United States
Environmental Protection
Agency
Research and Development

National Health and Environmental
*Effects Research Laboratory*
Gulf Breeze, FL 32561
EPA/600/S-98/001 April 1998

EPA

# ENVIRONMENTAL RESEARCH BRIEF

## Bioaugmentation with *Burkholderia cepacia* $PR1_{301}$ for *In Situ* Bioremediation of Trichloroethylene Contaminated Groundwater

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A pilot field study was conducted at the Moffett Federal Airfield, Mountain View, CA, to determine whether effective *in situ* aerobic cometabolic biodegradation of trichloroethylene (TCE) could be accomplished through bioaugmentation with a genetically modified strain of *Burkholderia cepacia* G4 (G4) together with feeding of lactate to serve as an energy and growth substrate for the organism. Strain G4 is highly effective at TCE cometabolism but requires either phenol or toluene to induce oxygenase enzyme activity. A strain of G4 was developed through NTG mutagenesis that constitutively expresses the oxygenase so that no inducer need be added. The modified strain, *B. cepacia* $PR1_{301}$, ($PR1_{301}$), can degrade TCE effectively while growing on simple substrates such as lactate. Strain-specific molecular probes were developed for monitoring the presence and movement of $PR1_{301}$ and were based upon rep-PCR analysis.

Laboratory microcosm studies using Moffett aquifer material indicated that the quantity of microorganisms that could be injected was limited by oxygen availability. Within these limits, addition of wild-type G4 grown on phenol or mutant strains grown on lactate were effective initially at TCE cometabolism when added daily to the columns. However, the strains with constitutive oxygenase expression did not maintain TCE degradative ability for long when lactate was used. Organism presence in the microcosm effluents was found as long as bioaugmentation continued, but not when it was discontinued. Following discontinuation of bioaugmentation, microcosms fed phenol improved in TCE cometabolism with time, reaching over 90% removal, although at the end of the study, no G4 or related mutant organisms could be found within the microcosm. In contrast, a control microcosm fed phenol with each exchange, but without bioaugmentation, removed about 60% TCE initially, but with time, TCE removal efficiency decreased to near zero.

Three field studies with bioaugmentation were conducted. In each one, bioaugmentation with $PR1_{301}$ along with lactate addition was initially reasonably effective at TCE removal. In addition, phenol was rapidly consumed to near detection limits when added following a short period of bioaugmentation with $PR1_{301}$, and lactate addition, demonstrating that an initial phase of bioaugmentation could be effective to establish a population of phenol or toluene degraders if these substrates were to be used at a site. However, in both the second and third field trials, bioaugmentation with lactate feed alone in time became ineffective at TCE removal, reaching near zero removal within a few weeks. The inability of $PR1_{301}$ to remain effective in bioaugmented groundwater was also demonstrated in a brief laboratory study using groundwater from the bioaugmented well after the conclusion of the field study. This suggested either predation of the introduced population or the inability of $PR1_{301}$ to effectively compete for the added lactate, or perhaps both, were the cause of the eventual failure of the system. In order for bioaugmentation for TCE cometabolism to be successful, methods for avoiding this competitive problem need to be found.

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## Introduction

Trichloroethylene (TCE) has been widely used as a solvent over the past 50 years. Because of uninformed disposal practices, it has become a major groundwater contaminant. There has been much interest in the potential of aerobic *in situ* biotransformation processes for the destruction of TCE and other chlorinated aliphatic hydrocarbons (CAHs) in groundwater since cometabolism of TCE was first demonstrated in soil columns where natural gas and oxygen were added to stimulate the growth of native microorganisms. In cometabolism, an enzyme (oxygenase), used by the microorganisms for initiating primary substrate oxidation, fortuitously transforms many CAHs. Field-scale evaluations of *in situ* biodegradation of CAHs have been undertaken since 1985 at the Moffett Federal Airfield (Moffett Field), Mountain View, CA. Initially, methane was used as a primary substrate for aerobic cometabolism of several CAHs. While the methane-consuming culture developed was highly successful at transforming some CAHs, removal efficiency was rather low with TCE. Therefore, other potential inducers were sought. One of the most promising was phenol. Phenol was then evaluated at Moffett Field over three seasons using indigenous microorganisms only and was found to be quite superior to methane for *in situ* TCE degradation, providing up to 90% removal at TCE concentrations of up to 1 mg/l.

However, the use of phenol might pose regulatory problems because of its known toxicity and taste and odor potential. A possible alternative is the use of bioaugmentation with strains of bacteria in which the oxygenase is constitutive, thus they could not only degrade TCE, but also could do so without the requirement for an inducing compound. Such bacteria may grow on harmless water-soluble substrates and still maintain their ability to transform TCE. However, such substrates may be less selective and the introduced organisms may encounter strong competition from indigenous organisms, resulting in little TCE degradation. A possible approach to overcome this limitation is to use daily bioaugmentation in an attempt to maintain a competitive advantage. This was the approach evaluated in this study. An additional interest is that stimulation of native organisms with a specific substrate for *in situ* bioremediation of TCE may enrich for a population of microorganisms that are unable to cometabolize the target compound or that will degrade the target compound slowly. Bioaugmentation through addition of bacterial cultures known to transform TCE rapidly may enhance native biodegradation or even provide the sole means of degradation in systems without indigenous TCE-degrading organisms.

Tn5 mutagenesis was previously demonstrated to result in the production of a constitutive TCE-degrading strain, but the insertion of additional genetic information, particularly antibiotic resistance, in this recombinant strain may subject its release to strict regulatory and public approval. As an alternative, a non-revertible regulatory mutant selected for spontaneous constitutive TCE transformation through N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis was produced in this study and tested both in the laboratory and at Moffett field.

### *Objectives of Study*

The objectives of this study were (1) to evaluate at laboratory and field scale the potential for bioaugmentation with a bacterial mutant containing a constitutive monooxygenase to enhance and improve *in situ* bioremediation of groundwater contaminated with TCE, (2) to determine the movement, fate, and effectiveness of introduced microorganisms in an aquifer, (3) to evaluate the value of introduced microorganisms and of bioaugmentation for enhancing *in situ* biodegradation, and (4) to evaluate the applicability of molecular tools in the monitoring, operation, and control of *in situ* bioreclamation systems.

