FIELD PUSH-PULL TEST PROTOCOL

FIELD PUSH-PULL TEST PROTOCOL FOR AEROBIC COMETABOLISM OF CHLORINATED ALIPHATIC HYDROCARBONS

for

Environmental Security Technology Certification Program

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1.0 FIELD PUSH-PULL TEST PROTOCOL OVERVIEW

A major problem limiting the widespread use of aerobic cometabolism for treating chlorinated aliphatic hydrocarbons (CAHs) contamination in groundwater is the need for site-specific data for use in feasibility assessment and remedial design. Currently, the approach used to obtain this information consists of preliminary laboratory microcosm tests performed on core samples followed by pilot-scale well-to-well recirculation tests (Semprini *et al.*, 1992). Although this approach has been successfully applied in a limited number of field demonstrations, it has several disadvantages that limit its routine use. For example, sediment samples are difficult to obtain and samples obtained by coring may be too small to provide representative information on subsurface conditions. Well-to-well recirculation tests interrogate a larger volume of the subsurface and thus have the potential to provide more representative information but are expensive and logistically complicated.

This protocol describes a newly developed field technology for evaluating the feasibility of using in situ aerobic cometabolic processes to treat groundwater contaminated with chlorinated solvent mixtures. The technology is called the single-well push-pull test. Push-pull tests have been previously used to obtain quantitative information on a variety of aquifer physical, chemical, and microbiological characteristics (Istok et al., 1997; Schroth et al., 1998; Istok et al., 1999; Schroth et al. 2001; Hageman et al., 2001), but have not previously been used to investigate aerobic cometabolic processes. This protocol describes the use of push-pull tests to evaluate the potential for aerobic cometabolism of CAHs using gaseous cometabolic substrates such as propane and soluble substrates, such as toluene. The protocol consists of 8 Sections. Sections 1 and 2 describe the overview of the protocol and introduction to the field test methodology. Section 3 describes how field push-pull tests are performed. Section 4 describes how to prepare test solutions. Section 5 summarizes the analytical methods used to measure concentrations of tracers, nutrients, substrates, chlorinated solvents and their transformation products in field samples collected during push-pull tests. Section 6 presents example results from field tests conducted at McClellan AFB, CA. Section 7 presents example results from field tests conducted at Fort Lewis, WA. Section 8 contains references.

2.0 INTRODUCTION

2.1 Introduction to Push-Pull Tests

A push-pull test consists of the controlled injection ("push") of a prepared test solution into an aquifer followed by the extraction ("pull") of the test solution/groundwater mixture from the same location (Figure 1). Tests may be performed in existing monitoring wells or multilevel samplers. The injected test solution consists of water containing one or more conservative (i.e., nonreactive) tracers and one or more reactive solutes; the type, combination, and concentration of reactive solutes are selected to investigate specific aquifer characteristics. During the *injection phase*, the test solution is injected into the aquifer where it flows approximately radially outward and penetrates a roughly cylindrical volume of aquifer material centered about the well (Figure 1A). During the *extraction phase*, flow is reversed and the test solution/groundwater mixture is pumped from the same location and concentrations of tracer, reactive solutes, and possible reaction products are measured as a function of time (Figure 1B). Tracer concentrations are used to adjust concentrations of reactive solutes and reaction products for dilution. Mass balances are computed by integrating dilution-adjusted concentrations during the extraction phase. Reaction rates are computed from the mass of reactive solute consumed and/or product formed.

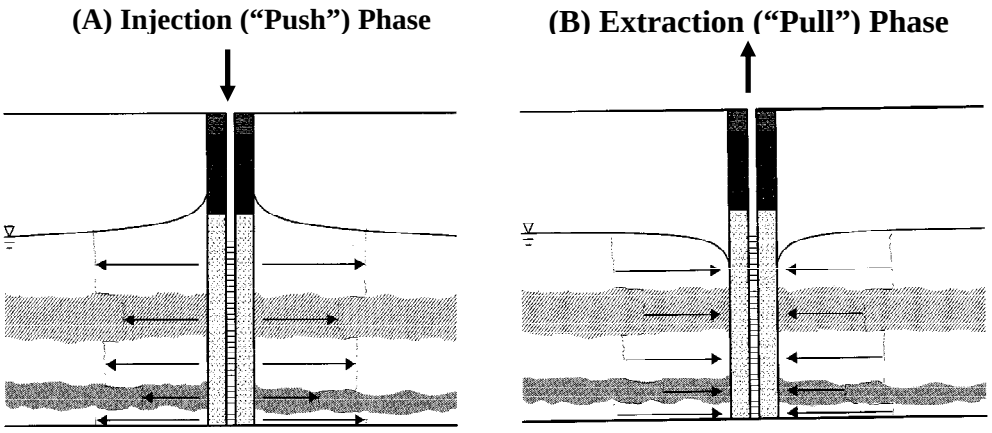

Figure 1. Injection and extraction phases of a "push-pull" test

If the durations of the injection and extraction phases are relatively small (~ hrs), the effects of regional groundwater flow are minimal and the flow and transport of the injected test solution is dominated by the induced gradient flow field created near the well by injection and extraction pumping. However, in some tests it may be desirable to include a *drift phase* (with no pumping) between injection and extraction phases to increase the residence time of the test solution in the aquifer and allow more time for reactions to proceed (Figure 2). During the drift phase, flow and transport of the injected test solution is dominated by the regional groundwater flow field. Drift phase durations may range from hrs to months, depending on the type of test and site conditions. For example, long drift phases are generally desirable to allow time to stimulate activity of indigenous microorganisms and to allow for injected reactants to be transformed in situ to detectable products. However, if the duration of the rest phase is too large, excessive dilution of the injected test solution may occur, lowering concentrations of tracer, reactants, and products below detection limits.

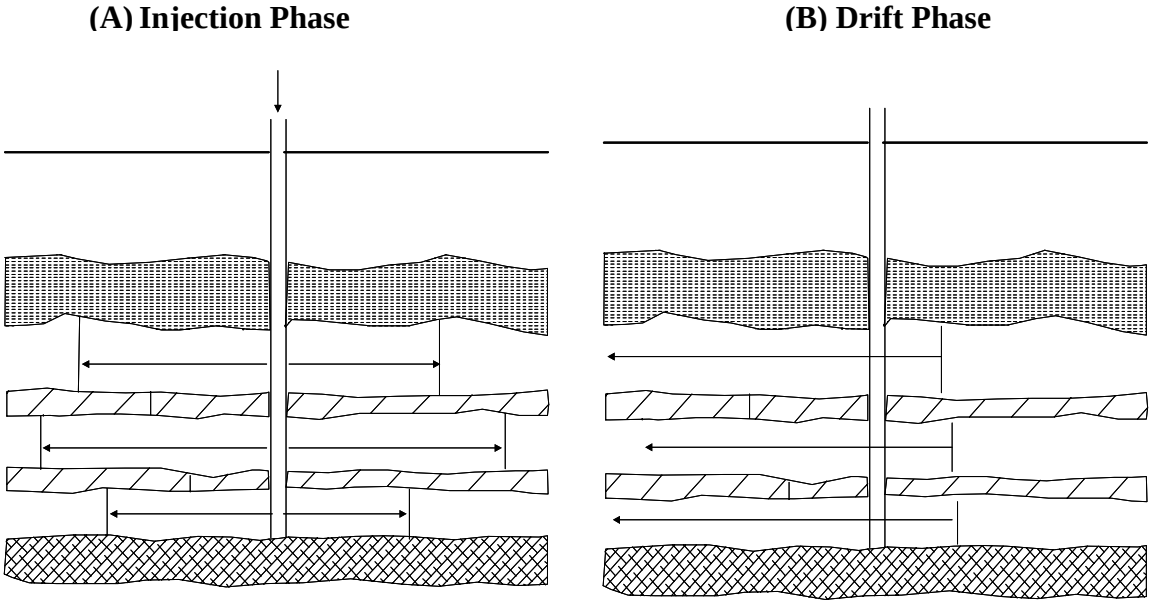

Figure 2. Transport of injected test solution away from well during a natural drift phase

2.2 Objectives of Push-Pull Tests for Aerobic Cometabolism

This protocol describes the use of push-pull tests to evaluate the potential for aerobic cometabolism of CAHs, such as trichloroethene (TCE) using gaseous cometabolic substrates such as propane and soluble substrates, such as toluene. A series of push-pull tests are described that can be used to obtain the following site-specific information:

1) To determine the transport characteristics of nutrients, substrates, and CAHs and their transformation products,
2) To determine whether indigenous microorganisms have the capability to utilize selected substrates and transform targeted contaminants,
3) To determine rates of substrate utilization and contaminant transformation, and
4) To optimize combinations of injected nutrients and substrates to maximize rates of contaminant transformation.

Transport characteristics (e.g., retardation factors) of substrates, contaminants, and, in some cases their transformation products are needed to compute substrate utilization and contaminant transformation rates and are also needed as input to site-scale groundwater flow and contaminant transport modeling and these are obtained using *Transport Tests*. Transport tests are conducted in a way that minimizes the potential for substrate utilization or contaminant transformation. *Biostimulation Tests* are designed to stimulate microbial activity. Rate of substrate utilization and contaminant transformation are determined using *activity tests,* which are conducted under conditions that promote the expression of indigenous microbial activity. In the protocol, transport tests are conducted first (Figure 3). Then a series of biostimulation tests is conducted to stimulate microbial activity. One or more activity tests are conducted to demonstrate aerobic cometabolic activity of the indigenous microorganisms by monitoring the rate of consumption of injected nutrients (e.g., nitrate) and gaseous substrates (e.g., propane and oxygen), or soluble

substrates (toluene and oxygen), the production of defined products from injected surrogate compounds (e.g. the production of ethylene oxide from injected ethylene and the production of ortho-cresol from injected toluene), and the production of defined CAH oxidation products (e.g. the production of cis-DCE epoxide). The final test is an inhibition test, where a mechanism based inhibitor of the monooxygenoase enzyme (acetylene or 1-butyne) is added to inhibit the transformations observed in the previous activity test.

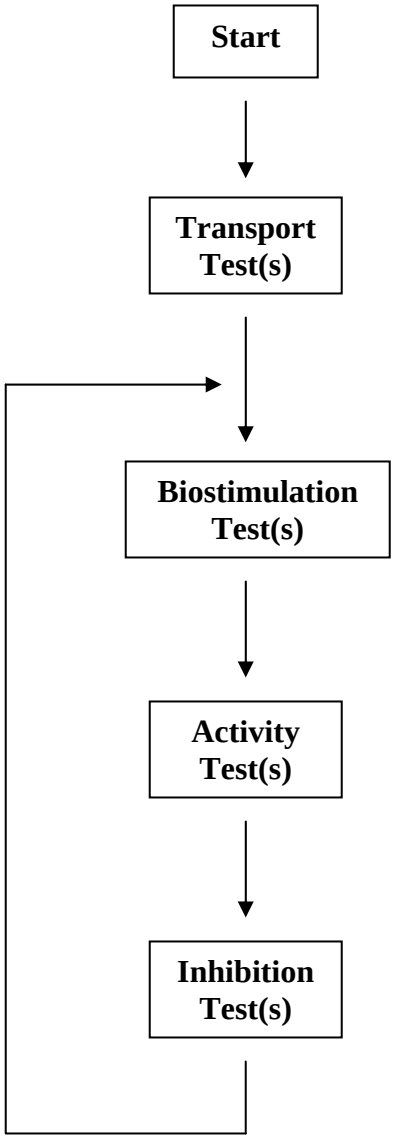

Figure 3. Push-pull test sequence

3.0 EXPERIMENTAL PROTOCOL FOR FIELD PUSH-PULL TESTS

3.1 Transport Tests

Test solutions for transport tests contain a tracer and additional solutes (substrates, CAH surrogates, or CAHs) for which transport information is desired. Note that it is also possible to simultaneously obtain transport information for additional solutes present in site groundwater components if these are *not* present in the injected test solution but are analyzed for during the extraction phase. Transport tests are conducted under conditions selected to minimize the opportunity for microbial transformation of injected solutes. This is usually accomplished by selecting injection and extraction pumping rates that minimize the total time that the test solution is in contact with the aquifer. For example, the composition of the injected test solution may be adjusted by removing a necessary nutrient (e.g., NO_3^-) or substrate (e.g., O_2). The volume of injected test solution is selected to interrogate a sufficient volume of aquifer so that representative results are obtained. Samples of the test solution are collected during the injection phase so that the initial concentrations of all solutes are known. Additional samples are collected during the extraction phase to develop breakthrough curves for all injected solutes and, if desired, solutes present in the site groundwater that were not included in the injected test solution. In a transport test, extraction pumping continues until approximately twice the injection volume has been recovered, which is usually sufficient to recover a substantial portion of the injected test solution.

Transport test data are interpreted using methodology presented in Schroth et al. (2001). This involves first fitting the advection-dispersion equation to the breakthrough curve for the conservative tracer (e.g., Br^-) to estimate aquifer dispersivity and then fitting the advection-dispersion equation to the breakthrough curve for a potentially retarded solute to estimate the solute's retardation factor. An example is in Section 6.1.

3.2 Biostimulation Tests

Biostimulation tests are designed to expose the indigenous microbial community to nutrients and substrates for extended periods of time (days to weeks) to stimulate growth and activity. The injected test solutions contain only tracer, nutrients, and gaseous substrates or soluble substrates (no surrogates or CAHs). This approach utilizes aqueous solutions to deliver dissolved substrates and nutrients to the aquifer. An alternate method for introducing gaseous substrates involves direct gas injection (gas sparging) wherein a defined gas mixture is injected into the well screen (Figure 4). Substrates are released to the aquifer by mass transfer from the injected gas stream and by dissolution of trapped gas bubbles that form during gas injection. This method was tested in our second demonstration at McClellan AFB.

The extraction phase of a biostimulation test consists of discrete sampling events instead of the continuous extraction phase pumping and sampling used for transport and activity tests. The frequency of the sampling events is selected to provide sufficient data to monitor changing concentrations of substrate during the test. Biostimulation tests are often repeated until the resulting increase in activity is large enough to be detected by an activity test (Figure 3). The biostimulation test data are interpreted using the method of Haggerty et al. (1998), which involves plotting dilution-adjusted solute concentrations as a function of sample residence times. Dilution adjustments are performed using measured concentrations of the bromide tracer (for solutes with retardation factors equal to one) or with retardation factors estimated from transport tests (for solutes with retardation factors greater than one). The sample residence time is defined as the elapsed time from the midpoint of the injection phase to the time the sample was collected. Examples are presented in Section 6.1 and 7.3.

