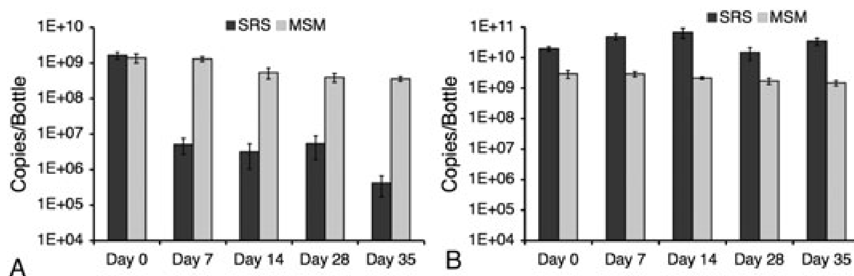

Figure 4. Average copies/bottle of (A) JS666-specific isocitrate lyase gene and (B) universal eubacterial 16S rRNA gene from microcosms constructed from Savannah River Site (SRS) soils + MSM, or MSM-only. Microcosms were inoculated with JS666 at 3.5 × 10^9 copies/bottle and given no external carbon source. Bottles were sampled in triplicate; error bars represent standard deviations.

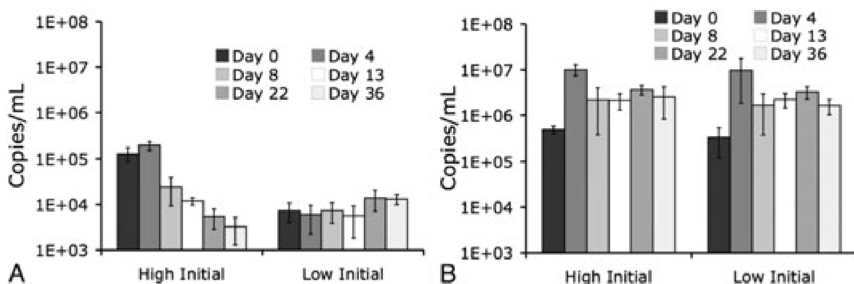

Figure 5. Average copies/bottle of (A) JS666-specific isocitrate lyase gene and (B) universal eubacterial 16S rRNA gene from microcosms constructed from SJCA groundwater, purged of residual cDCE. Microcosms were inoculated with JS666 at 3.5 × 10^8 (high initial) and 1.8 × 10^7 (low initial) copies/bottle and given no external carbon source. Bottles were sampled in duplicate; error bars represent standard deviations.

As a field study is currently underway, another starvation study was conducted with SJCA groundwater to determine the persistence of JS666 without cDCE as a substrate and to provide another material for the investigation of possible microbiostasis. In this study, groundwater purged with filtered air to remove residual cDCE was inoculated at two levels. Again, probe signal for both the isocitrate lyase and the universal eubacterial 16S rRNA genes was monitored over an extended period of starvation. As with our earlier starvation study conducted with SRS soil, there was an obvious downward trend in JS666 DNA-target levels in SJCA groundwater following inoculation (Figure 5A) but not of the eubacterial 16S rRNA gene levels (Figure 5B). As with the previous study, JS666 declined to stasis levels on the order of 10^4 copies/mL (or 10^5 copies/bottle), regardless of the inoculum level, which suggests that this could be the common level to which JS666 converges in environmental systems. This also lends support to the theory of microbial biostasis in natural environments. Moreover, these low levels did produce viable, active microcosms (and growth of JS666) when cDCE was subsequently administered (Giddings et al. 2010), despite cell levels being at or below detection limits.

We have determined that the method outlined here is likely to be sufficient to monitor distribution of JS666 at bioaugmented sites, and the precision is adequate to track a target whose concentration is expected to vary many orders of magnitude in application. Additionally, we found within environmental, mixed cultures, that the DNA target does not persist long after cell death (Figures 4 and 5), especially within the time scale of most remediation efforts, demonstrating that positive result from the probe is a strong indicator that degradation can occur if suitable environmental conditions are provided. Finally, absent suspected predation, the probe accurately and precisely tracks growth. Collectively, the studies appear to validate the utility of the molecular probe for site assessment in a bioaugmentation context.

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