### *Overview of Study*

The study was conducted by researchers at the Western Region Hazardous Substance Research Center (WRHSRC), Stanford University; the Center for Microbial Ecology (CME), Michigan State University; the University of West Florida; and the U.S. Environmental Protection Agency, Gulf Breeze Environmental Research Laboratory. The microorganism developed at the University of West Florida and used for bioaugmentation was *B. cepacia* G4 $PR1_{301}$ ($PR1_{301}$), a non-recombinant derivative of *B. cepacia* G4 (G4) that constitutively expresses toluene ortho-monooxygenase (TOM) and is highly effective at TCE cometabolism. Molecular probes for monitoring the fate and effects of PR1 were developed at CME. The laboratory microcosm studies and field studies were conducted by WRHSRC, with molecular probe analysis of field samples to determine the movement and fate of $PR1_{301}$ conducted by CME.

## Methods

### *Development of $PR1_{301}$ and Probes for Its Detection*

A non-recombinant strain ($PR1_{301}$,) capable of constitutive TCE degradation was developed for this study. Bacterial cultures used for $PR1_{301}$, development were grown on two formulations of media based on a basal salts minimal medium (BSM), BSM-lactate (BSM, 20 mM lactate) and BSM-phenol-TTC (BSM, 0.025 mg/ml triphenyl tetrazolium chloride (TTC), 0.2 mg/ml proteose peptone, 2 mM phenol). NTG mutagenesis and enrichment for Tol-, Phe- mutants using a toluene vapor feeder was performed as previously described. Tol- and Phe- mutants were detected using their TTC dye-reduction assay with the following changes: phenol (2 mM) was used as the primary carbon source instead of toluene vapor, and 0.2 mg/ml proteose peptone was added to the BSM-phenol-TTC purified agar plates. Mutagenized cells were diluted to give approximately 150 colonies per 100 $\mu L$ plated. Assays for TOM through the oxidation of trifluoromethyl phenol (TFMP) to the yellow trifluoromethyl heptadienoic acid (TFHA) were performed as previously described.

Rapid strain-specific nucleic acid probes for detecting *B. cepacia* G4 (G4), including the above mutant $PR1_{301}$ strain as well, were developed for use in monitoring movement and survival of $PR1_{301}$ and other G4 strains in laboratory and field microcosms. The probes were made by cloning DNA fragments amplified from genomic DNA of G4 using rep-PCR and primers specific for repetitive extragenic palindromic (REP) sequences. The specificity of the probes was determined by hybridization against DNA fragments amplified from G4 and 80 genetically distinct bacterial isolates from the Moffett field aquifer. Two out of four probes tested were found to specifically hybridize to DNA fragments of the expected size in the rep-PCR fingerprint of G4, but not to the other strains tested. One of these probes, a 650 bp fragment, produced a hybridization signal when as few as 10 CFU of G4 were present in a mixture with $10^5$ CFU nontarget strains, indicating that the sensitivity of these probes was comparable to those of other PCR-based detection methods. The probes were used to discriminate groundwater and microcosm samples that contained G4 from those that did not.

False positive results were obtained with a few samples, but these were readily identified by using hybridization to the second probe as a confirmatory step. The general applicability of the method was demonstrated by constructing probes specific to three other environmental isolates.

## Laboratory Microcosm Studies

In preparation for field studies, the effects of bioaugmentation on the aerobic cometabolism of TCE in groundwater were initially investigated in the laboratory using small-column aquifer microcosms (17 ml total volume, 5 - 6.5 ml pore volume) containing Moffett field aquifer material. In initial studies, non-sterile non-bioaugmented microcosms fed phenol as a primary substrate mimicked observed *in situ* behavior at the Moffett field site, cometabolizing approximately 60 µg/l TCE while fed 6.5 mg/l phenol. High density single bioaugmentation with G4 increased TCE removal in sterile aquifer material, while producing mixed results in non-sterile material. Low density semi-continuous bioaugmentation enhanced TCE transformation in non-sterile microcosms. Phenol-fed microcosms augmented with either G4 or $PR1_{301}$ transformed twice as much TCE as the non-augmented phenol-fed microcosm. In addition, should primary substrate addition be a regulatory concern, TCE degradation was observed without primary substrate addition through bioaugmentation using organisms expressing the TCE-transforming enzyme.

In subsequent long-term studies, aquifer microcosms were repeatedly bioaugmented and fed solutions containing 6.5 mg/l phenol or 15 mg/l lactate and 250 µg/l TCE every two to three days. The effectiveness of TCE cometabolism by an indigenous phenol-fed microbial population declined significantly during a 280-day experiment. This behavior, possibly due to the negative selective pressure of TCE cometabolism which leads to the formation of toxic products, had not been observed previously in shorter-term TCE transformation experiments. The addition of G4 or $PR1_{301}$ to microcosms along with phenol or lactate initially allowed for substantial TCE degradation but led to the eventual depletion of dissolved oxygen and a decline in TCE transformation. After termination of bioaugmentation, dissolved oxygen levels recovered in all microcosms, and those microcosms that continued to receive phenol returned to or surpassed previous TCE transformation levels, while unfed and lactate-fed microcosms lost degradative activity. The introduced organisms, however, did not appear to be responsible for the recovered TCE degradation in the phenol-fed, formerly bioaugmented microcosms. The source of activity in these microcosms was not identified but is likely to have been efficient TCE-transforming indigenous organisms selected by the operating conditions within the microcosms. G4 and PR1 were never found present in the effluents from the non-bioaugmented columns but were repeatedly found in column effluents during 83 days of active bioaugmentation in bioaugmented columns. However, within 10 days after bioaugmentation was stopped, neither G4 nor PR1 were again detected. The greatly improved TCE biodegradation that occurred in the previously bioaugmented phenol-fed columns through the remainder of operation to day 237 raised the question of whether the bioaugmented organisms may be growing in the column. However, when they were dismantled and the column contents were analyzed for G4, none was found present. We were unable to confirm why the improved performance occurred in the phenol-fed bioaugmented columns after bioaugmentation was stopped. The lack of effective long-term TCE biodegradation in lactate-fed bioaugmented microcosms raised initial questions about the potential for successful field application.