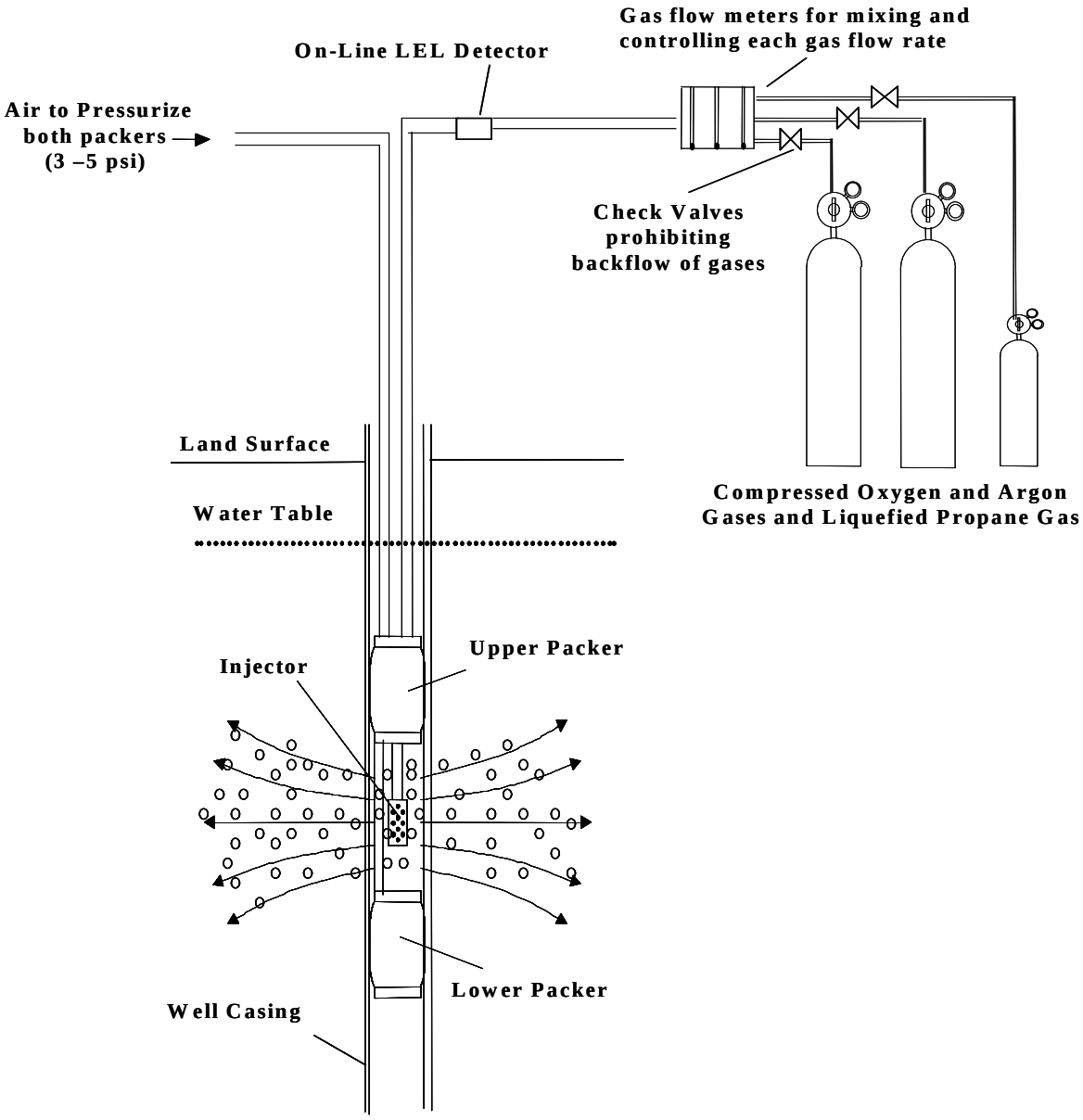

Figure 4. Direct gas injection to introduce substrates to the subsurface

3.3 Activity Tests

Unlike transport tests, activity tests are conducted under conditions that allow microbial activity to be detected. Thus, injected test solutions contain all nutrients and substrates required for a particular reaction to proceed. Samples are collected during the injection phase of an activity test so that initial concentrations of all solutes are known. A drift phase with no pumping is typically included between the injection and extraction phases. The duration of the drift phase is selected to be long enough to permit detectable consumption of injected substrates (e.g., O_2, propane or toluene), surrogates (e.g., ethylene, isobutene), or CAHs (e.g., cis-DCE) and detectable production of surrogate or CAH transformation products (e.g., ethylene oxide or isobutene oxide). The duration of the drift phase must also be selected to be sufficiently short that a substantial portion of the injected test solution can be recovered during extraction phase pumping. Regional groundwater flow will eventually transport injected test solutions away from the well and reduce measured solute concentrations below detection limits. The effect of regional flow can be mitigated by injecting a larger volume of test solution, reducing the duration of the drift phase, or increasing the extraction phase pumping rate.

Activity test data are interpreted using the method of Haggerty et al. (1998), which involves plotting dilution-adjusted solute concentrations as a function of sample residence times. Dilution adjustments are performed using measured concentrations of the bromide tracer (for solutes with retardation factors equal to one) or with retardation factors estimated from transport tests (for solutes with retardation factors greater than one). The sample residence time is defined as the elapsed time from the midpoint of the injection phase to the time the sample was collected. Examples are in Section 6.1 and 7.3. Activity tests are typically conducted before and after biostimulation tests so that increase in microbial activity resulting from biostimulation may be detected and quantified. Typically rates of nutrient and substrate utilization and surrogate and/or

CAH transformation increase following biostimulation and thus it may be desirable to decrease the duration of the rest phase as microbial activity increases.

3.4 Inhibition Tests

The final test to be performed is the inhibition test. The inhibition test is the same as an activity test, except a mechanistic based inhibitor of the monooxygenase enzyme of interest is added along with the substrates of interest. For propane utilizers, acetylene is used as the inhibitor of the oxygenase enzyme, while 1-butyne was used as an inhibitor of the toluene ortho-monooxygenase enzyme. Test procedures are exactly the same as used in the activity test so direct comparison between the tests can be made. If effective inhibition is achieved, the results from the inhibition test should be similar to those observed in the transport test.

4. TEST SOLUTION PREPARATION

4.1 Conservative Tracer and Nutrients

Although many conservative (i.e., nonreactive) tracers have been used in groundwater studies, bromide at a concentration of 100 mg/L is recommended as a conservative tracer for push-pull tests. This concentration was selected as a compromise between analytical detection limits (~ 1 mg/L for Br^- by ion chromatography) and the desire to avoid injecting test solutions with densities substantially larger than that of site groundwater. Bromide is added as potassium bromide (KBr). If background Cl^- concentrations are below a few mg/L, Cl^- (added as NaCl) is an acceptable alternative. Alternative tracers may be used if their conservative transport behavior is demonstrated (e.g., by performing a transport test with coinjected bromide) and if their chemical and microbial stability can be assured for the duration of activity and biostimulation tests. Nitrate in the form of sodium nitrate ($NaNO_3$) may be added as a nutrient in some tests. Both KBr and $NaNO_3$ are highly water-soluble; thorough mixing of added KBr

and $NaNO_3$ is accomplished during the gas sparging used to introduce gaseous substrates and surrogate compounds to the test solution (see next section).

4.2 Gaseous Substrates and Surrogate Compounds

Gaseous substrates (propane and oxygen) and surrogate CAHs (propylene and ethylene) are introduced into the test solution by bubbling (sparging) the test solution contained in plastic carboys with a defined mixture of compressed gases (Figure 5). Sparging also serves to thoroughly mix the test solution with respect to added KBr and $NaNO_3$. Specified aqueous concentrations of substrate and surrogate CAHs are achieved by controlling the flow rate of each gas to the sparging lines. The flow rates are selected to achieve a desired partial pressure of each gas in the carboy headspace; from the partial pressures, the aqueous concentration of each gas may be determined using the solution temperature and Henry's law constant for the gas. Gas flow rates are controlled and gases are mixed using gas flowmeters (Cole-Parmer Instrument Co., Vernon Hills, IL), which are calibrated for each specific gas used. For all tests it is necessary to avoid creating an explosive gas mixture in the carboy headspace. To avoid this problem, a portion of the test solution is contained in one carboy and sparged with the flammable gases (propane, ethylene, and/or propylene) and a portion is contained in a second carboy and sparged with oxygen (Figure 5). The two portions of test solution are combined by pumping from each carboy into a single injection line and mixed with a mixing coil prior to injection. The resulting dissolved gas composition of the injected test solution is therefore controlled by the partial pressure of each gas in the two carboys and the two pumping rates. Samples of the injected test solution are collected using a syringe and sampling valve during the injection phase and analyzed for aqueous gas concentrations so that the composition of the injected test solution is well known. A photograph of a typical field set up is shown in Figure 6.

4.3 Liquid Substrates and Surrogate Compounds

The test solution is prepared with groundwater extracted from the well port where push-pull tests solution is to be injected. Bromide is used as a non-reactive tracer. Reactive solutes include the dissolved growth substrate (toluene), hydrogen peroxide (DO), non-toxic dissolved surrogate isobutene, and nitrate as a nutrient. Groundwater needed for making the inject solution is pumped from the wells using a Masterflex peristaltic pump (Barnant Co., Barrington, IL). The test solution is prepared by adding bromide, nitrate and hydrogen peroxide in a plastic carboy and thoroughly mixed. Toluene is added to a collapsible Teflon bag and to achieve a desired concentration. Isobutene solution is prepared in a plastic carboy the same method as described in section 4.2. The different injection solutions are mixed together at different flow rates to achieve the desired injection concentration. Figure7 shows schematic of equipment used to introduce liquid substrates and surrogates in single push-pull field tests.

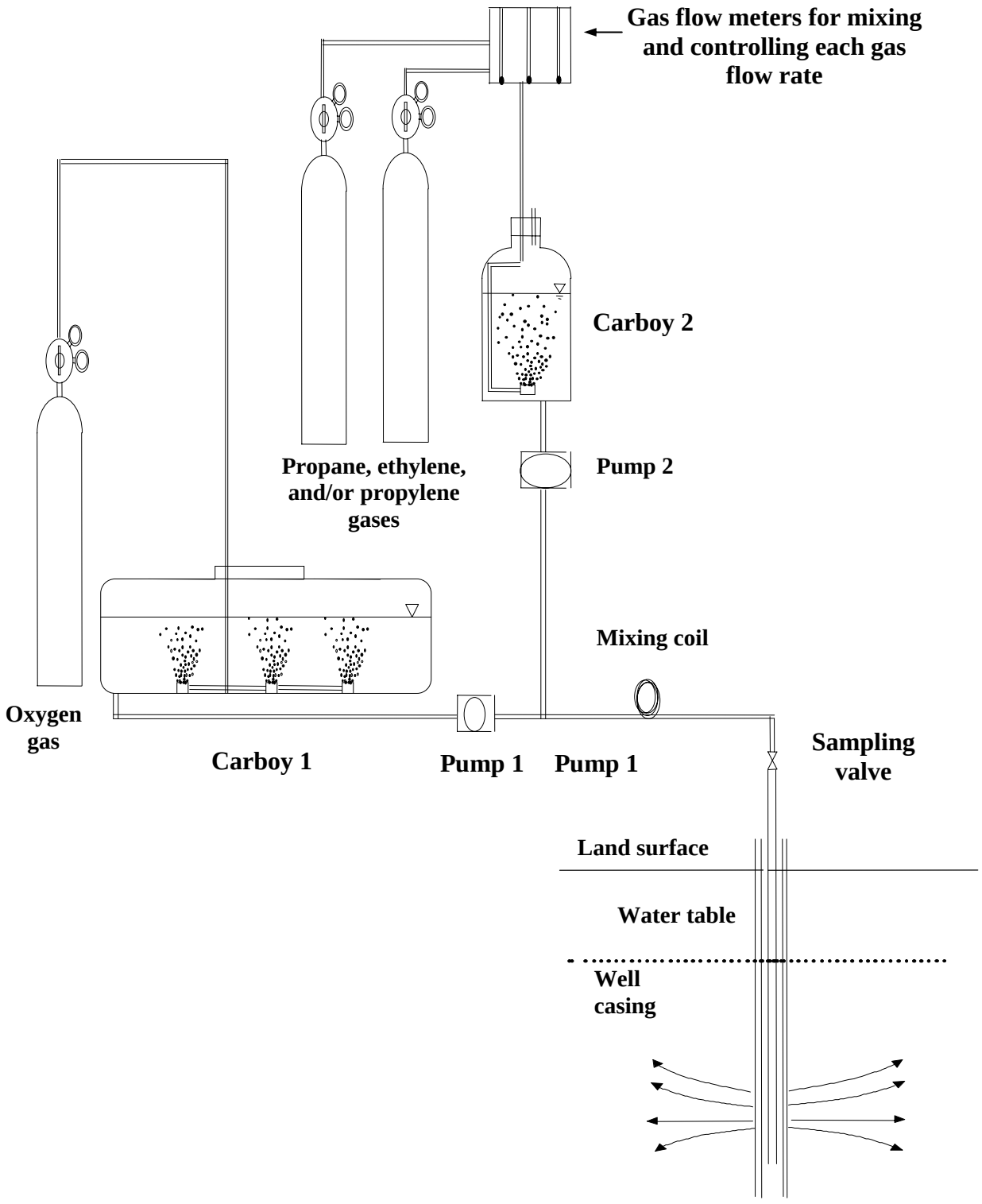

Figure 5. Equipment used to introduce gaseous substrates and surrogates into injected test solutions.