## *Field Bioaugmentation Studies with $PR1_{301}$*

The field study of bioaugmentation for *in situ* bioremediation of TCE was conducted at Moffett Field. This location was the site of several previous studies of *in situ* biodegradation of CAHs. In all cases, the previous studies made use of indigenous microorganisms. The last previous study conducted at this site was concerned with injection of phenol and toluene to stimulate indigenous microorganisms for cometabolism of TCE. It was found that the dominant organisms utilized toluene ortho-monooxygenase (TOM) for primary substrate and TCE oxidation. Since the organism proposed to be used for bioaugmentation, *B. cepacia* $PR1_{301}$ also produces TOM, a new series of injection and monitoring wells was developed to avoid the potential interference to this study from native microorganisms previously stimulated with phenol and toluene addition. However. the analytical system and all other features of the test site were similar to those used in the past.

The *in situ* evaluation was performed using the same methodology as in our previous studies. Here, a series of stimulus-response tests were performed under induced gradient conditions of injection and extraction of groundwater. The stimulus was the injection of groundwater that was blended with the microorganism and chemicals of interest. The response was the concentration history of the chemicals at the monitoring locations. The profile of the field system is illustrated in Figure 1. This system consisted of an injection well (2SSEI) and an extraction well (P2) located 9 m apart with three monitoring wells in between. Both the injection and extraction wells consist of standard (ca. 5-cm diam.) polyvinyl chloride pipe installed by using a hollow-stem auger. The wells contained 1.5-m long slotted screens, installed 4.5-6.0 m below the ground surface, and fully penetrated the aquifer zone that contained sand and gravel. The sampling wells consisted of 3.18-cm diam. stainless steel wire-wound sand points with 0.6 m screens. The screen sections were located 4.7-5.3 m below the ground surface, in the center of the sand-gravel layer of the aquifer. These three wells were spaced 1, 2, and 3.5 m from the injection well. In addition, two special monitoring wells 2SSE (FP1) and 2SSE (FP2) fully penetrated the aquifer and were located 0.5 m and 1.5 m, respectively, from the injection well. Into these wells were inserted a series of nylon bags containing glass beads that fully covered the depth of the groundwater aquifer. These beads were used for organism colonization to determine the presence of the injected strain $PR1_{301}$. The formation groundwater is moderately saline, having a total dissolved solids content of 1,500 mg/l, and was contaminated by some chlorinated aliphatic hydrocarbons (CAHs), mainly 1,1,1-trichloroethane (TCA), but was devoid of chlorinated ethenes the subject of this study. Thus, the target compound, TCE, was added to injection water in a controlled manner. Nitrate was present in the native groundwater (25 mg/l as measured by ion chromatography) and served as a source of nitrogen nutrient. Total phosphorus concentrations were low (<0.1 mg/l, as measured by induced-coupled argon plasma spectrometry), but near solubility limits of common phosphorus minerals, which were probably the source of the needed phosphorus.

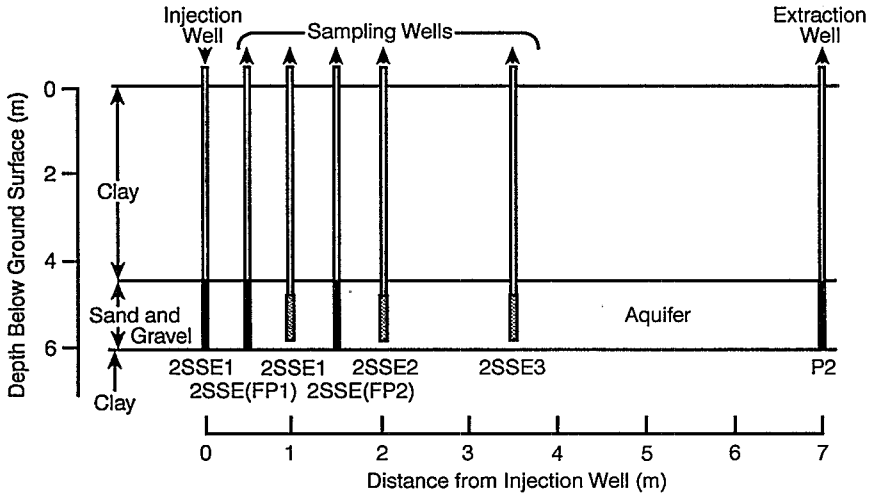


**Figure 1.** Cross-sectional view of *in situ* bioaugmentation site at Moffett Field.

## *Chemical Introduction into the Aquifer*

Chemicals were introduced into the injected water continuously or in an automatic programmed manner. The extracted water used for injection was treated before chemicals were added by filtration through a nominal 1 μm filter, and UV disinfection. Oxygen was introduced using a counter-current column for gas transfer into the injection water. TCE, phenol, and lactate were added to injection water by pumping water solutions containing these chemicals. The injection water and sampled waters all passed through stainless-steel tubing, which prevented passage of gasses and TCE through tubing walls. Thus, excellent mass balances of all chemicals were maintained through the system.

## *Field Analytical System*

Water samples for analysis were obtained from the monitoring well locations and from injected and extracted water by automated pumping to an automated data acquisition and control system (DAC). This permitted the continuous measurement of the principal chemical constituents, which were bromide tracer, phenol, toluene, TCE, dissolved oxygen (DO), and pH. The instruments operated by the DAC system are an ion chromatograph for the bromide tracer analysis, a reverse phase high performance liquid chromatograph (HPLC) for phenol and toluene analyses, an anion exclusion HPLC with conductivity detector for lactate, a gas chromatograph equipped with an electron capture (GC-ECD) and a Hall conductivity detector (GC-Hall) for TCE analysis or a PID detector for toluene analysis, a dissolved oxygen meter (Yellow Springs, OH), and a pH meter. The lower concentration limits for the analyses were DO, 0.1 mg/l; bromide, 0.5 mg/l; TCE, 0.5 μg/l; lactate, 0.5 mg/l; and phenol, 1 μg/l. All data were compiled automatically and stored in a database on a personal computer at the test site.

## *Growth of Bioaugmentation Culture*

A standard procedure was used for the growth of $PR1_{301}$ used in bioaugmentation for the first two field trials, and a modified procedure was used for the third field trial. During the first two field trials a culture of $PR1_{301}$ was transferred from a broth tube to 1 liter of media contained in a 2-liter flask. The media consisted of 4 μg/l of sodium lactate in mineral media. These were introduced into the flask through a foam plug, and the mixture was aerated for 48 hr at room temperature in the instrument-control room in the field. Aseptic conditions for the microorganism were maintained throughout. The culture grown in the flask was then transferred to 16 liters of similar media, but containing 1 μg/l of sodium lactate in a carboy in which pure oxygen was bubbled for a 24-hr period. Samples were taken for suspended solids, lactate, and PRI analyses. The contents of the carboy were then pumped daily into the groundwater through the injection well at the normal injection flow rate of 1.5 liter/min.

During the winter season of the second field study, all lactate in the carboy was not always used, and suspended solids, representing PR1 growth, were somewhat lower than during the normal season. For this reason, during the third field study, conducted in the summer, organisms were grown under more controlled conditions in the laboratory and then transported to the field. The transportation time was less than 30 minutes. Similar procedures were followed except that air was used for mixing and growth of microorganisms in the carboys rather than pure oxygen. In addition, two 16-liter carboys of microorganisms were added per day rather than one in order to obtain a more definitive study of the effectiveness of bioaugmentation.

## *Field Study Results*

Three separate studies were conducted of bioaugmentation with $PR1_{301}$. It was originally planned to conduct one single long-term study, but heavy rains occurred twice during the study, resulting in excessive groundwater flow and hydraulic head so that the study had to be interrupted. Nevertheless, sufficient data were obtained from each separate study to allow evaluation of the effectiveness of bioaugmentation. In addition,

some aspects of the studies were repeated to provide more conclusive results.

## Field Bioaugmentation Evaluation

A summary of the experimental variables used during the three separate field tests is given in Table 1. In all cases, 10 liters/min of groundwater was extracted from the aquifer and 1.5 liter/min of air stripped, filtered, and disinfected groundwater, augmented with phenol or lactate, and TCE was added to the injection well. The primary substrates (lactate or phenol) were pulse-injected three times a day as in previous studies. Here, either lactate or phenol was added over a period of 15 to 30 min to provide time-averaged concentrations over the 8-hr period as indicated in Table 1. DO, bromide, and TCE were added continuously at the concentrations indicated. Bioaugmentation through daily injection of 16 liters during the first two field studies or 32 liters during the second was carried out during the periods indicated.

The first study was conducted for 26 days or a little over 600 hr. The second study was conducted over 34 days, or about 800 hr. The third study was conducted for 40 days, or about 950 hr. During the first study, conditions were maintained constant the entire period with lactate used throughout as the primary substrate. During the second study, lactate was added as the primary substrates for the initial 130 hr, the primary substrate was then switched to phenol for up to 387 hr, and then the primary substrate was witched back to lactate. In the final study, lactate was used exclusively during bioaugmentation. However, bioaugmentation was stopped after 530 hr. Phenol was substituted for lactate as the primary substrate after 830 hr.

## First Field Study

Both daily bioaugmentation and lactate addition at a time-averaged concentration of 13 mg/l began at time zero. Figure 2 indicates the normalized bromide concentration measured at the three monitoring and the one extraction wells. Based upon time for 50% arrival, the time of movement of injected water from the injection well to the first monitoring well 2SSE1 was about 6 hr. Movement to the second (2SSE2) and third (2SSE3) monitoring wells was about the same or about 18 hr. After about 50% bromide arrival occurred, the bromide concentration increased more rapidly at 2SSE2 than at 2SSE3, as expected. The peculiar behavior of similar times for 50% arrival, but divergent times for 100% arrival at the second and third monitoring wells had occurred previously in the first or south leg of wells constructed at the Moffett Field site some 10 years earlier. More normal arrival times were obtained in a southeast leg that was constructed between the first study and this study. This peculiar behavior apparently resulted from heterogeneities occurring in the aquifer. The important feature, however, is that bromide concentration approached 100% with time at all monitoring wells, a requirement to be able to adequately evaluate removal efficiency for TCE. The concentration of bromide at the extraction well was 12% to 13% of that in the injection water, which is as it should be with an extraction rate 8 times that of the injection rate. The bromide tracer studies indicate the newly constructed leg was satisfactory for the bioaugmentation study to proceed.

Figure 3 illustrates lactate concentration at the various monitoring wells. Lactate was injected in pulses three times per day to give the average time-averaged injection concentration of 13 mg/l. Some removal of lactate began immediately, as the concentration at the second and third monitoring wells never exceeded 3 mg/l, declining to non-detectable levels after about two days of injection. Some lactate, too, was found at the first monitoring well location for the first 130 hr of injection, and then became non-detectable after that time. The lactate was consumed readily by the microflora either added or already existing in the aquifer, with most removal occurring within the short distance between the injection well and the first monitoring well.

The pH at the three monitoring wells was similar and varied from about 7.1 at the beginning of bioaugmentation to about 6.9 - 7.0 at the end. The injection well gage pressure remained the same at about 4.9 pounds per square inch throughout the study until the rain began. This indicates that excessive bioclogging did not occur throughout the bioaugmentation period.

Figure 4 illustrates DO concentration in the injection well, the monitoring wells, and the extraction well. Most DO demand occurred between the injection and the first extraction well with steady-state DO consumption after 400 hr equaling about 16

**Table 1.** Experimental Variables for the Three Field Studies of Bioaugmentation

| Field Study | Hours of Study | Bioaugmentation (g/d) | Primary Substrate | Injection Concentration, mg/l | | | |
|---|---|---|---|---|---|---|---|
| | | | | Primary Substrate | TCE | DO | Br |
| 1 | 0 - 624 | 5.0 ± 0.9 | Lactate | 13 | 0.08 | 32 | 60 |
| | 0 - 130 | 3.5 ± 1.3 | Lactate | 13 | 0.10 | 32 | 60 |
| 2 | 130 - 387 | 3.5 ± 1.3 | Phenol | 6 | 0.10 | 32 | 60 |
| | 387 - 800 | 3.5 ± 1.3 | Lactate | 13 | 0.10 | 32 | 60 |
| 3 | 0 - 520 | 10.5 ± 1.3 | Lactate | 13 | 0.10 | 32 | 60 |
| | 520 - 830 | 0 | Lactate | 13 | 0.10 | 32 | 60 |
| | 830 - 950 | 0 | Phenol | 6 | 0.10 | 32 | 60 |