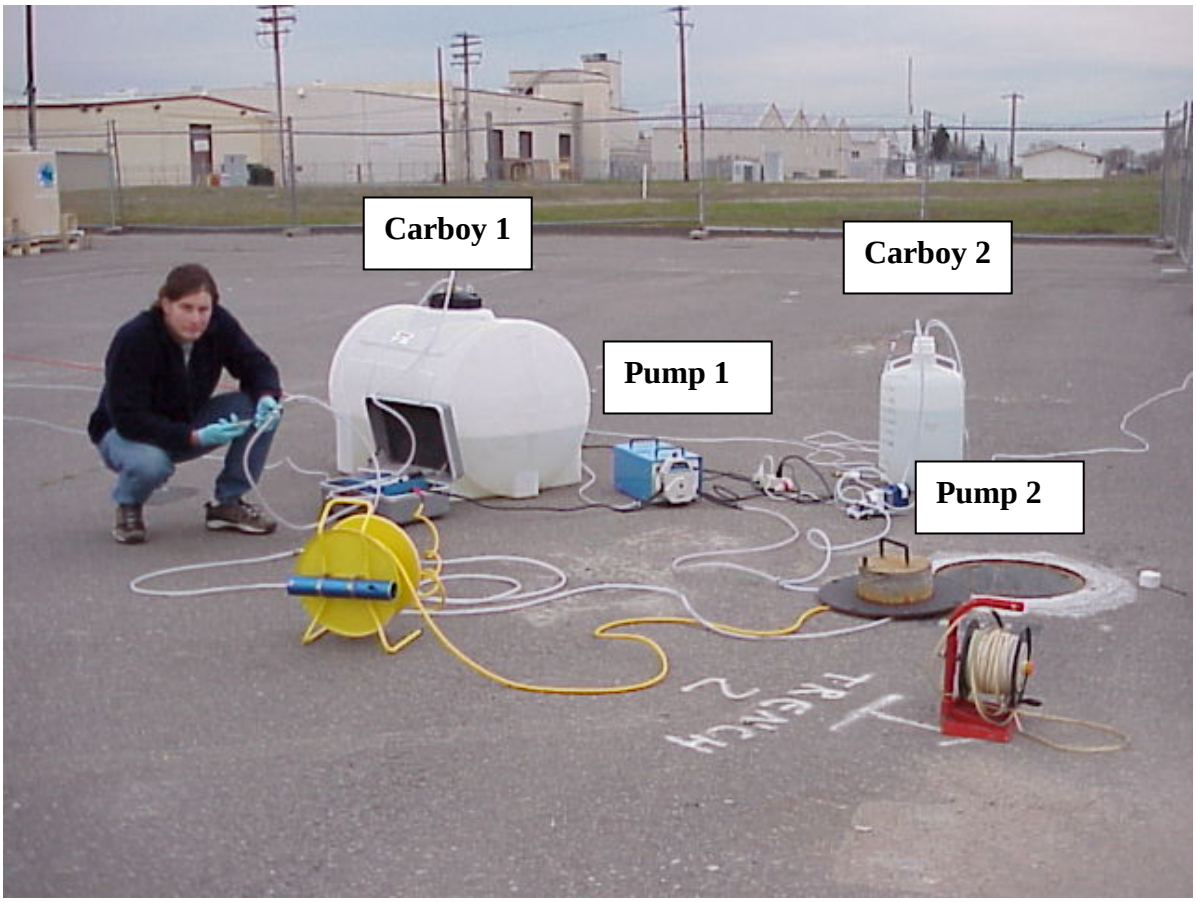

Figure 6. Typical field setup for push-pull tests

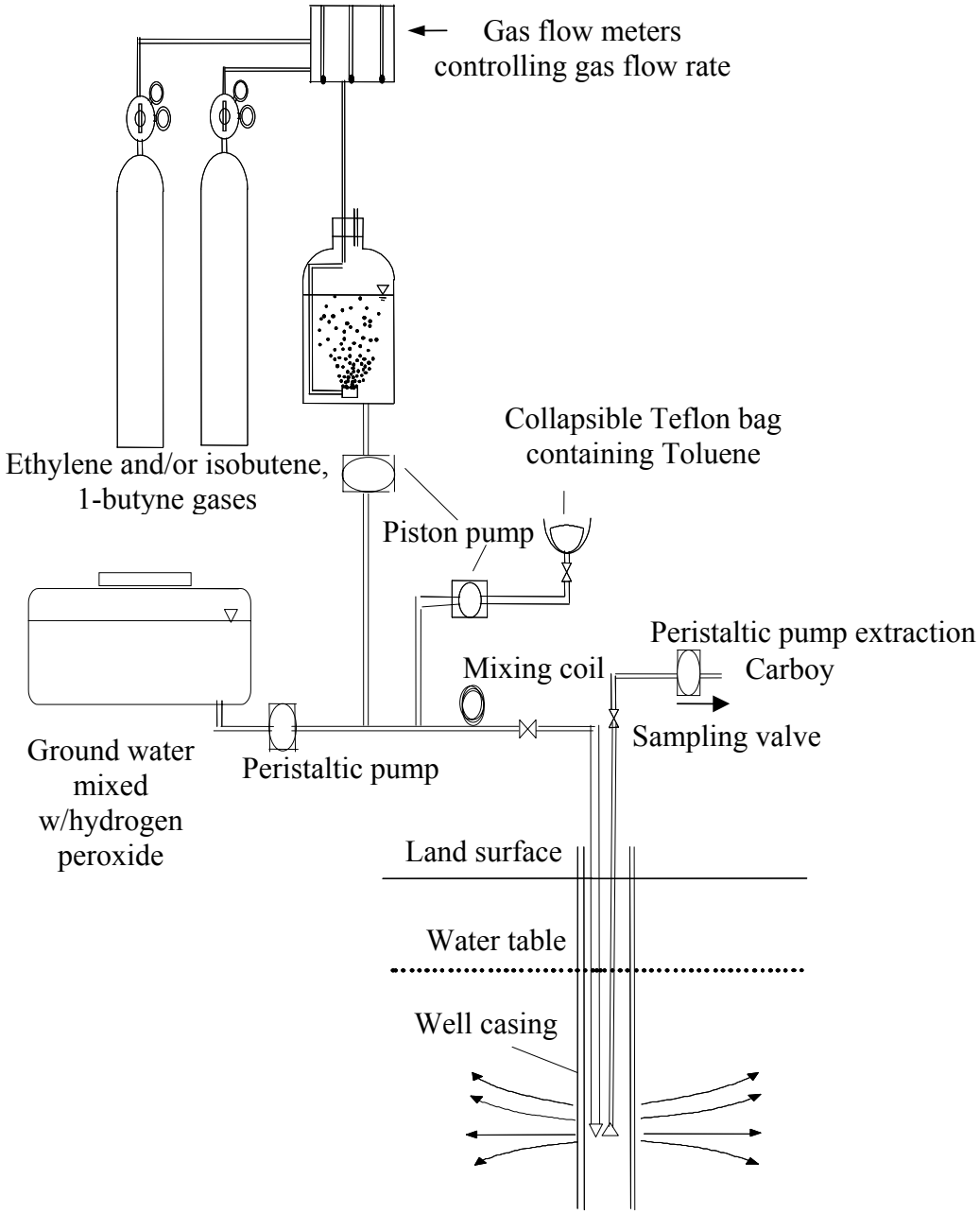

Figure 7. Schematic of equipment used to introduce liquid substrates and surrogates in single push-pull field tests.

5.0 ANALYTICAL METHODS

5.1 Sample Collection

Liquid samples are required for analysis of injected tracer, nutrient, substrate, surrogates, CAHs, and their transformation products. A sampling valve equipped with syringe adapter is used to collect samples during the injection and extraction phases of all tests. To collect a sample, a gas-tight syringe is fitted to the sampling valve, purged several times, and then aspirated to obtain a liquid sample. The time of sample collection is also recorded. The contents of the syringe are dispensed into sample vials as follows: A 1 mL sample is collected in a plain glass vial for tracer (Br^-) and nutrient (NO_3^-) analyses by ion chromatography (IC). A 2 mL sample is collected in a syringe for dissolved oxygen analysis in the field by oxygen electrode. A 40 mL sample without headspace is collected in brown bottles equipped with a septa and a screw cap for substrate, CAHs, and transformation product analyses by gas chromatography (GC). IC and GC samples are stored at 4 ºC until analyzed.

5.2 Determination of Inorganic Anions by Ion Chromatography

Concentrations of inorganic anions (Br^- and NO_3^-) are determined with a Dionex DX-500 (Sunnyvale, CA) ion chromatograph equipped with electrical conductivity detector and a Dionex AS14 column. The eluent consisted of 3.5 mM Na_2CO_3 and 1.0 mM $NaHCO_3$ and the eluent flow rate was 1.5 mL/min. A 0.6-mL sample was transferred to Dionex Polyvials ™ with filter caps for auto-sampler injection; the auto-sampler was programmed to deliver an injection volume of 50 µL. Run time was approximately 10 minutes. External calibration was performed using five standards with anion concentrations between 5 and 100 mg/L; the approximate quantitation detection limit is 1 mg/L.

5.3 Determination of Dissolved Oxygen by Oxygen Electrode

Dissolved oxygen is determined in the field using a Clark (Yellow Springs, OH) style oxygen electrode and meter (Figure 8). The electrode is mounted in a glass water-jacketed vessel to maintain a stable electrode temperature; the temperature of the water is recorded with a mercury thermometer. The electrode contacts the sample within a small (1.8 mL) volume chamber mounted inside the vessel. To perform a dissolve oxygen measurement, a water sample collected from the sampling valve is dispensed from the syringe into the chamber (filling it to overflowing), which is then closed with a glass plug. A small stir bar within the chamber and an external magnetic stirrer are used to mix the sample during measurement. After the meter reading stabilizes, the oxygen saturation value from the meter is recorded. The sample is then removed from the chamber using a plastic syringe.

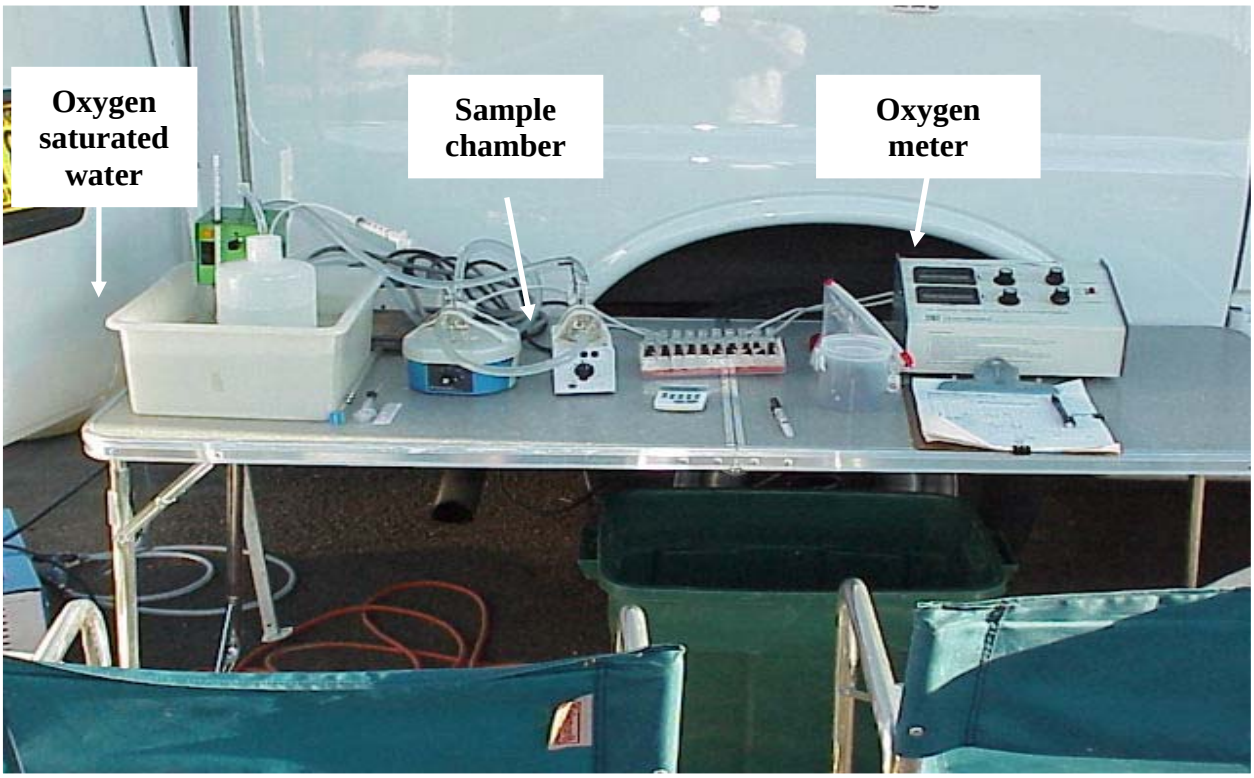

Figure 8. Field equipment used to measure dissolved oxygen concentrations.

To convert oxygen saturation values to concentration units (mg/L), the oxygen saturation of a reference sample is measured immediately after each sample measurement using the same procedure. The reference sample consists of oxygen saturated distilled water, which is prepared by sparging a 1 L bottle with oxygen gas. The dissolved oxygen concentration of a sample is determined using the measured oxygen saturation for the sample, the measured oxygen saturation for the reference sample, the measurement temperature, and a handbook value for oxygen solubility in distilled water at the measurement temperature.

Hydrogen peroxide concentrations are monitored using thiocyanate colorimetric method developed by CHEMetrics, Inc. The thiocyanate method consists of ammonium thiocyanate and ferrous iron in acid solution. Hydrogen peroxide oxidizes ferrous iron to the ferric state, resulting in the formation of a red thiocyanate complex. This method covers hydrogen peroxide concentrations of 0-1000 mg/L.

5.4 Determination of Gaseous and Liquid Substrates, Surrogate Compounds, and CAHs by Gas Chromatography

Test samples are collected in 40-mL VOA vials with a Teflon/neoprene septum and a polypropylene-hole cap (Supelco, Bellefonte, PA). Samples are not preserved with acid, since the transformation of potential cometabolic by-products, ethylene oxide, propylene oxides, and isobutene oxide, are acid catalyzed. Samples for laboratory analysis are stored at 4 ºC and analyzed within one week.

Gaseous substrates, surrogates, and CAHs and their transformation products: Gaseous substrates, surrogates, and CAHs and their transformation products are determined by a modified EPA 8000 purge and trap GC analysis. A 1 or 5 mL aqueous sample is taken from a VOA vial using a S. G. E. gas tight luer lock syringe (Supelco Co, Bellefonte, PA). The sample is then added into a purge tube installed in HP 7695 Purge & Trap. A Tenax/silica gel/charcoal trap is

used as a purge trap (Supelco, Bellefonte, PA). A sample purge time of 15 min is used, rather than the standard 5 min, to increase the removal of the less effectively trapped compounds, such as ethylene, and to detect low concentrations of the less volatile metabolic products, such as ethylene epoxide and propylene epoxide. Chromatographic separation is achieved with a 30-m megabore GSQ-PLOT column from J&W Scientific (Folsom, CA) installed on a HP6890 series GC connected to a photo ionization detector (PID) followed by a flame ionization detector (FID) operated at 250 °C. The GC is operated at the following conditions: initial oven temperature, 40 °C for 3 min; 4 °C/min up to 70 °C; 5 °C/min up to 220 °C. The GC is operated in the splitless inlet mode with a carrier gas (He) flow of 15 mL/min, a H_2 flow to detectors of 35 mL/min, an air flow to the detectors of 165 mL/min and a FID detector makeup gas (He) flow of 15 mL/min. The retention time of each compound under this GC method is as follows: ethylene (3.3 min); propylene (9.8 min); propane (10.2 min); ethylene oxide (14.9 min); propylene oxide (21.9 min); cis-DCE (28.8 min); and TCE (33.7 min). The sensitivity of PID and FID on each compound is different, so that each compound is quantified by a more sensitive detector. Ethylene, propane, ethylene oxide, propylene oxide are quantified by FID, and propylene, cis-DCE and TCE are quantified by PID. Calibration curves for the compounds are developed using external standards.

Liquid substrates, surrogates, and CAHs and their transformation products: Purge-and-trap method is used in determining the dissolved concentrations of toluene, ortho-cresol, ethylene, isobutene and their transformation products, and CAHs. Five mL of aqueous samples from the VOA vials are introduced into an HP 7695 purge-and-trap system, and the volatile compounds were sorbed onto a Vocarb-3000 trap (Supelco, Bellefonte, PA). Optimizing P&T Cycle Time Experimentation yielded a time of 11 minutes to provide the optimal sample purge for the determination of toluene and o-cresol. Under equivalent conditions, a 5-minute purge time did

not adequately separate cis-DCE and isobutene oxide. A 2-minute desorption time of 250°C accommodated sharp initial peaks and provided good separation. Chromatographic separations are achieved with two 30-m megabore GSQ-PLOT and HP-624 columns from Agilent (New Castle, DE) installed on a HP6890 series GC connected to a photo ionization detector (PID) followed by a flame ionization detector (FID). The GC is operated splitless inlet mode with He carrier gas flow, a H_2 flow to FID detectors of 35 mL/min, an air flow to the detectors of 165 mL/min and a detector makeup gas (He) flow of 15 mL/min. For the GSQ-PLOT column, the GC is operated at the following conditions: column flow 15 mL/min; initial oven temperature, 50°C; 4 °C/min up to 150 °C hold for 3 min; 10 °C/min up to 220 °C. The retention time of each compound under this GC method is as follows: ethylene (3.1 min); isobutene (9.71 min); isobutene oxide (19.97 min); cis-DCE (20.24 min); TCE (25.9 min); Toluene (31.81 min); and o-Cresol (34.06 min). The HP624 megabore column is used to better separate the isobutene oxide and cis-DCE with the close retention times of 19.97 min and 20.24 min on GSQ-PLOT column, respectively. Using the HP624 column also supplements the identification of o-cresol and isobutene oxide with authentic standards which are assayed with chromatographic separation. For the HP624 column, the GC is operated at the following conditions: column flow 5 mL/min; the initial oven temperature, 40 °C for 5 min; 3 °C/min up to 100 °C hold for 2 min. The retention time of each compound under this GC method is as follows: isobutene (7.04 min); 1-Butyne (7.62 min); trans-DCE (12.28 min); isobutene oxide (12.69 min); cis-DCE (14.87 min); TCE (19.19 min); Toluene (23.28 min); and o-Cresol (25.15 min). Calibration curves for the compounds are developed using external standards.

6.0 EXAMPLE RESULTS FROM FIELD PUSH-PULL TESTS CONDUCTED AT THE McCELLAN AFB, CA

To illustrate additional details about the push-pull test methodology, a series of single-well-push-pull tests were performed to assess the feasibility of in-situ aerobic cometabolism of chlorinated aliphatic hydrocarbons (CAHs), such as trichloroethylene (TCE) and cis-1,2-dichloroethylene (cis-DCE), using propane and toluene as growth substrates. Propane tests were performed in the saturate zone at the McClellan Air Force Base, CA, while toluene tests were performed at Fort Lewis, WA (Section 7). The sequence of field tests followed the flow chart given in Figure 3; additional experimental details for each test are given in Tables 1 and 2. A transport test was conducted first followed by a series of biostimulation tests and then a series of activity tests. Detailed descriptions of test methodology and test results for each test type are described in the following sections.

6.1 Transport Tests

Push-pull tests were performed in two monitoring wells (MW2 and MW3) at McClellan AFB, CA. The aquifer at this site is mainly contaminated with cis-DCE (20 – 40 μg/L) and TCE (200 – 400 μg/L), and is aerobic (~ 6.2 mg/L dissolved oxygen). The aquifer consists primarily of alluvial deposits, and is unconfined with a water table depth ranging from 30 m to 32 m below ground surface. Tests were conducted in two monitoring wells (MW2 and MW3) constructed of 5.1 cm polyvinyl chloride casing with a 2.9 m long well screen.

Transport Tests were conducted in each well. These tests were followed by a biostimulation period consisting of five sequential additions of propane and dissolved oxygen to each well; followed by a series of activity tests and acetylene blocking tests (Table 1). Field equipment consisted of compressed or liquified gases, gas flow meters, two carboys (500 L and 50 L), a collapsible metalized-film gas-sampling bag (Chromatography Research Supplies, Addison, IL), a peristaltic pump to inject the test solution into the well, and a submersible pump

to extract groundwater from the same well (Figures 5 and 6). Site groundwater was used to prepare three solutions: 1) 500-L with known concentrations of bromide (KBr, Spectrum Chemical Mfg. Corp. Gardena, CA) to serve as a nonreactive tracer, nitrate ($NaNO_3$, Mallinckrodt Chemical, Inc. Paris, KY) as a trace nutrient, and oxygen as an electron acceptor; 2) 50-L with known concentrations of one or more dissolved gases [(propane (99.5%), ethylene (>99.9%), and/or propylene (>99.0%); Airgas Inc., Randor, PA] to probe for microbial activity; and 3) 5-L with known concentrations of dissolved acetylene (99.6%, Airgas Inc., Randor, PA) in a collapsible metalized-film gas-sampling bag. Specified dissolved gas concentrations in the 500 L and 50 L carboys were achieved by controlling the flow rates of each gas to ceramic sparging stones placed in the bottom of the carboys. Gas flow rates were controlled using rotameters fitted to a gas proportioner multitube frame that contained direct reading flow tubes (Cole-Parmer Instrument Co., Vernon Hills, IL). After dissolved gas concentrations had stabilized, the contents of the carboys and metalized bag were combined to obtain the desired solute concentrations using calibrated peristaltic and piston pumps and injected into the well. The composition of the test solution was monitored during injection by collecting samples from the well using a submersible pump (GRUNDFOS Pumps Co, Fresno, CA).