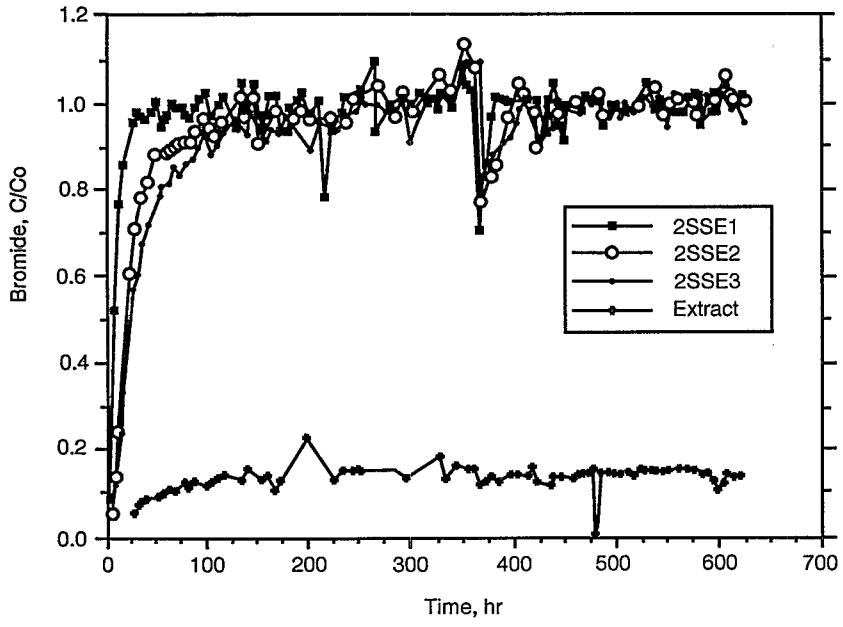


**Figure 2.** Normalized bromide tracer concentration at the various monitoring wells following continuous injection of 60 mg/l beginning at time zero during the first field study.

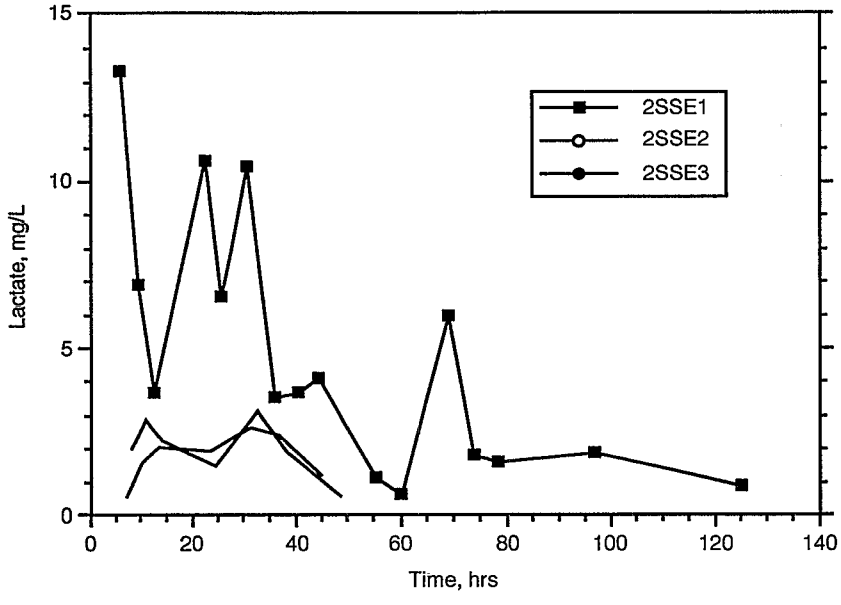


**Figure 3.** Lactate concentration versus time at the various monitoring wells following 13 mg/l continuous lactate addition beginning at time zero during the first field study. Beyond 130 hours concentrations were all below the detection limit.

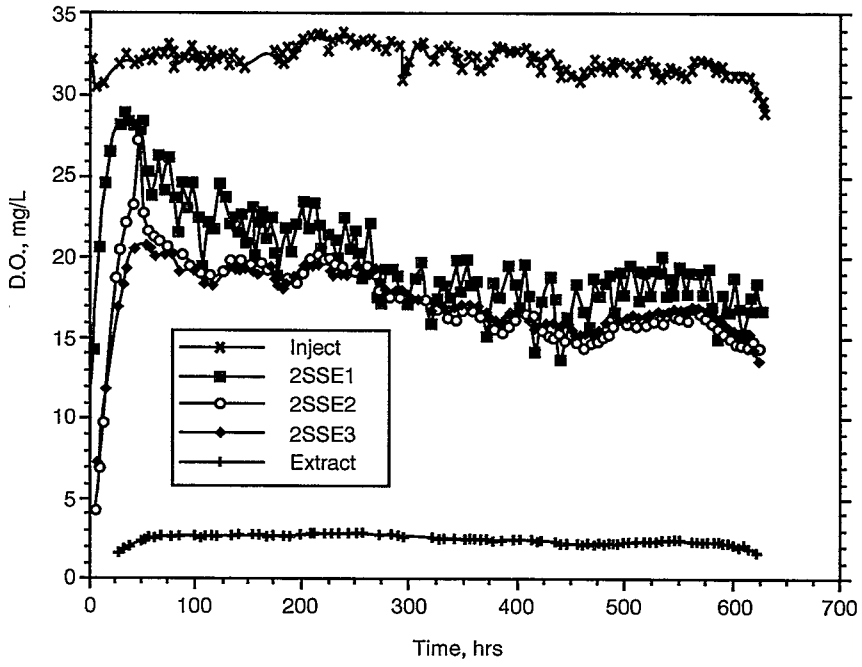


**Figure 4.** Dissolved oxygen concentration at the various monitoring locations during the first field study. Dissolved oxygen in native groundwater was near zero.

mg/l. The oxygen demand from complete oxidation of the 13.5 mg/l of added lactate would be about 15 mg/l. Some oxygen demand may have resulted from oxidation of natural organics or inorganics occurring in the aquifer, but most of the excess probably was due to oxygen demand by the injected microorganisms. As not all of the lactate in the carboy was used, there would have been some oxygen demand from the remaining lactate as well.

The normalized TCE concentrations at the three monitoring wells and the extraction well are indicated in Figure 5. The TCE injection concentration averaged about 70 µg/l. It is somewhat difficult to interpret this information because of the very strong sorption of TCE to aquifer material that occurs at this test site. TCE needs to reach steady state removal before adequate evaluation of removal effectiveness can be made. Since most injected organisms would probably reside between the injection well and the first monitoring well, and most of the lactate was used here, the greatest portion of TCE removal should occur within this zone as has been found from previous studies. During the first 80 hr, TCE at 2SSE1 appeared to approach a steady-state concentration of about 22% of the injected concentration. However, after this time, TCE concentration increased significantly and approached 80% of the injected value between 280 and 450 hr. After that, removal appeared to increase with the TCE concentration at 2SSE1 equaling about 60% of the injected concentration. These data would indicate that bioaugmentation was moderately successful. At about 600 hr a rainstorm occurred that ended the first field study. Based upon these results, it appeared that bioaugmentation at the given level with $PR1_{301}$, and feeding of lactate resulted in successful removal of about 50% or more of the injected TCE.

Monitoring was conducted for $PR1_{301}$, through samples taken daily from the three monitoring wells. Once bioaugmentation began, $PR1_{301}$ was detected in samples taken from the first monitoring well during the first six days of bioaugmentation. None was detected in water taken from the second or third monitoring wells. However, after six days $PR1_{301}$ was no longer detected until almost the end of this field trial, and then was found on only two other occasions. These two other occasions occurred after 480 hr of operation at times when TCE removal appeared to increase somewhat. No $PR1_{301}$ was detected on the glass beads removed at the end of this field trial.

## *Second Field Study*

Once the field system had returned to normal following the end of the rains, the second field trial was begun. As in the first study, bioaugmentation was started at time zero with addition of organisms once per day. The lactate was fed at the same time-averaged concentration as previously. The objective of the first five days was to see whether removals observed during the first study would be seen again. Within the first two days, TCE removal between the injection and the first monitoring well appeared to level off at about 50% as indicated in Figure 6. The average injected TCE concentration was about 80 µg/l.

After 125 hr the primary substrate was changed to a time averaged 6 mg/l phenol to determine whether bioaugmentation might be used to introduce a population of phenol degrading organisms that would prevent phenol spread in an aquifer if it were the primary substrate of choice for field implementation. This concentration of phenol had been found from previous studies to provide between 50% and 75% removal of TCE.

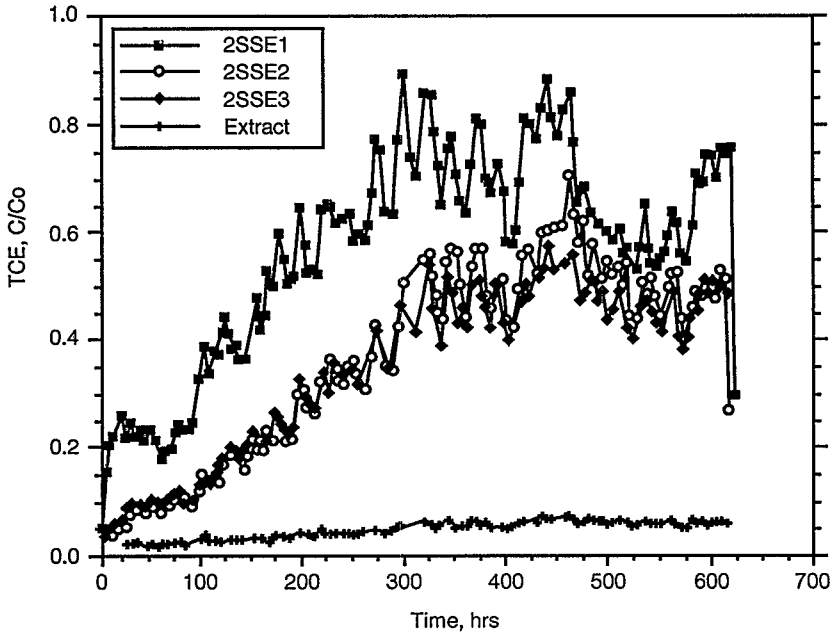


**Figure 5.** Normalized TCE concentrations at the various monitoring locations during the first field study. Added TCE concentration was 70 µg/l.

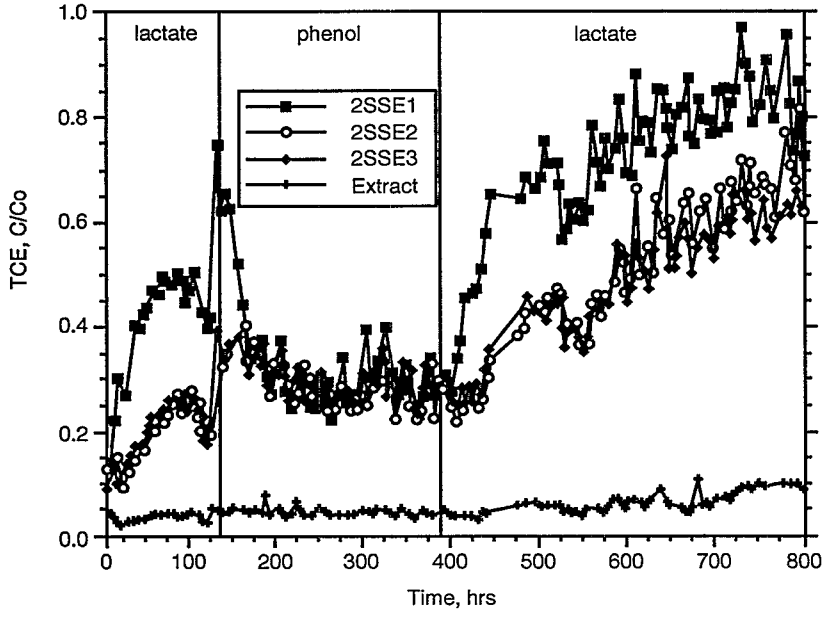


**Figure 6.** Normalized TCE concentrations at the various monitoring locations during the second field study. Added TCE concentration was 80 µg/l.

Figure 6 indicates that as soon as phenol was added, stable TCE removal of about 70% was obtained between the injection and the first monitoring well. The stable removal over the next 250 hr demonstrated the benefit of phenol addition along with bioaugmentation.