Samples of the injected test solution were collected by pumping the groundwater from the wells using a Grundfos pump placed down-hole in the screened interval of the well. Thus, the actual concentration of entering the aquifer was monitored. This down-hole sampling method provided very reproducible concentrations of the dissolved gases in the injected fluid.

Transport Test. A short-duration transport test was conducted in each well to compare the relative mobility of bromide, nitrate, and dissolved propane, oxygen, propylene, and ethylene in the aquifer prior to subsequent tests (Table 1). Two hundred sixty liters of test solution (prepared as described above) were injected at 2 L/min. After a 16 hr rest phase with no

pumping, the test solution/ground water mixture was extracted from the well at a rate of 2.5 L/min. Samples collected during the extraction phase were analyzed and used to prepare breakthrough curves for each injected solute.

In Figure 9A, extraction phase breakthrough curves for bromide, propane, ethylene, propylene, and DO are plotted as 1-C* that is, $1-[(C - C_{BG})/(C_o - C_{BG})]$. where C is the measured solute concentration in a sample collected after injection, C_o is average concentration of the same solute in the injected test solution, and C_{BG} is the background (pre-injection) concentration of the same solute in the ambient groundwater. The transport characteristics of all the substrates were very similar to bromide, showing no retardation. Based on mass balances on the injected solutes, the percent recovery of bromide was 99%, while the recovery of other injected solutes were slightly higher or similar to bromide (see Table 2). Nitrate and dissolved oxygen had recoveries greater than 100% since they are present in the native groundwater. The results demonstrate that the solutes can be effectively injected and recovered using the push-pull method that was developed, even at the aquifer depth of 30 m at the McClellan site. The dilution adjusted concentrations are all near unity (Figure 9B) indicating no reaction or retardation.

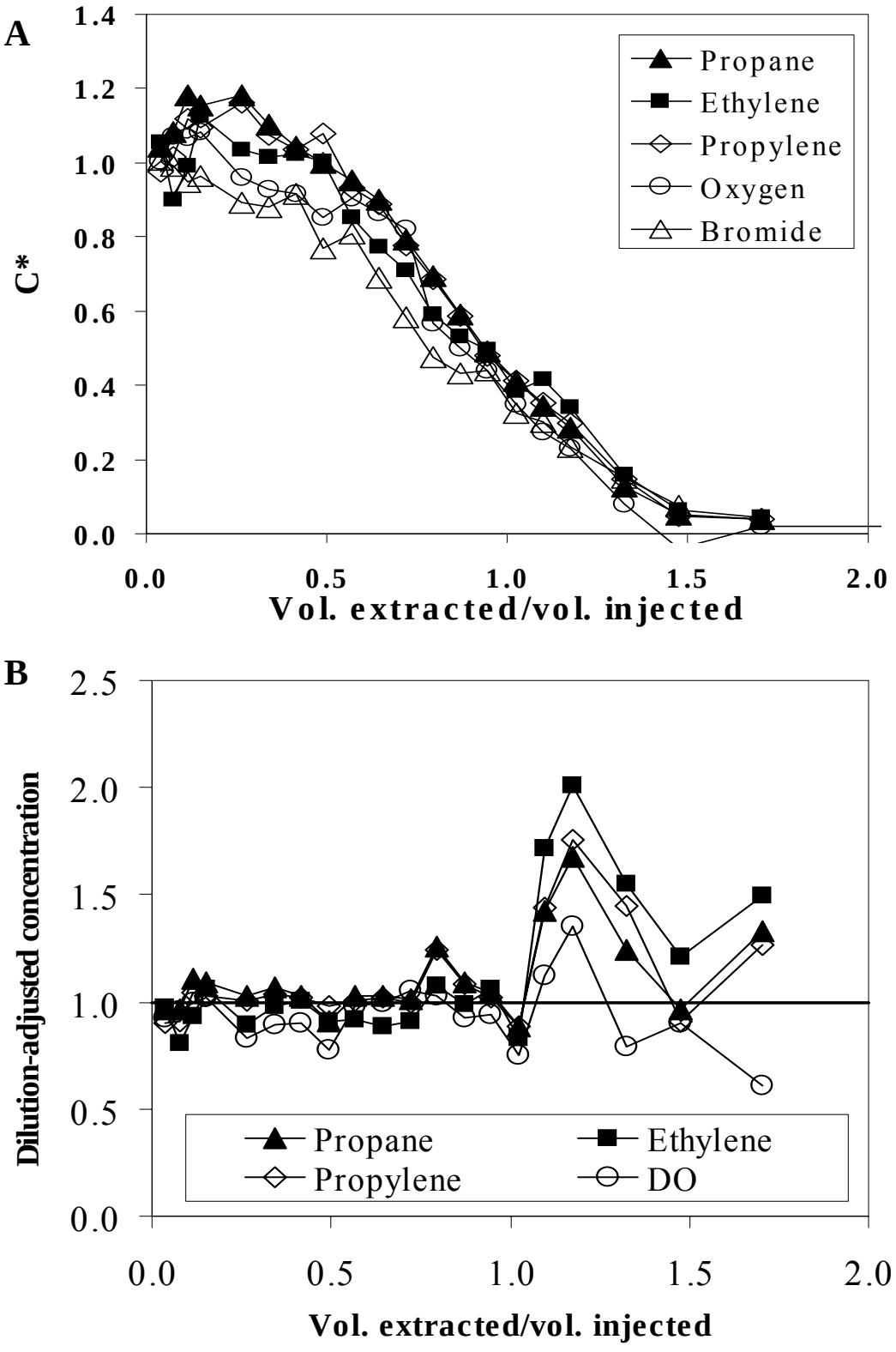

Figure 9. Extraction phase breakthrough (A) and dilution adjusted (B) curves in a push-pull transport test conducted at the McClellan AFB, CA (MW2) field.

Table 1. Test Solution Composition for Push-pull Tests Conducted at the McClellan AFB, CA (MW2) Field.

Test Type	Injection Volume (L)	Propane (mg/L)	Propylene (mg/L)	Ethylene (mg/L)	[1]Oxygen (mg/L)	[2]NO_3^-N (mg/L)	Br^- (mg/L)	[3]cis-DCE (µg/L)	[3]TCE (µg/L)
Transport Test	264	2.0 ± 0.1	4.0 ± 0.2	4.1 ± 0.2	22 ± 0.8	[3]NI	34 ± 1.5	3.7 ±1.0	27 ±5.1
Biostimulation Period (5 sequential additions)	498 ± 15	7.6 ± 3.0	[7]NI	NI	30 ± 3.5	7.7 ± 0.6	108 ± 20	2.5 ±0.5	28 ±2.5
[5]1st Propane Activity Test	238	2.4 ±0.1	NI	NI	30 ±0.8	1.9 ±0.1	40 ±1.5	4.4 ±1.1	54 ±1.1
2nd Propane Activity Test	250	1.3 ±0.1	NI	NI	16 ±0.6	4.4 ±0.2	22 ±0.1	2.1 ±0.2	36 ±3.2
Ethylene Activity Test	255	NI	NI	0.67 ±0.02	17 ±0.45	5.8 ±0.3	68 ±1.5	1.3 ±0.01	32 ±2.0
3rd Propane Activity Test	251	1.6 ±0.1	NI	NI	18 ±1.0	6.0 ±0.2	122 ±4.3	1.4 ±0.1	31 ±2.6
Propylene Activity Test	255	NI	1.6 ±0.1	NI	16 ±0.6	4.9 ±0.1	228 ±3.5	1.4 ±0.2	33 ±2.1
4th Propane Activity Test	317	1.6 ±0.2	NI	2.0 ±0.18	35 ±0.95	3.8 ±0.1	37 ±1.4	5.2 ±0.8	44 ±4.2
[6]Acetylene Blocking Test	346	1.2 ±0.9	NI	2.2 ±0.15	31 ±2.2	7.3 ±0.4	77 ±3.2	4.8 ±0.3	35 ±1.0

[1]: Background average dissolved oxygen concentration of 6.3 mg/L. [2]: Background average NO_3^- (as N) concentration of 1.1 mg-N/L. [3]: Average concentrations of cis-DCE and TCE concentrations in the injected test solution (C_0). [4]: Average values obtained during 1st through 5th Biostimulation tests. [5]: The 1st propane activity test was performed just prior to the 2nd biostimulation test. [6]: Tests were performed in MW3 only. Injected acetylene concentration was ~ 0.5 mM (10 mg/L). [7]: NI indicates not included.

Table 2. Summary of Quantities of Injected and Extracted Solutes Mass, Percent Recovery, and Zero-order Rate for Push-pull Tests Using MW2

Test Type	Quantities	Propane		Ethylene		Propylene		Br^-	
		MW2	MW3	MW2	MW3	MW2	MW3	MW2	MW3
Transport Test	% recovery	104	105	99	99	103	105	99	98
	rate(μmol/L/hr)	≈ 0	≈ 0	≈ 0	≈ 0	≈ 0	≈ 0	-	-
1st Propane Activity Test	% recovery	94	94	-	-	-	-	96	88
	rate (μmol/L/hr)	0.09	≈ 0	-	-	-	-	-	-
2nd Propane Activity Test	% recovery	31	7	-	-	-	-	[2]107	92
	rate (μmol/L/hr)	1.1	0.8	-	-	-	-	-	-
Ethylene Activity Test	% recovery	-	-	[1]59 (3.1%)	[1]75 (3.8%)	-	-	102	90
	rate (μmol/L/hr)	-	-	0.51	0.35	-	-	-	-
3rd Propane Activity Test	% recovery	44	17	-	-	-	-	99	90
	rate (μmol/L/hr)	1.0	1.8	-	-	-	-	-	-
Propylene Activity Test	% recovery	-	-	-	-	[1]75 (2.3%)	[1]69 (0.45 %)	92	88
	rate (μmol/L/hr)	-	-	-	-	0.34	0.46	-	-
4th Propane Activity Test	% recovery	-	40	-	[1]60 (5.2%)	-	-	-	107
	rate (μmol/L/hr)	-	0.82	-	1.2	-	-	-	-
Acetylene Blocking Test	% recovery	-	90	-	[1]86 (0.12 %)	-	-	-	107
	rate (μmol/L/hr)	-	≈ 0	-	≈ 0	-	-	-	-

[1]: Numbers in parenthesis indicate percentage of the oxide mass extracted to the mass of ethylene transformed. [2]: When bromide recovery is greater than 100%, a value of R_{tracer} in an equation 1 s assumed as 1.00.

6.2 Biostimulation Tests

During the Biostimulation Period, five sequential additions of propane and oxygen were performed in each well to stimulate the activity of indigenous propane oxidizing bacteria. Test solutions were prepared and injected as described above and contained known concentrations of bromide, dissolved propane and oxygen, and nitrate (Table 1). Since commercial grade propane can contain ethylene and propylene, high purity propane (99.5%) was used to insure the stimulation of propane-utilizing microorganisms, and not ethylene-utilizing or propylene-utilizing microorganisms. Periodic sampling of the test solution/groundwater mixture was used to quantify rates of propane and oxygen utilization.

Measured solute concentrations in the test solution are summarized in Table 1; computed mass of solutes injected and extracted are in Table 2. The extraction phase consisted of discrete sampling events distributed over 3-25 days following test solution injection. For each sampling event, groundwater samples were collected and analyzed propane, dissolved oxygen, nitrate, and bromide. In the first biostimulation test, the trends in concentration changes of the three compounds were very similar, showing gradual decreases over 25 days (Figure 10). In tests four and five the rates of propane, oxygen (DO), and nitrate utilization increased. The simultaneous decrease in concentrations of the injected electron donor (propane), electron acceptor (oxygen) and nutrient (nitrate) provide evidence that the biostimulation tests were successful in stimulating activity of propane oxidizing bacteria in the subsurface.

The concentration trends in Figure 10 can be more clearly seen if the data are adjusted for the dilution of the test solution with site groundwater. Because the transport test confirmed that propane, oxygen, and nitrate are transported identically to bromide in the absence of microbial

utilization, concentrations of these solutes in each sample were adjusted for dilution by dividing measured concentrations for the relative bromide concentration (i.e. the measured bromide concentration divided by the bromide concentration in the injected test solution) for that sample. In biostimulation test five, the normalized concentrations decreased following injection and the rate of utilization increased in subsequent tests (Figure 11). These results suggest the stimulation of propane-utilizing microorganisms was achieved in the repeated push-pull tests. By the fifth test (Figure 11B), propane was completely consumed, while oxygen was partially consumed. Incomplete utilization of oxygen and difficulty in clearly observing nitrate utilization resulted from the background oxygen and nitrate concentrations of regional groundwater that mixed with the injected solution.

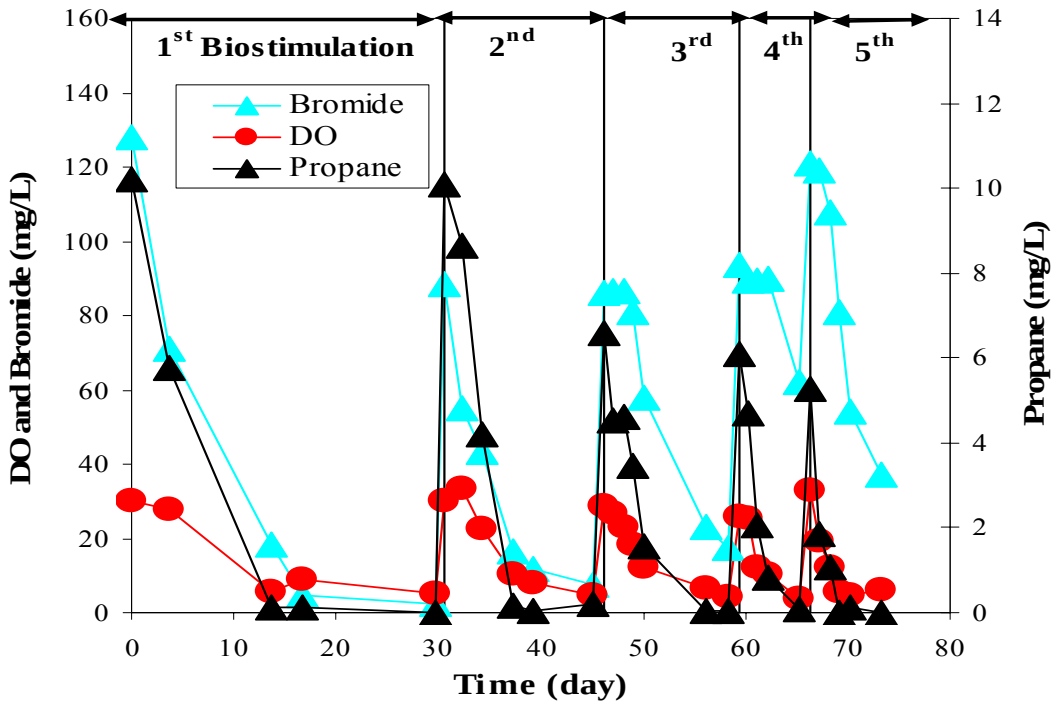

Figure 10. Measured propane, oxygen (DO), nitrate, and bromide concentrations during five field biostimulation tests conducted at the McClellan AFB, CA (MW2) field.