Phenol measurements indicated that during the first 24 hr, about 0.5 mg/l phenol was detected at the second and third monitoring wells, but by the second day concentrations had dropped below the detection limit of about 1 µg/l. Some phenol was found at the first monitoring well. This brief study indicates that indeed bioaugmentation can be very effective for rapidly establishing a phenol-using population to prevent the undesired spread of phenol in the aquifer when first added. During the previous study when phenol was first injected into this aquifer, about two weeks were required for an indigenous population to grow sufficiently for significant phenol degradation to occur. Then, several days were required for the phenol concentration to drop below the detection limit. In this study, phenol had never been injected into the aquifer at this location and so the immediate response with phenol removal was undoubtedly a result of the bioaugmentation. Thus, such bioaugmentation would be very beneficial at the start of *in situ* bioremediation where toxic compounds such as phenol or toluene may be injected.

After 390 hr, a switch was made back from phenol to lactate to determine whether the fairly good TCE removal obtained with lactate bioaugmentation could be maintained. The response shown in Figure 6 indicates TCE concentration then increased at all monitoring locations and, within a few hundred hours, approached the injection concentration. Therefore, these results indicate that bioaugmentation was no longer effective. DO measurements indicated DO consumption between the injection point and the second monitoring points was about 24 mg/l, which is significantly higher than the 16 mg/l obtained in the first study. This probably resulted because all of the lactate in the carboys was not consumed during this period so that a greater amount of oxygen demanding material was introduced into the aquifer. However, DO remained above 8 mg/l at all monitoring wells throughout the study and so was sufficient for TCE oxidation.

An important question arose as to why the bioaugmentation was not effective at the end of the second field study. One hypothesis was that the outside temperatures had decreased as this was the winter season, and conditions for growth were not as optimal as in the fall study. As a result, the quantity of microorganisms injected per day dropped from 5 grams to 3.5 grams. The aquifer temperature itself remained constant at about 18°C. Additionally, sporadic rain occurred after about 300 hr and this was associated with an increase in injection pressure. While this might be a result of bioaugmentation, our results from the previous study suggest that it was more due to the rainfall causing an increase in the static hydraulic pressure in this confined aquifer. Heavy rainfall occurring around 800 hr terminated the second field study.

As before, samples were obtained from the various monitoring wells for analysis for the presence of $PR1_{301}$. $PR1_{301}$, was detected in the first monitoring well every day for the first four days, and then it was not detected except for two times during phenol injection at around 300 hr. None was found during the last period when lactate was added after 390 hr. $PR1_{301}$ glass beads obtained at the end of the lactate feed and at the end of the phenol feed.

### *Third Field Study*

A third field study was begun the following summer when there was no concern that rainfall might occur to adversely affect the results. In an attempt to better confirm the previous TCE removal associated with bioaugmentation and lactate feed, cultures were grown in the laboratory under more ideal conditions, and the amount of $PR1_{301}$ for injection was doubled. Here, two 16-liter carboys containing a total of about 10 g dry weight of $PR1_{301}$ was used per day. Laboratory measurements of TCE degradation rates by the culture were obtained daily to ensure that the injected population was efficient in TCE biodegradation.

TCE removal during the third study is indicated in the normalized TCE concentration graph shown in Figure 7. During the first 124 hr, TCE removal looked very good, mimicking the removal obtained initially in the first two studies, and seemed to reach a steady state at about 80% to 90%. Then after 130 hr, the TCE concentration began to increase in a manner suggesting TCE removal had stopped, and that only sorption was affecting TCE removal. The upward TCE trend continued through 520 hr when bioaugmentation was stopped. It continued to increase while only lactate was added until 830 hr, when the primary substrate was switched to 6 mg/l phenol. TCE removal then began, with the TCE concentration at all locations continuing on a downward trend reaching about 50% removal when the third field study was stopped after 950 hr of operation.

DO concentration measurements indicated DO consumption increased throughout the study, and by 500 hr equaled about 30 mg/l, leaving only about 7 mg/l at 2SSE1 and 2.5 mg/L at 2SSE2. This increase in oxygen consumption with time, greater than that from the previous studies, can be attributed primarily to oxidation of the biomass that had been added to the systern. When bioaugmentation stopped, DO concentration began to rise immediately. In spite of the large DO demand, sufficient DO was always present in the aquifer and so was not the cause of the poor TCE cometabolism observed during bioaugmentation and lactate addition.

## Conclusion

### *Laboratory Study of $PR1_{301}$ Survival*

The relatively good TCE removal with bioaugmentation and lactate addition found in the first field study and during the first few days of the second and third studies, followed by poor TCE removal during later periods of the last two studies, suggested that a population of predators to $PR1_{301}$ may have developed in the field. This was also suggested by the fact that arrival of $PR1_{301}$ at the monitoring wells occurred during the first few days of bioaugmentation, but rarely after that. In order to obtain some confirmation of the possibility of a predator population causing this problem, samples of groundwater and aquifer organisms were obtained at the end of the field study by rapid groundwater withdrawal and its collection from the injection well where the population was expected to be the highest. This resulted in a groundwater sample containing about 2,000 mg/l total suspended solids. Microscopic observation of the extracted water with about 2,000 mg/l total suspended solids, indicated a high population existed of motile bacteria that were not representative of $PR1_{301}$. A few small ciliated protozoa were also observed.