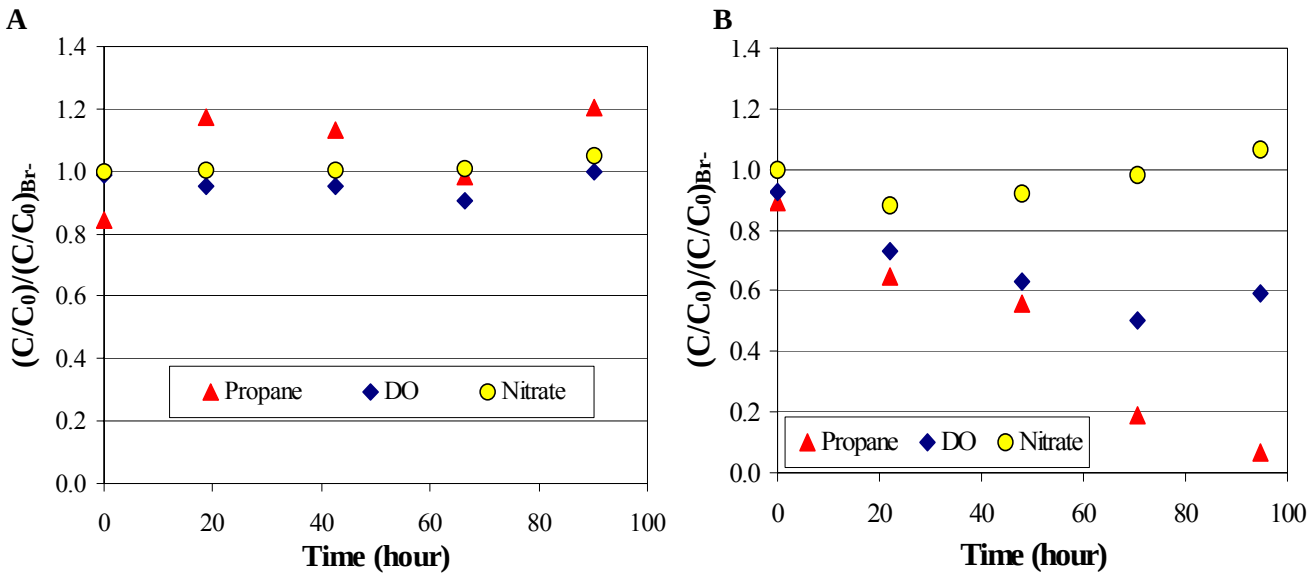

Figure 11. Normalized concentrations of propane, oxygen (DO), and nitrate during the 3rd biostimulation (A) and 5th biostimulation (B) propane biostimulation push-pull tests (MW2) "drift test."

6.3 Activity Tests

Following the Biostimulation Period, a series of five activity tests were conducted to quantify rates of propane utilization, ethylene and propylene transformation, and cis-DCE and TCE transformation (Table 1). Test solutions were prepared and injected as described above. After rest phase of 12 to 16 hr with no pumping, the test solution/ground water mixture was extracted from the well at a rate of 2.5 L/min. Samples collected during the extraction phase were analyzed and used to prepare breakthrough curves for each injected solute and transformation products formed in situ.

Propane Activity Test. After injecting groundwater containing propane, oxygen, nitrate and bromide (Table 1), the solution was permitted to react in the aquifer for 12.1 hrs and then extracted over a period of 6.5 hrs. Propane utilization was not detected during the 1st propane activity test as normalized concentrations of injected propane, oxygen, and bromide were all

similar and dilution adjusted concentration were also in unity (Figure 12A and 12B). However, substantial propane and oxygen utilization were observed during the 2nd propane activity test (Figure 12C and 12D). Similar results were observed in tests at MW3 (data not shown). Estimated zero-order rates of propane utilization were also similar between wells MW2 and MW3 (Table 2).

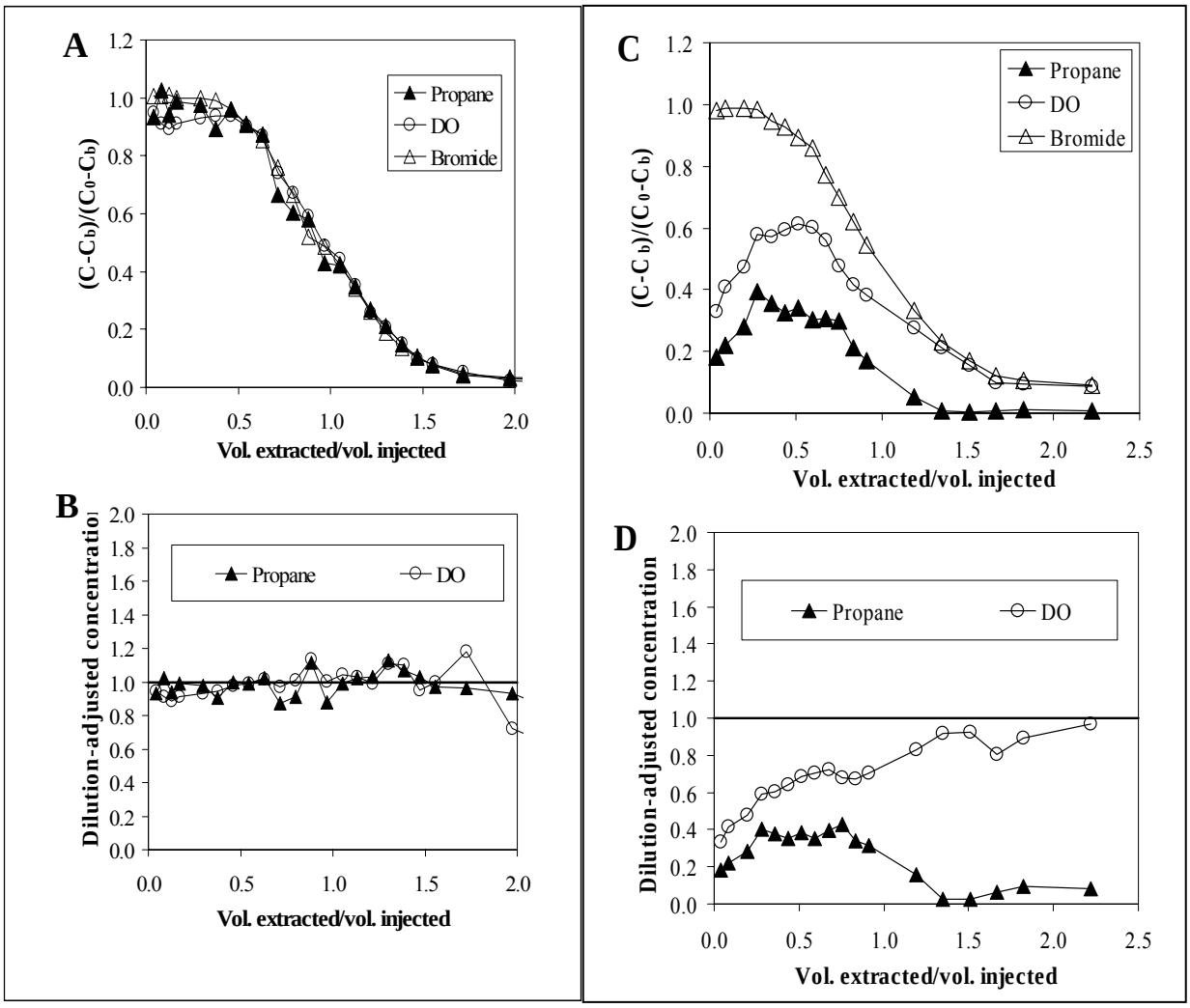

Figure 12. Extraction phase normalized concentrations in well MW2 during (A, B) 1st propane activity test, and (C, D) 2nd propane activity tests.

Ethylene Activity Test. The ethylene activity test was performed to demonstrate cometabolism by propane-utilizers, with ethylene acting as a surrogate compound for the CAHs. After injecting the solution containing ethylene, oxygen, nitrate and chloride (Table 1), the solution was permitted to react in the aquifer for 12.4 hrs and then extracted over a period of 7.3 hrs. Chloride was used as a conservative tracer rather than bromide to identify the test solution from the previously injected solution. As shown in Figure 13A and 13D, ethylene was transformed at a much slower rate than propane was utilized. Very little uptake of nitrate was observed. During the extraction phase, a byproduct having the same retention time on the GC as ethylene oxide was detected (Figure 13B). The build-up of the product was associated with ethylene transformation via cometabolism. In Figure 13C, extraction phase breakthrough curves for cis-DCE, TCE, and bromide are plotted as 1-C*. The transformation of cis-DCE and TCE proved more difficult to assess, since they were present in the injected groundwater at concentrations lower than were present in the aquifer. The results indicate decreases below the bromide curve at later time, which indicates some transformation may be occurring. Their transformation at earlier time might have been inhibited by the presence ethylene. Extensive transformation, however, of cis-DCE and TCE was not observed in the 24-hr activity test. It is possible that the presence of ethylene inhibited cis-DCE and TCE transformation.

Propylene Activity Test. A propylene activity test was performed to demonstrate cometabolism by propane-utilizers, with propylene acting as a surrogate compound for CAHs. The injected test solution containing propylene, oxygen, nitrate and bromide, was permitted to react in the aquifer for 13.2 hrs and then extracted over a period of 6.7 hrs. As shown in Figure 14A and 14D, propylene was transformed at a slower rate than that of propane or ethylene. Very little uptake

of nitrate or oxygen was observed. During the extraction phase, a byproduct having the same retention time on the GC as propylene oxide was detected (Figure 14B). The build-up of the product was associated with propylene cometabolism. In Figure 14C, extraction phase breakthrough curves for cis-DCE, TCE, and bromide are plotted as 1-C*. Neither cis-DCE or TCE transformation was indicated from the activity data at early time, with the trends following those of bromide. Some decreases in the normalized cis-DCE and TCE, below the bromide curve, were observed at later time that might be associated with transformation. The presence of propylene at early time may have inhibited their transformation. The activity test results clearly showed that propane-utilizers stimulated with repeated push-pull tests were able to cometabolize ethylene and propylene resulting in the formation of the by-products ethylene oxide and propylene oxide.

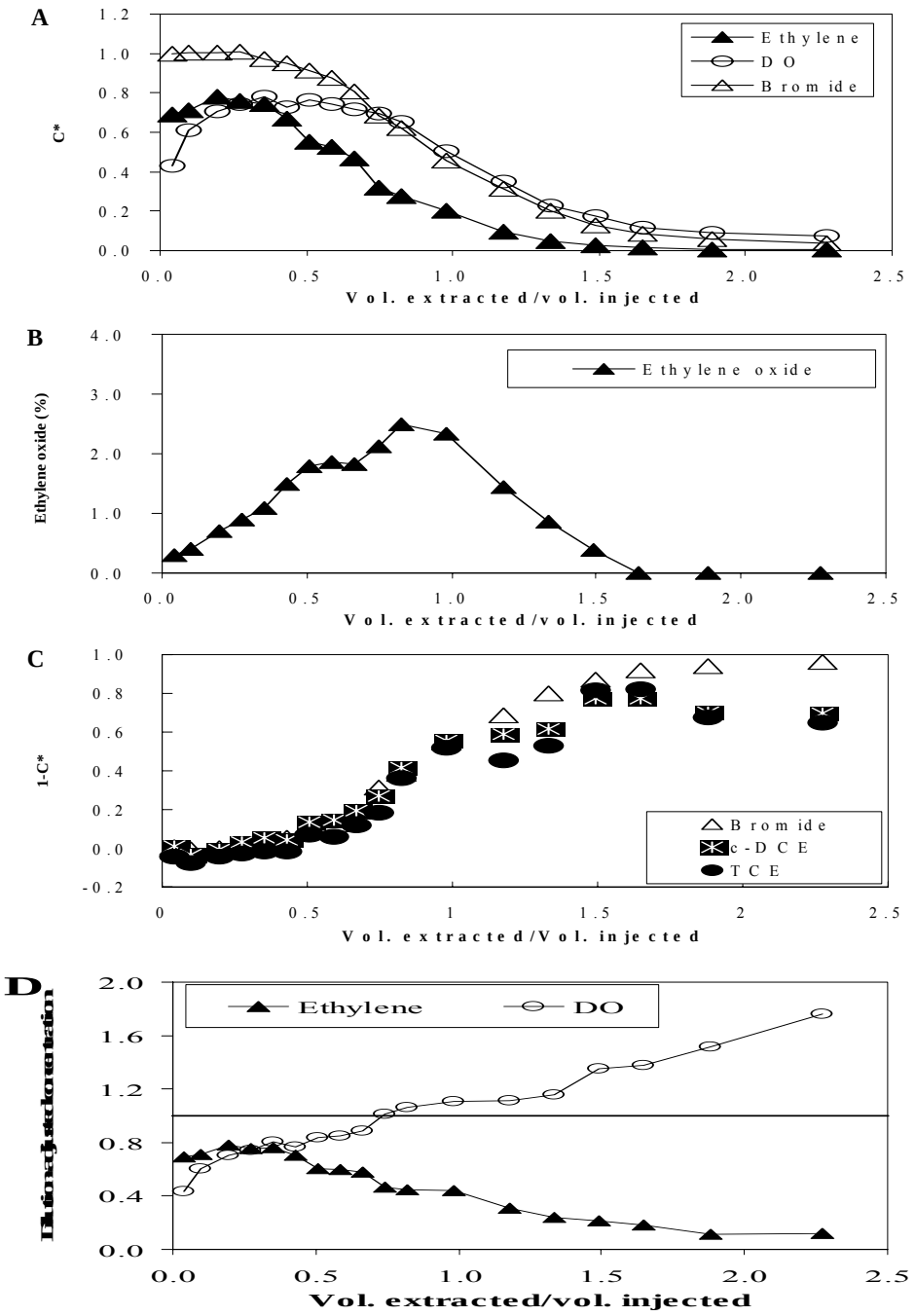

Figure 13. (A) Normalized concentrations for ethylene, oxygen, and bromide in well MW2 during the ethylene activity test, (B) ethylene oxide concentrations in the extracted groundwater as a percentage of average ethylene concentration in injected test solution (C) normalized cis-DCE and TCE concentrations in the extract groundwater, and (D) dilution adjusted concentrations of ethylene and DO .

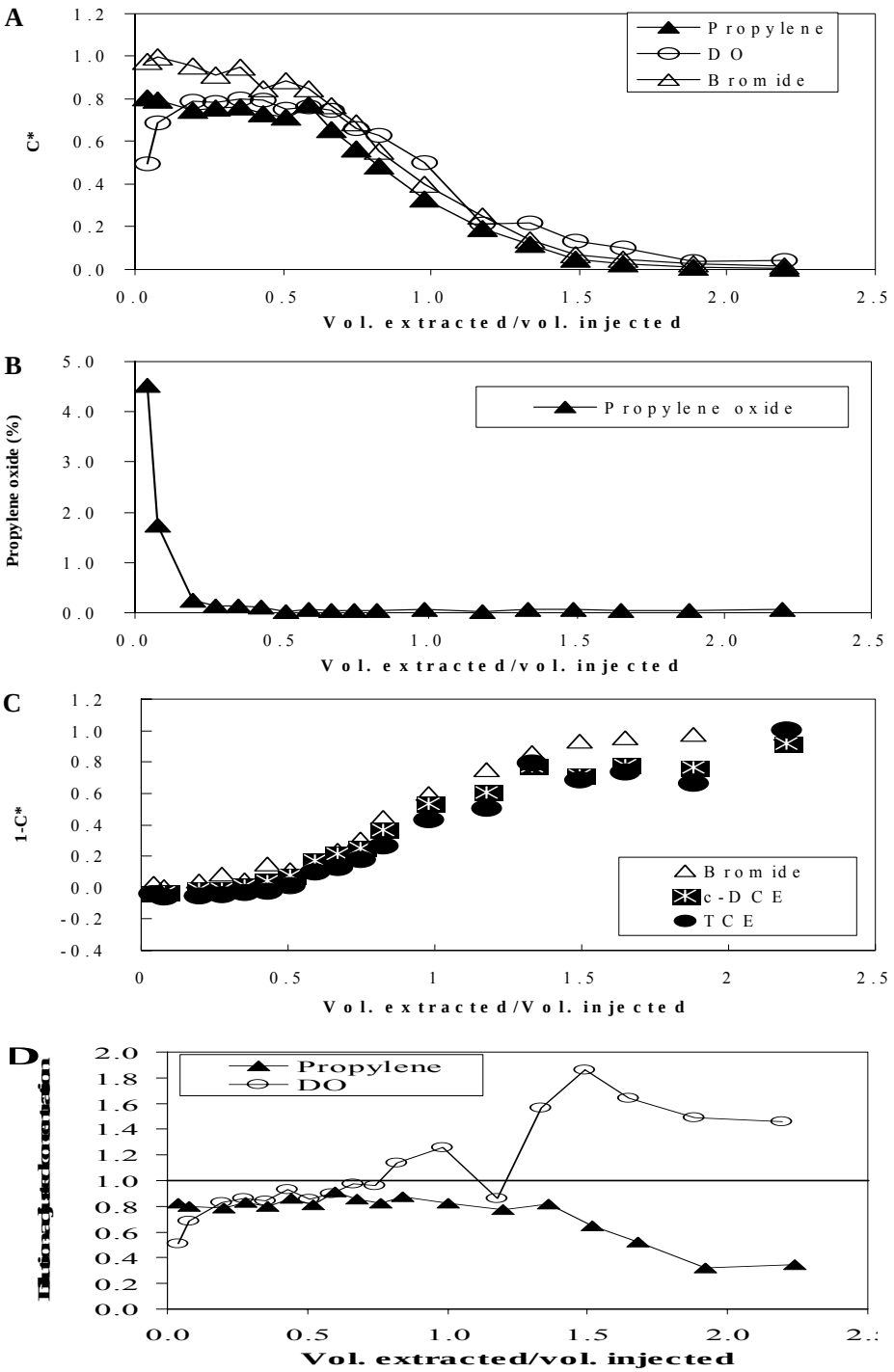

Figure 14. (A) Normalized concentrations for propylene, DO, and bromide in well MW3 during the propylene activity test and (B) propylene oxide concentrations in the extracted groundwater as a percentage of average propylene concentration in injected test solution (C) normalized cis-DCE and TCE concentrations in the extract groundwater, and (D) dilution adjusted concentrations of propylene and DO.