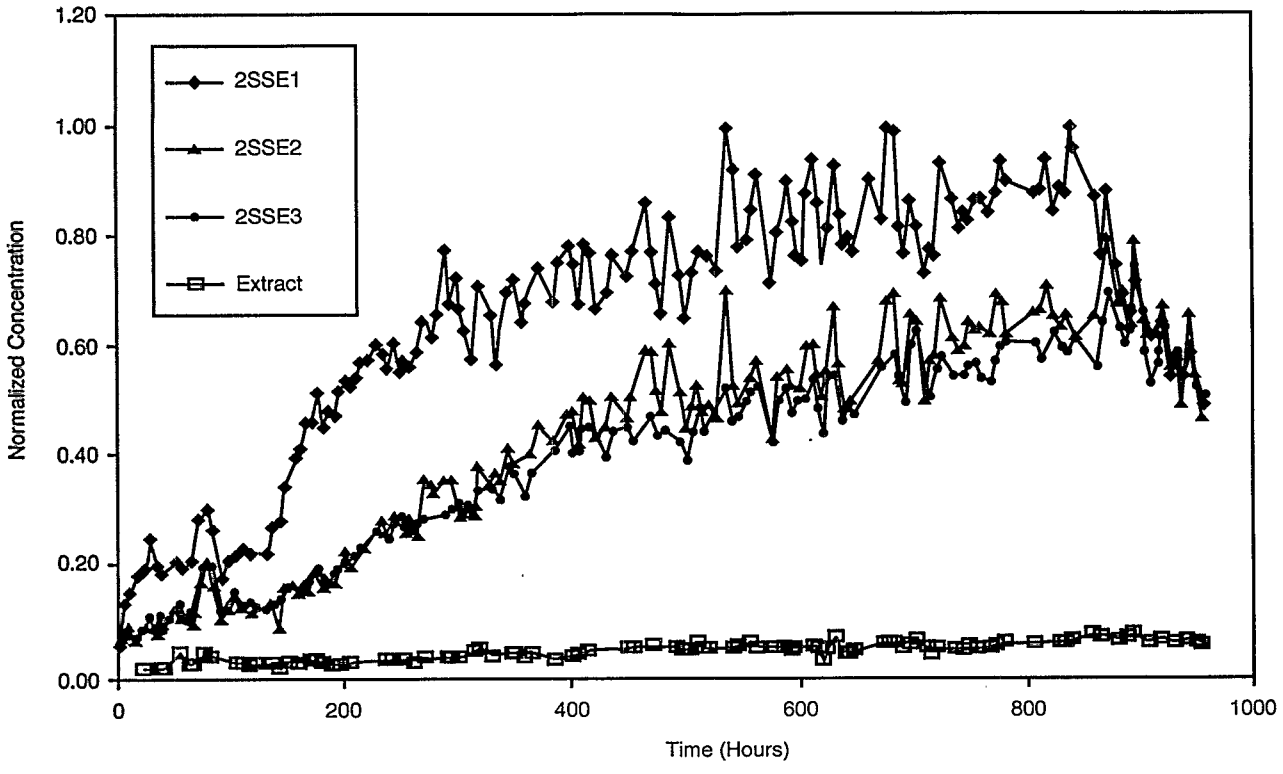


**Figure 7.** Normalized TCE concentrations at the various monitoring locations during the third field study. Added TCE concentration was 100 µg/l. Bioaugmentation was conducted for the first 520 hr only, 13 mg/l lactate was added for the first 830 hr, and 6 mg/l phenol was added during the last 120 hr.

Two separate studies were conducted to compare the ability of $PR1_{301}$ cultures to persist in the above groundwater. In the first, several individual bottles, each containing 25 ml of $PR1_{301}$ grown as usual in the 16-liter carboys (yielding 320 mg/l total suspended solids) together with 75 ml of the above groundwater, were incubated at room temperature. Controls with buffered nutrient water and organisms and other buffer controls without organisms were prepared similarly. All samples were mixed at room temperature. Periodically, individual bottles were supplemented with 3.9 mg/l TCE and the rate of consumption over time was monitored. In the second study, 800 ml of the above groundwater was mixed in a 2-liter flask with 800 ml of culture grown as usual in 16-liter carboys. A 2-liter flask control contained 800 ml of buffered nutrient water and 800 ml of culture, but no groundwater. Both flasks were mixed at room temperature. Daily, 75 ml was withdrawn from each flask for analysis of TCE decomposition rate and was replaced with 75 ml of buffer water containing 16 mg/l sodium lactate, similar to the concentration added in the field study. For withdrawn samples, 25 ml was mixed with 75 ml of buffer water, 2.6 mg/l TCE was added, and the rate and extent of TCE utilization with time was measured. A control containing 100 ml of buffer water and 2.6 mg/l of TCE was also evaluated for possible physical loss of TCE.

In the first field study, the TCE degradation rate in groundwater cultures was about 20% higher than cultures in buffer water, perhaps because the groundwater contained some active $PR1_{301}$. However, by day four, the rate with the groundwater culture was only about 88% of that in buffer water, and by day ten the groundwater culture removed no TCE while the buffer water culture was still removing TCE at about 10% of the initial rate. These data suggest that the groundwater was detrimental to the activity of $PR1_{301}$. The second study was perhaps more definitive as lactate was added in order to maintain some activity among the cultures similar to what was done in the field. Here again, the TCE utilization rate on day zero was about 8% higher with the groundwater culture, but within three days, the groundwater culture was no longer consuming TCE but the buffer water culture maintained a TCE degradative ability throughout the last nine days of the study at a rate of about 23% of that obtained on day zero. This again indicates that groundwater conditions were detrimental to $PR1_{301}$, activity. The results are not conclusive as to whether predation caused the activity loss or whether $PR1_{301}$ simply lost out in the competition for lactate and for this reason could not maintain a high level of activity for long. In any event, the laboratory study confirmed that $PR1_{301}$ could not maintain activity well in the groundwater environment. Three field studies with bioaugmentation were conducted. In each one, bioaugmentation with $PR1_{301}$, along with lactate addition was initially reasonably effective at TCE removal. In addition, phenol was rapidly consumed to near detection limits when added following a short period of bioaugmentation with $PR1_{301}$ and lactate addition. These results demonstrated that an initial phase of bioaugmentation could be effective to establish a population of

phenol or toluene degraders if these substrates were to be used at a site. However, in both the second and third field trials, bioaugmentation in time with lactate feed alone became ineffective at TCE removal, reaching near zero removal within a few weeks. The inability of $PR1_{301}$, to remain effective in bioaugmented groundwater was also demonstrated in a brief laboratory study using groundwater from the bioaugmented well after the conclusion of the field study. This suggested either predation of the introduced population or the inability of $PR1_{301}$ to effectively compete for the added lactate, or perhaps both, were the cause of the eventual failure of the system. In order for bioaugmentation for TCE cometabolism to be successful, methods for avoiding this competitive problem need to be found.

## Acknowledgments

This study was supported through Cooperative Agreement CR822029 from the Gulf Ecology Division, U.S. Environmental Protection Agency. Special thanks are extended to Dr. Laurence H. Smith and Dr. Alfred M. Spormann for their assistance in culture growth during the third phase of the field study.

## Disclaimer

The U.S. Environmental Protection Agency through its Office of Research and Development partially funded and collaborated in the research described here under Cooperative Agreement No. CR819630 to North Carolina State University. It has been subjected to the Agency's peer and administrative review and has been approved for publication as an EPA document. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

## Quality Assurance Statement

This project was conducted under an approved Quality Assurance Program Plan. The procedures specified in the plan were used without exception. Information on the plan and documentation of the quality assurance activities and results are available from the principal investigator.

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