Zero-order rate estimation

In order to calculate the rates of substrate utilization and surrogate compound transformation, a method for estimating zero-order reaction rates developed by Istok et al. (1997) was adapted. For the field test, the total quantities of all injected solutes (TM_i) in μmol, were calculated using equation 1

$$TM_i = C_s * V_{inj} \quad (1)$$

where C_s is measured solute injection concentration (μM) and V_{inj} is volume of the injected solution (L). The total quantity of all recovered solutes (TM_e) in μmol was obtained by integrating breakthrough curves using equation 2

$$TM_e = \sum (V_{ext}^* * C_{ext}^*) \quad (2)$$

where V_{ext}^* is a volume of test solution/groundwater mixture extracted between the measurements and C_{ext}^* is an average concentration between the measurements. Recovery percentages (R) for injected solutes were computed using equation 3

$$R = \frac{TM_e}{TM_i} * 100 \quad (3)$$

The zero-order reaction rate (r_0) in μmol/L/hr for reactants was calculated using equation 4

$$r_0 = \frac{TM_i - \{TM_e / 0.01 R_{tracer}\}}{(V_{inj})(t^*)} \quad (4)$$

where R_{tracer} is recovery percentage for tracer and t* is mean residence time (hr). The 0.01 factor in equation 4 converts from percentage into fractional numbers. The mean residence time (t*) was defined as the elapsed time from the midpoint of the injection phase to the centroid of the bromide breakthrough curve. Table 2 summarizes the masses of injected and extracted solutes,

the percent recovery of the injected solutes upon extraction, and zero-order rate estimates using the method previously described. In the transport test, recovery percentages for dissolved gaseous substrates, oxygen, and nitrate were slightly higher or similar to those achieved with bromide. The result demonstrates that the solutes can be effectively recovered using the push-pull method that was developed. The results also indicate that partitioning due to entrapped gas or sorption was minimal in the aquifer. Thus, in the transport test the zero-order reaction rate was approximately zero. These results indicate that there was no biological and abiotic loss of substrates in the aquifer.

In the propane activity test, the recovery percentage of bromide was slightly higher than that of oxygen and nitrate, and much higher than propane. The propane degradation rate calculated in Table 2 is a conservative estimate, because all the propane that was injected was degraded within 12.1 hrs (Figure 10). Thus, the actual rate is likely larger than that reported. During the extraction phase, oxygen present in the native groundwater was introduced into the extraction groundwater. Despite this introduction, a high zero-order reaction rate of oxygen was estimated. The zero-order rate is higher than propane, which is consistent with the great stoichiometric amounts required for the oxidation of propane.

In the ethylene activity test, the recovery percentage of chloride (conservative tracer) was slightly higher than that for ethylene, and was similar to that for nitrate. The zero-order reaction rate for ethylene transformation was slower than propane degradation rate. The oxygen recovery percentage was much higher than that for chloride, indicating that the oxygen consumption was minimal and oxygen from the ambient groundwater was introduced into the extraction solution.

In the propylene activity test, slightly lower recovery percentage for propylene and nitrate was observed than bromide. A higher oxygen recovery percentage was again observed. The zero-order reaction rate for propylene transformation was smaller than rates of propane degradation and ethylene transformation.

6.4 Acetylene Blocking Tests.

The 4th propane activity test was performed with both propane and ethylene present in the injected groundwater. Simultaneous utilization of propane, ethylene, and oxygen were observed (Figure 15A), and ethylene oxide was again produced with a ratio ethylene oxide formed to ethylene transformed of ~ 5.2 % (Figure 15C and Table 2). The zero-order rate of ethylene oxidation was about a factor of three greater than achieved in the earlier test in MW3, while the propane utilization rate was similar to that achieved in the in the 2nd propane activity test. It may be that the presence and utilization of propane resulted in an increase in the rate of ethylene oxidation. Ethylene concentrations were also a factor of three higher, which likely affected the zero-order rate estimate. The presence of ethylene may have also inhibited the rates of propane utilization, since the zero-order rate of propane utilization is slower than achieved in the 3nd propane activity test. Since the activity tests were performed sequentially, it is difficult to make strong conclusions related to inhibition and the causes of the changes in rates. Transformation of cis-DCE and TCE proved more difficult to assess, since they were present in the injected groundwater at concentrations lower than were present in the aquifer. However, normalization with respect to the background concentrations indicated that cis-DCE was likely transformed (Figure 15B), however TCE was not.

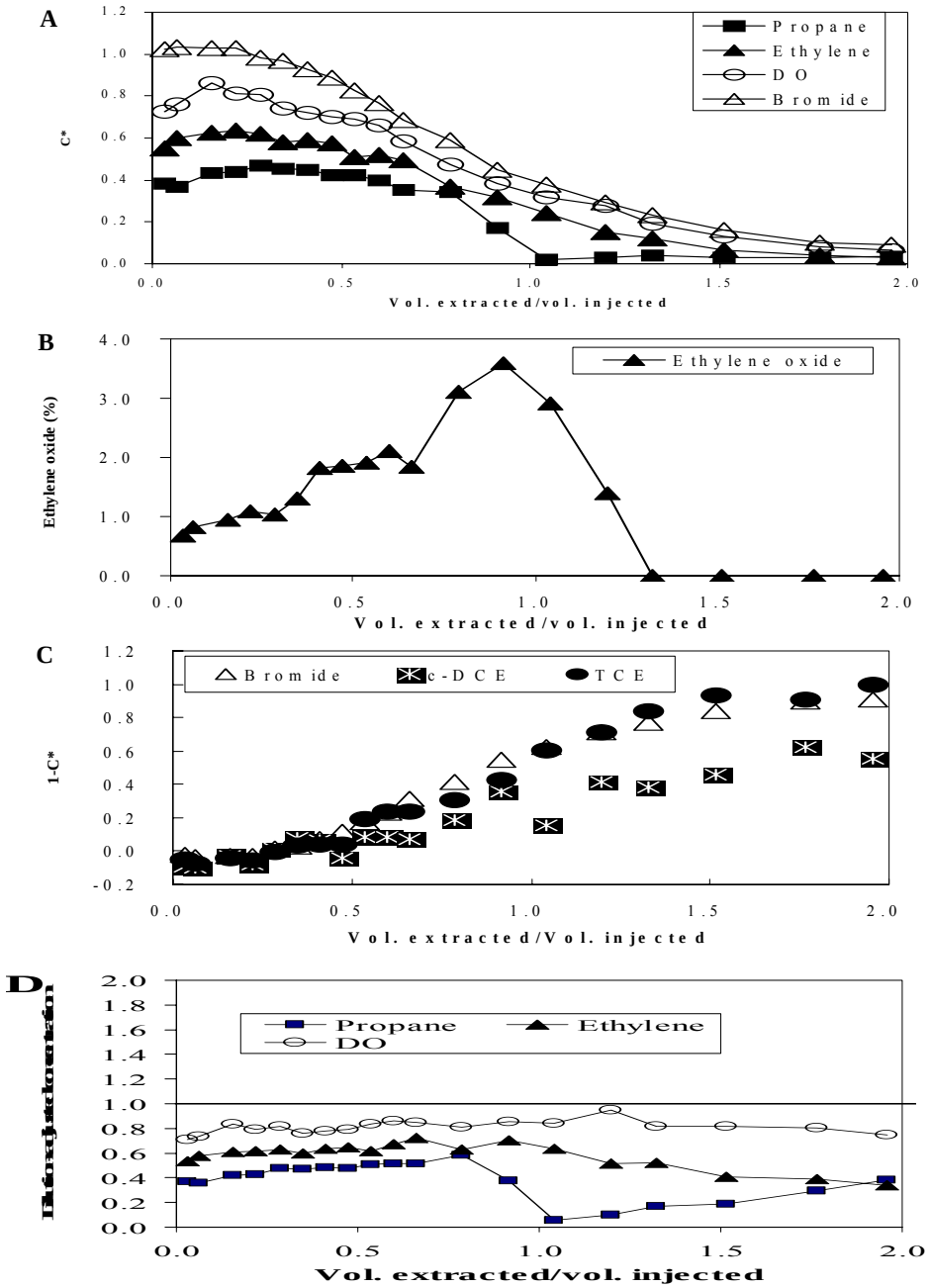

Figure 15. Extraction phase breakthrough curves from well MW3 during the 4th propane activity test (A) injected solutes (B) ethylene oxide concentrations expressed as a percentage of average ethylene concentration in injected test solution, (C) cis-DCE and TCE concentrations in the extract groundwater, and (D) dilution adjusted concentrations of propane, ethylene, and DO.

In a final test the utilization of propane and the transformation of cis- DCE and ethylene were inhibited by acetylene, a known inhibitor of the propane monooxygenase enzyme. An acetylene blocking test was then performed using the same conditions was the 4th propane activity test, but with acetylene added to the injection solution. Acetylene was injected at a concentration of ~ 0.5 mM (10 mg/L). In the presence of acetylene, substrate utilization was essentially completely inhibited (Figure 16A, 16B), and very little ethylene oxide was produced (Figure 16C). The ratio ethylene oxide formed to ethylene transformed was ~ 0.12 % (Table 2). Zero-order rates of propane-utilzation and ethylene oxidation decreased by a factor of 4.7 and 2.4, respectively, in the acetylene blocking test compared to the 4th propane activity test (Table 2). The strong inhibition by acetylene indicates that a propane monooxygenase enzyme is likely responsible for propane degradation and the cometabolism of ethylene. Concentrations of cis-DCE and TCE in the injected and extracted fluids were also measured during the activity tests.

The relationship between ethylene and propylene as surrogates for CAH transformation can also be evaluated. In Figure 17, extraction phase breakthrough curves for propane, ethylene, cis-DCE, TCE, and bromide are plotted as 1-C*, that is, $1-[(C - C_{BG})/(C_o - C_{BG})]$. This method of plotting was used because, unlike the other substrates, cis-DCE and TCE concentrations were lower in the injected test solution than in the background groundwater as a result of the sparging of groundwater with oxygen and the other gas prior to injection. For a non-reactive compound, such as bromide, this method of normalization should result in zero values during the early phase of an extraction and should increase to unity as during the latter phase of extraction. A reactive component with an injection concentration much greater than background (i.e. propane or ethylene) should yield values greater than zero, but it was less than unity during the early phase

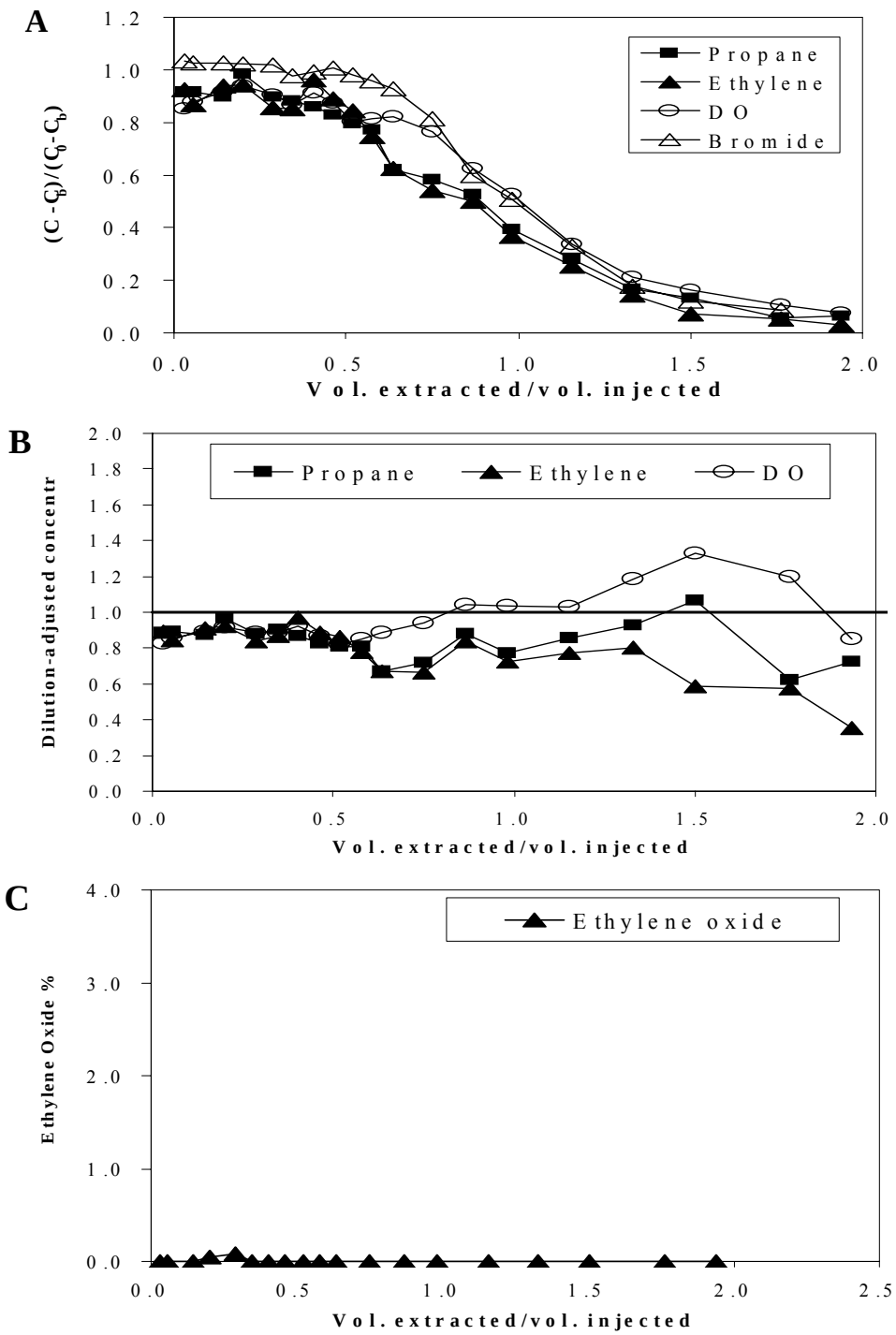

Figure 16. Extraction phase breakthrough curves from well MW3 during the acetylene bbocking test (A) injected solutes (B) dilution adjusted concentrations propane, ethylene, and DO (C) ethylene oxide concentrations expressed as a percentage of average ethylene concentration in injected test solution.

of extraction, and then increased to unit as extraction proceeds. For reactive compounds with high background concentrations in the aquifer (cis-DCE or TCE) compared to the injection concentration, negative values could result during the early phase of extraction, with values potentially remaining below unity as extraction proceeds.

During the 4th propane activity test, the propane and ethylene values were greater than zero during the early phase of extraction, and increased to unity as extraction continued, indicating significant degradation of propane and ethylene occurred during the rest phase. cis-DCE values were lower than those of bromide, indicating that cis-DCE was cometabolically transformed during the test. TCE values were essentially identical to those of bromide, suggesting that no detectable TCE transformation occurred (Figure 17). During the acetylene blocking test, values for all solutes showed similar trends as bromide. Here cis-DCE values approached unity towards the end of the test, indicating cis-DCE transformation was also inhibited by acetylene.

Several factors likely contributed cis-DCE only being marginally transformed and no detectable TCE transformation. The residence time for the activity tests was only 24 hrs and the reactions rates were too slow to see significant changes. The presence of ethylene and propylene may have also inhibited the rates of cis-DCE and TCE transformation. It was not until later time that ethylene and propylene were reduced to low concentrations. At this time, there was significant mixing with the native groundwater that contained cis-DCE and TCE, thus making it difficult to detect TCE and cis-DCE transformation. cis-DCE appeared to be more rapidly transformed than TCE. This result is consistent with the results of the cometabolic air sparging demonstration that was conducted at the same site (Tovanabootr et al. 2001) and the results of

microcosm studies performed with aquifer solids and groundwater from the site (Timmins et al. 2001). One possible improvement in the protocol is to conduct some tests where cis-DCE or TCE is added to the test solutions above background concentrations. Results of such tests are presented in the Ft. Lewis demonstation.

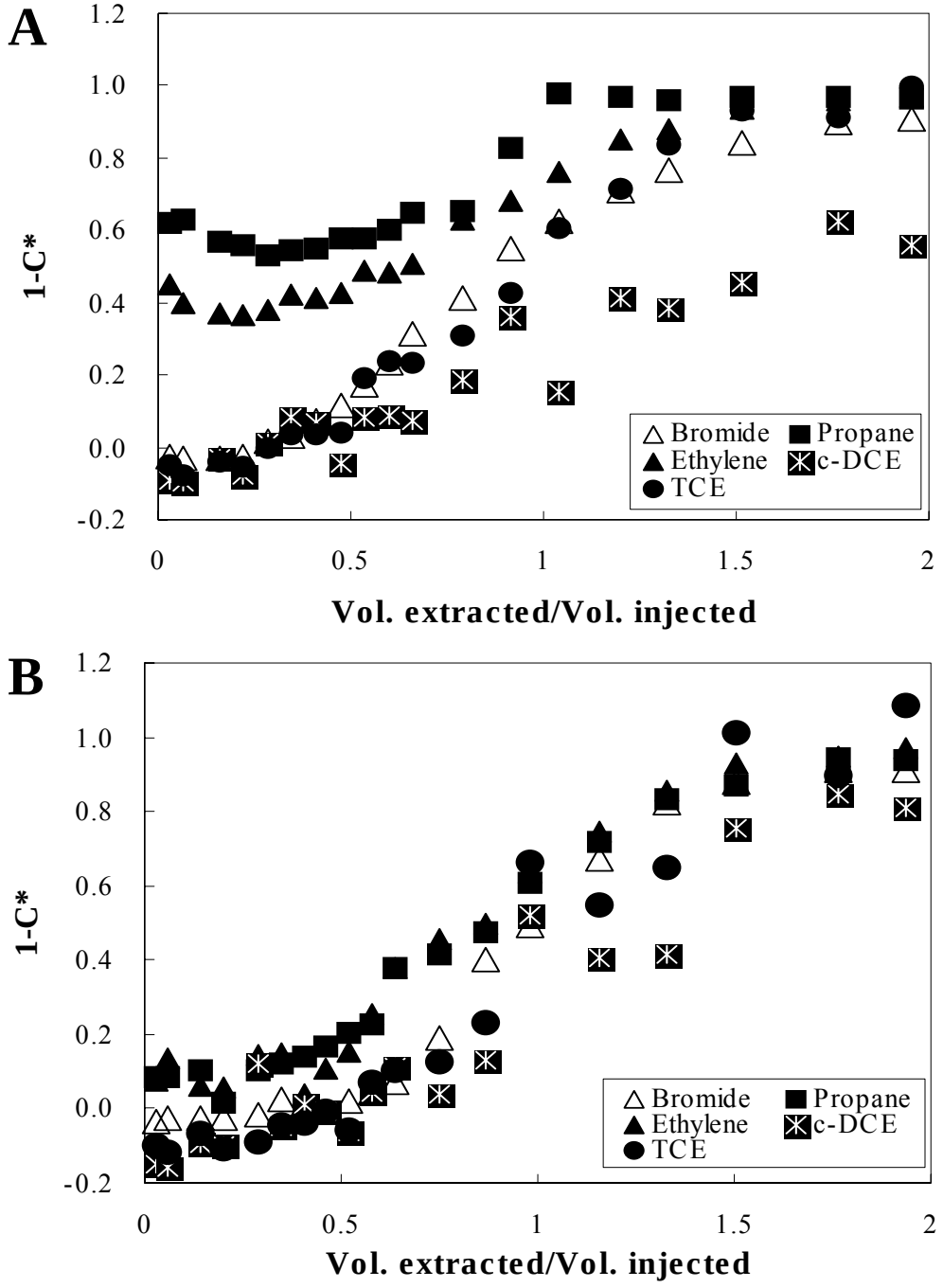

Figure 17. Extraction phase breakthrough curves during from well MW3 (A) the 4th propane activity test, and (B) acetylene blocking test.

7.0 EXAMPLE RESULTS FROM FIELD PUSH-PULL TESTS CONDUCTED AT FORT LEWIS, WA

Our third site demonstration evaluated aerobic cometabolism of chlorinated aliphatic hydrocarbons (CAHs) using toluene as a cometabolic substrate. The demonstration was performed at Fort Lewis Logistics Center, WA. The effectiveness of dissolved substrate addition to stimulate indigenous toluene-utilizers was evaluated in multi-level monitoring wells. Tests were conducted in a shallow alluvial aquifer in the area of Fort Lewis known as the East Gate Disposal yard (EGDY), formerly known as Landfill 2. The EGDY site consists of approximately 29 acres of which 13.5 acres is fenced (U.S. Army, 2002). The EGDY was used as a disposal site for TCE between 1940 and 1970 (U.S. Army, 2002). The depth of groundwater at the site is approximately 10 feet and groundwater velocities across EGDY range from 0.25 to 0.75 feet per day (ESTCP, 2001). LC191 and LC192 were multi-port monitoring wells selected for the push-pull tests. The multi-port monitoring wells were of interest since they allow for the use of smaller injection volumes, which simplified test logistics. Groundwater samples from these wells had TCE and cis-DCE concentrations ranging from 50-500 ug/L, nitrate about 3 mg/L, and dissolved oxygen concentrations were around 5-6 mg/L (Table 3).

The series of transport, biostimulation, and activity tests were performed in Ports 1 and 2 (P1 and P2) (25 ft and 35 ft depths, respectively) of each well. Test solutions were prepared with groundwater extracted from each port and amended with a suite of solutes and injected into the same location. Bromide was used as a non-reactive tracer in all tests. Reactive solutes included the dissolved growth substrate (toluene), hydrogen peroxide, as a source of dissolved oxygen,

non-toxic surrogates (isobutene), and nutrient (NO_3^-). All of these compounds and cis-DCE and TCE present in the groundwater were measured during the injection and extraction phases.

Table 3. Background Groundwater Sample Composition from Injection wells from the Fort Lewis field data.

Well Location	cis-DCE (µg/L)	TCE (µg/L)	Oxygen (mg/L)	NO_3^- (mg/L)	Cl^- (mg/L)	SO_4^{2-} (mg/L)
LC191-P1	281 ± 22	118 ± 9	5.6 ± 0.6	3.1 ± 0.1	6.3 ± 0.3	9.9 ± 0.3
LC191-P2	161 ± 13	112 ± 9	4.9 ± 0.5	1.85 ± 0.09	4.3 ± 0.2	10.3 ± 0.3
LC192-P1	60 ± 5	460 ± 37	6.6 ± 0.7	3.00 ± 0.1	3.9 ± 0.2	9.7 ± 0.3
LC192-P2	47 ± 3	514 ± 41	6.4 ± 0.5	2.51 ± 0.12	3.2 ± 0.16	11 ± 0.3

A series of push-pull tests was performed to evaluate transport characteristics, biostimulation, and transformation activity of the injected solutes. A single transport test was conducted in each well port prior to biostimulation. For these tests the injected solution was allowed to reside in the aquifer for about 20 hrs, and was then extracted at a rate of 1 L/min for 3.3 hrs. Biostimulation activity tests were performed like transport tests by injecting toluene, hydrogen peroxide, and nitrate in order to increase the biomass of toluene-utilizing microorganisms. Biostimulation was monitored by measuring dissolved toluene, nitrate, and oxygen in the selected well port. After biostimulation was achieved, push-pull activity tests were performed evaluating the reactivity of the stimulated microorganisms on surrogate compound (isobutene). Inhibition tests were performed in another phase where 1-butyne (as an inhibitor) was added and utilization and the inhibition of the transformation of toluene, isobutene, and

CAH was monitored. In biostimulation, activity, and inhibition tests, nutrients (modified G4 Minimal Media) (Yeager, 2001), were added to P1 and P2 at LC192 and LC191, respectively.

Two types of tests were performed upon the injection of the test solution. Activity tests were performed as previously described. In the Fort Lewis tests, the rest phase was about 20 hr (no pumping). Approximately 200-L of groundwater was then extracted at a flow rate of 1 L/min. A second type of activity test, called a natural drift test, was performed. These tests are similar to activity tests except groundwater was not extracted after a rest period. Samples instead were collected periodically from the injection location as the test solution drifted downgradient (Figure 2). The natural drift tests were performed at Fort Lewis, since groundwater velocities were faster than McClellan. The test permitted for longer reaction times in the aquifer. Rate estimates between activity test and the natural drift test can be also compared. Samples collected from both types of tests were analyzed for injected tracer and potentially reacting solutes, as well as reaction products formed in situ. Section 5.4 above summarizes the analytical methods used to measure concentrations of tracer, nutrients, substrates, chlorinated solvents and their transformation products in the test samples. A summary of injected solute concentrations for transport, biostimulation, activity, and inhibition tests is listed in Table 4. Detailed descriptions of methodology and results for each test type are described in the following sections.

Table 4. Test Solution Composition for Push-pull Tests Conducted at Fort Lewis.

Test Type	Injection Volume (L)	[1]Toluene (mg/L)	[1]Isobutene (mg/L)	1-[1]Butyne (mg/L)	[2]cis-DCE (µg/L)	trans-DCE (µg/L)	[2]TCE (µg/L)	[1]H_2O_2-O (mg/L)	[3]NO_3^- (mg/L)	Br^- (mg/L)
Transport Test	125	10.4 ±0.4	5.7 ±0.4	[4]NI	40-242	NI	92-379	40 ±4	38.3 ±2	95.9 ±4
[5]Biostimulation (6 natural drift additions)	200	19.9 ±0.4	NI	NI	6-80	NI	123-380	40 ±4	43.8 ±2	110 ±4
Biostimulation Test (push-pull)	105	10.2 ±0.2	NI	NI	10-60	NI	100-300	40 ±4	45.2 ±2	108 ±4
Activity Test (push-pull)	125	2.2 ±0.1	3.1 ±0.1	NI	448	NI	150-300	40 ±4	39.7 ±2	103 ±4
Activity Test (natural drift)	125	3.3 ±0.1	2.96 ±0.1	NI	540	510	180-290	40 ±4	38.0 ±2	109 ±4
Inhibition Test	125	3.0 ±0.1	3.1 ±0.1	20 ±0.4	490	496	180-280	40 ±4	38.0 ±2	103 ±4

[1]: Average concentrations in the injected test solution (C_0) in Ports 1 and 2 in LC191 and LC192. [2]: Range of cis-DCE and TCE concentrations in the injected test solution (C_0). [3]: Nitrate as a nutrient was added to Port 1 in LC191 and in Port 2 in LC192, while nutrients (modified G4 minimal media) were added to Port 2 in LC191 and Port 1 in LC192. [4]: NI indicates not Injected. [5]: In 4th biostimulation test, 500 mg/L cis-DCE was added.

7.1 Transport Test

Transport characteristics of injected solutes, including bromide, toluene, isobutene, DO, and NO_3^- push-pull tests were evaluated in transport tests as previously discussed. Experimental methods were essentially identical to the McClellan tests except smaller injection volumes were used. The injection system is shown in Figure 7. Groundwater (125-L) needed to make the injection solution extracted from the LC191 and LC192 wells ports at a flow rate of ~ 2 L/min using a Masterflex peristaltic pump (Barnant Co., Barrington, IL). The test solution (100-L) was prepared by adding bromide (125 mg/L), nitrate (50 mg/L) and hydrogen peroxide (105 mg/L). Groundwater (20-L) in the 50-L carboys was purged at controlled flow with isobutene gas for 1 hr to achieve aqueous concentrations of approximately 35 mg/L. The isobutene gas in the headspace is recirculated using a Masterflex peristaltic pump for 1 hr to help equilibrate the system. Groundwater (5-L) was added to a collapsible Teflon bag and toluene was added to achieve a concentration of 250 mg/L.

The injection solutions were pumped at different flowrates and mixed together to achieve the desired injection concentration. The rates were as follows: 100-L solution, ~1 L/min; 20-L solution, 0.2 L/min; and 5 L toluene solution, 50 mL/min. A series of metering pumps were used. The aqueous injected concentrations are presented in Table 4. The solution was injected into the aquifer over a 1.67 hr period. After a residence period of 20 hrs, approximately 200 L was extracted (over a period of 3.3 hrs) at a flow rate of 1 L/min. Samples of the injected test solution were taken from the well using a peristaltic pump placed at the same level in the well as injection line end. After extracting 1 L (3 times of injection line volume) of groundwater the samples were taken.

Extraction phase breakthrough curves, as a function of relative concentration (C/C_o), for bromide, toluene, isobutene, and nitrate are plotted in Figure 18A for P1 in well LC192. Extraction breakthrough curves for toluene, isobutene, and nitrate tests were very similar to the bromide tracer, indicating conservative transport of all injected solutes prior to biostimulation. The dilution adjusted concentrations of $[(C/C_o)/(C/C_o)Br^-]$ of toluene, isobutene, and nitrate are also shown in Figure 18B. The dilution adjusted concentrations are all near unity indicating no reaction or retardation. These results at the other three test locations were the same as those observed at LC192 in P1.

A summary of measured concentrations and computed masses achieved in the transport tests for toluene, isobutene, DO, nitrate, and bromide for all four test locations are shown in Table 5. Mass balances indicated 30-50% of injected masses were recovered at LC191 in P1 or P2 well ports, while 56-65% were recovered at LC192 in P1 or P2 (Table 5). The similar percent recoveries indicated similar transport characteristics of the conservative tracer and reactive solutes at both LC191 and LC192 well ports. The lower recovery at the LC191 well ports indicates higher groundwater velocities compared to the LC192 well ports. The shallower LC191 P1 had a lower recovery than the deeper P2 well port, indicts a faster groundwater in the shallower zone. The aquifer at the test site is alluvial, and thus spatial variability to hydraulic conductivity is expected. Groundwater extraction was also occurring in the aquifer, which could have resulted in spatial variability in groundwater velocities. The bromide tests indicated that differences in groundwater velocity existed at the different test locations, and groundwater was flowing faster in the shallower zone (~ 25 ft) compared to the deeper zone (~ 35 ft).

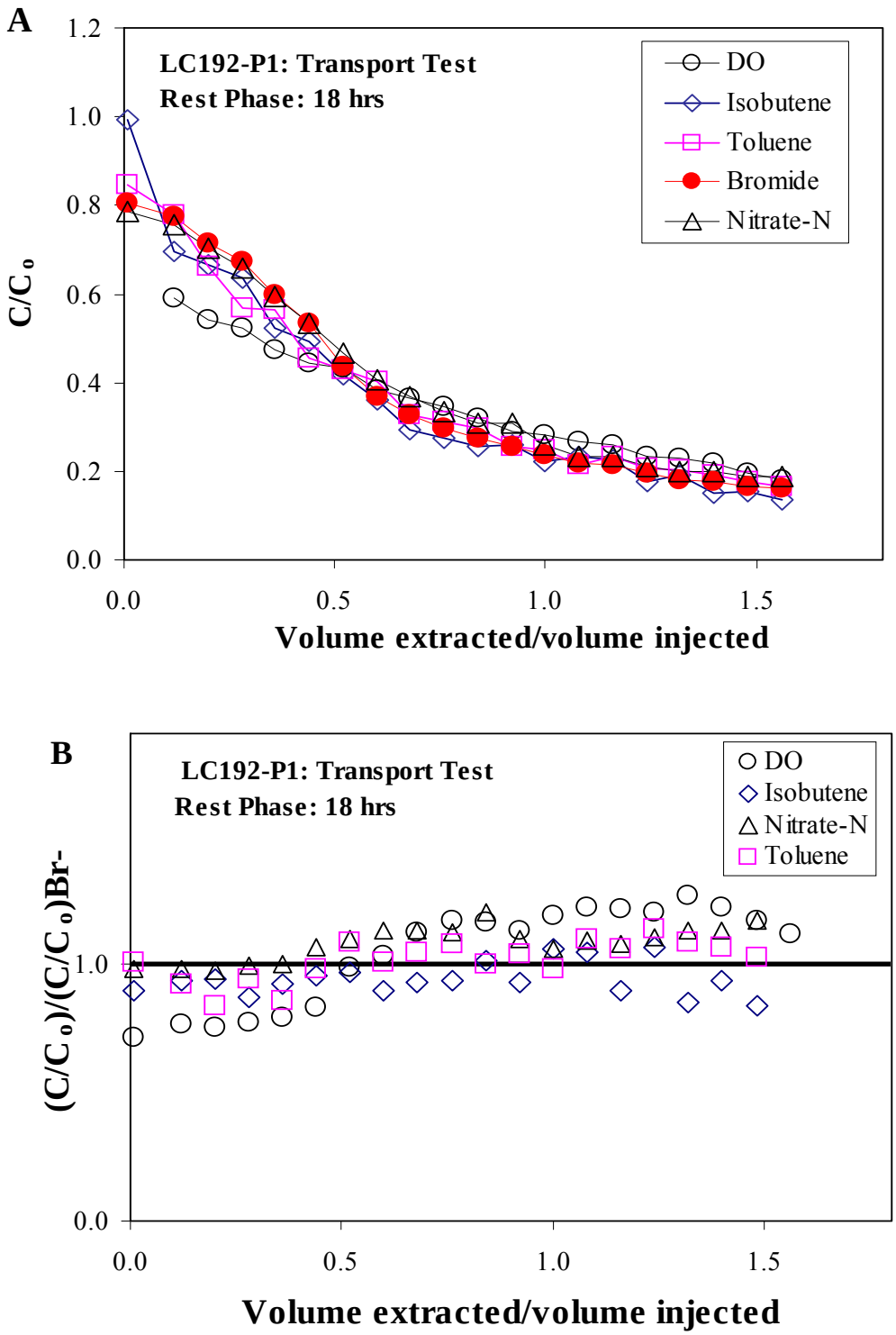

Figure 18. Extraction phase breakthrough curves for push-pull transport tests at Port 1 in LC192 (A). Dilution-adjusted concentrations are presented in (B).

Table 5. Summary of Quantities of Injected and Extracted Solute Mass and Percent Recovery in Transport Tests.

Test Location	Quantities	Toluene	Isobutene	DO	NO_3^--N	Br^-
Transport LC191-P1	Injected mass (mmol)	14.73	11.84	156.2	76.9	148.6
	Extracted mass (mmol)	4.44	4.33	45.8	24.0	48.9
	Mass recovery (%)	30.1	36.5	29.3	31.1	32.9
Transport LC191-P2	Injected mass (mmol)	15.01	12.89	156.2	71.9	153.7
	Extracted mass (mmol)	6.72	6.15	64.7	29.8	70.9
	Mass recovery (%)	44.8	47.7	41.4	41.4	46.1
Transport LC192-P1	Injected mass (mmol)	13.67	13.63	156.2	81.0	148.4
	Extracted mass (mmol)	7.97	7.65	90.8	45.6	88.3
	Mass recovery (%)	58.3	56.17	58.1	56.3	59.5
Transport LC192-P2	Injected mass (mmol)	13.08	12.41	156.2	79.4	148.7
	Extracted mass (mmol)	8.10	7.09	94.0	44.0	101.8
	Mass recovery (%)	61.9	57.1	60.2	55.4	66.1

7.2 Biostimulation by Injecting Dissolved Substrates

Biostimulation tests were performed by injecting a test solution containing dissolved toluene substrate, hydrogen peroxide, bromide, and nutrients in order to increase biomass of toluene-utilizing microorganisms. Injected solute compositions for biostimulation tests are summarized in Table 4. Nutrients, modified G4 Minimal Media, Yeager, (2001), were added to P1 and P2 at LC192 and LC191, respectively, while only nitrate was added to P1 and P2 at LC191 and LC192 wells. The modified minimal media contained (per 105 liters) 3.15 g NH_4NO_3, 2.0 g $MgSO_4$.7H2O, 0.11 g $CaCl_2.2H_2O$, 0.11 g Na_2EDTA, 0.05 g $FeCl_3$, 0.05 M KH_2PO_4/K_2HPO_4, 0.15 g H_3BO_3, 0.11 g $MgSO_4$.7H2O, 0.03 g $ZnSO_4$.7H2O, 0.01 g $CoCl_2.4H_2O$, 0.008 g $CuSO_4.5H_2O$, and 0.005 g $Na_2MoO_4.2H_2O$. For biostimulation tests, groundwater (100-L) containing 105 mg/L bromide, 42 mg/L nitrate, and 89 mg/L of hydrogen peroxide and a 5-L of 210 mg/L toluene in a collapsible Teflon bag was injected to increase biomass. The test solution was injected into the aquifer and then was transported under natural-gradient conditions. Over a period of one month five additions of groundwater amended with toluene (~20 mg/L) were made to increase biomass of toluene-utilizing microorganisms. Samples were taken from the injected test solutions immediately after each addition and again after one week, during that time the solution was subject to the natural-gradient conditions in the aquifer. All samples were analyzed for toluene, DO, nitrate, cis-DCE and TCE. Results from these experiments showed that complete utilization of toluene and significant reduction in cis-DCE concentrations. DO, nitrate, and TCE concentration were near groundwater background concentrations (Table 3). Interestingly, a trace amount of o-cresol was observed in the samples collected after one week of residence in the aquifer.

Biostimulation activity tests were then performed using the same procedures as the earlier transport tests, where the injected solution was allowed to reside in the aquifer for 20 hrs and then extracted over a period of 3.3 hrs. Biostimulation test results showed decreases of injected toluene concentration and the production of o-cresol as an intermediate oxidation product, indicated the stimulation of toluene-utilizing microorganisms contain an ortho-monooxygenase enzyme. o-cresol was identified by retention time comparisons with an authentic o -cresol standard. Under the GC operating conditions as described in section 5.4, the retention time for o-cresol was 25.12 min. Toluene and o-cresol formation concentration in P1 and P2 at LC191 and LC192, respectively are plotted in Figures 19A and 19B. A small fraction of utilized toluene was observed as o-cresol. The o-cresol represent range from 0.1 to 0.3% of the total toluene mass injected (Table 6). The small mass of o-cresol produced could be due to the rate of formation and microbial utilization of the o-cresol. Toluene oxidation to o-cresol by the toluene ortho-monooxygenase pathway was also observed by Hopkins et. al., (1995) and Fries et. al., (1997) at the Moffett field site.

Extraction breakthrough curves of normalized concentrations and dilution-adjusted curves for P1 in LC192 are presented in Figures 20A and 20B, respectively. Figure 20A shows a decrease in concentrations of injected solutes, toluene, nitrate, and DO compared to the bromide tracer after approximately 25 hrs of residence in the aquifer. The decrease in toluene concentrations are most evident, especially when compared with the transport tests conducted prior to biostimulation (Figure 18A). Figure 20B shows decrease to less than unity in toluene, nitrate, and DO concentrations. Toluene utilization is most pronounced, especially when compared to the transport test results shown in Figure 18B. Dilution-adjusted DO concentrations

increased at the end of the test, since DO is present in the regional groundwater that mixed with the injected solution. In Figure 20C, extraction phase breakthrough curves for cis-DCE, TCE, and bromide are plotted as 1-C*. TCE concentrations in injected and extracted solutions remained almost the same since the groundwater was not sparged during this experiment and 1-C* was about one. cis-DCE concentrations in injected solution and background remained low during 24 hrs of test period, and 1-C* were about unity. For bromide this normalization resulted less than one and in the early phase of an extraction and increased to unity during the latter phase of extraction. Reactive compounds such as toluene, DO, and nitrate with an injection concentration much greater than background resulted in values greater than zero, but less than unity, during the early phase of extraction, and then increased to unit as extraction proceeded.

Mass balance calculations indicated a similar bromide mass recovery between transport and biostimulation tests conducted in all test ports (Tables 5 and 6). Nitrate mass recoveries in the biostimulation tests were 21.6 and 27.3 in P1 and P2 in LC191 and 32.9 and 43% in P1 and P2 at LC192, respectively (Table 6), which were less than the nitrate recoveries in the transport tests (Tables 5 and 6). Similarly, toluene mass recoveries in the toluene activity tests range from 57 to 83% of the bromide recoveries compared to 90 to 98% in the toluene transport tests (Table 5). The toluene activity tests provided evidence of the stimulation of toluene-utilizing microorganisms. o-cresol formation was observed indicating that organisms expressing an ortho mono-oxgygenease enzyme were formed, and decrease toluene breakthrough curves, demonstrate toluene utilization occurred. A summary of measured concentrations and computed mass recoveries for toluene, DO, nitrate, and bromide for biostimulation activity test are summarized in Table 6.

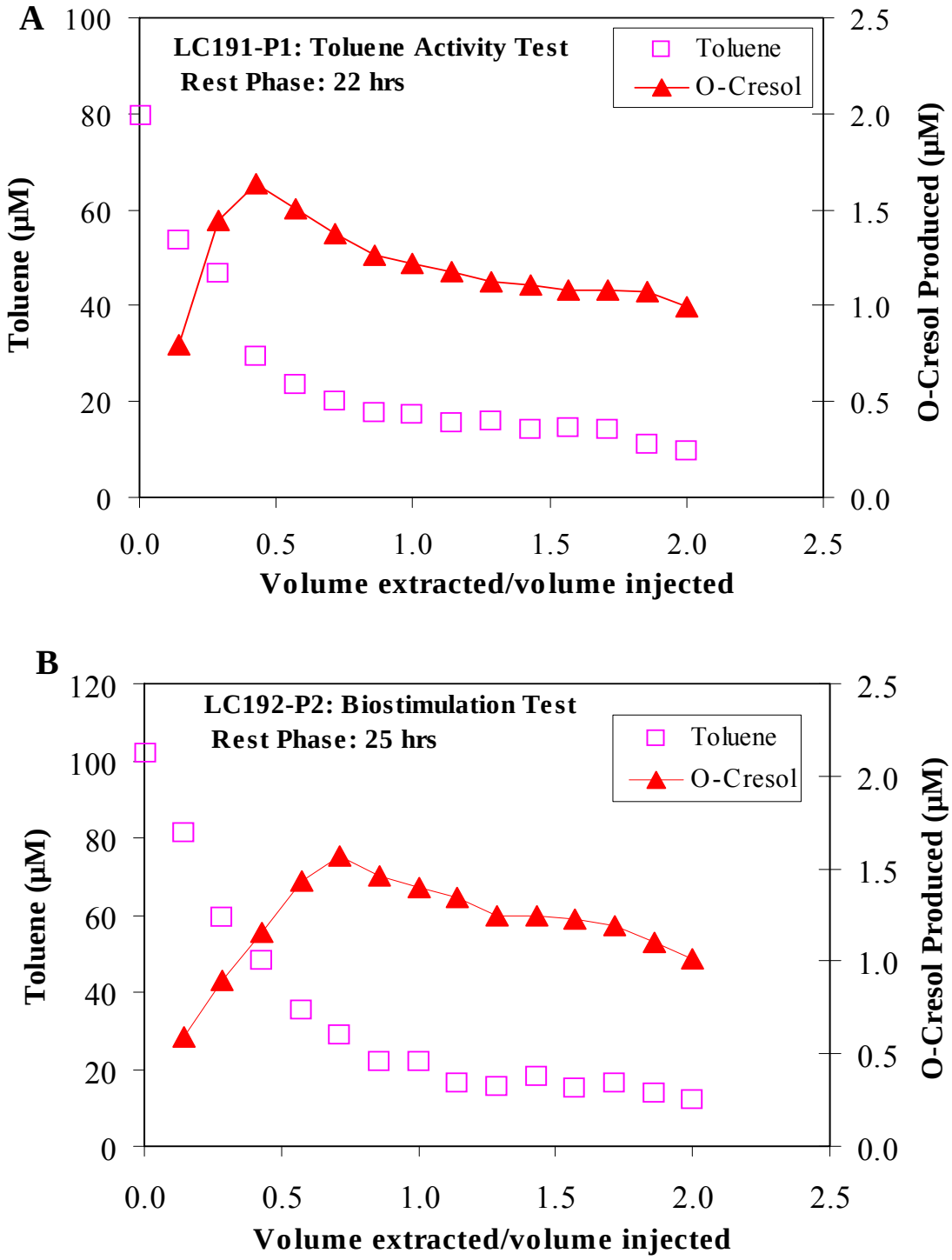

Figure 19. Toluene and o-cresol concentrations in the extracted groundwater during the toluene biostimulation push-pull test.

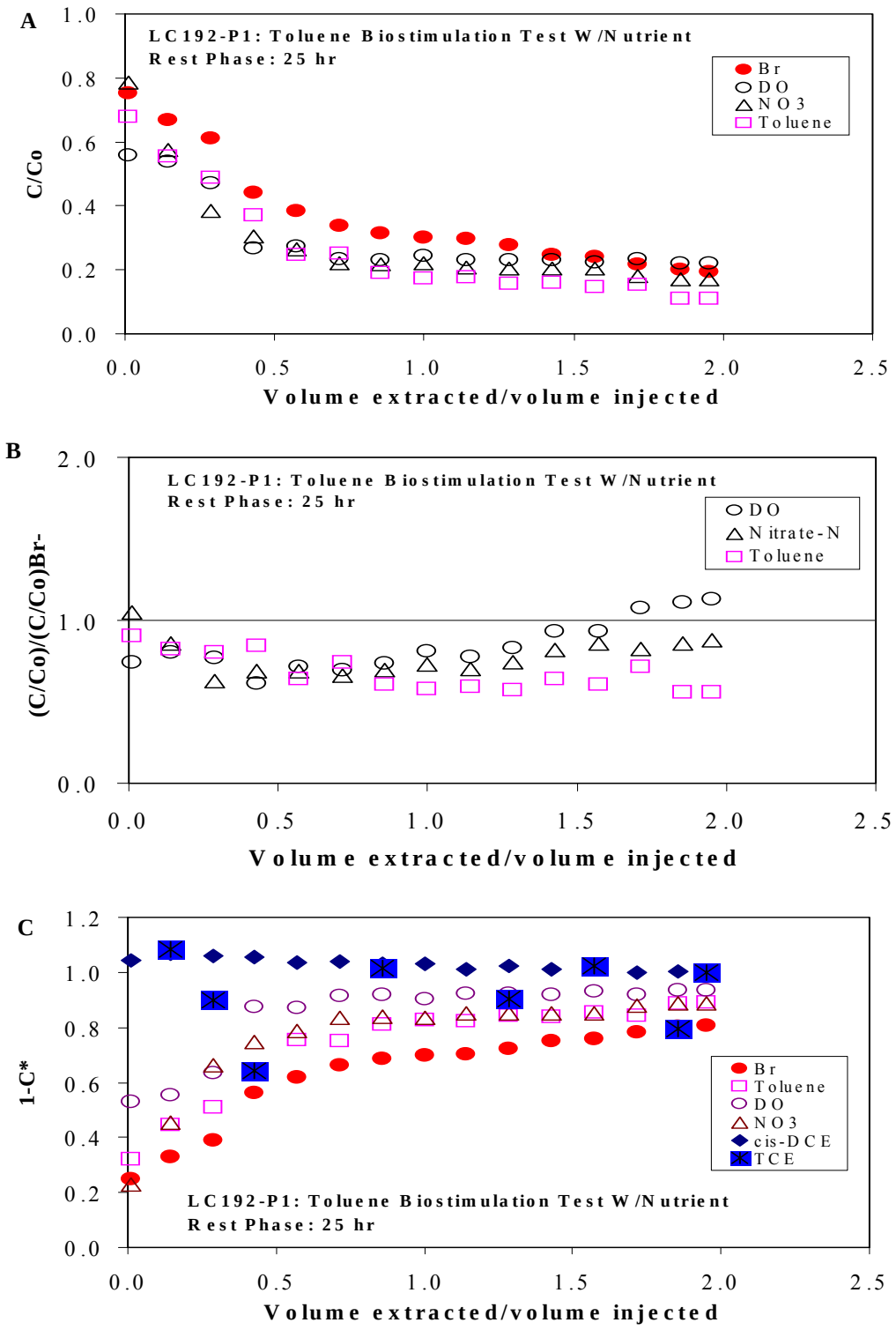

Figure 20. Extraction phase breakthrough curves of toluene for push-pull biostimulation tests at Port 1 in LC192 (A). (B) Dilution-adjusted concentration of toluene, DO, and nitrate and (C) Background adjusted concentration.

Table 6. Summary of Quantities of Injected and Extracted Solutes Mass, Percent Recovery, and Zero-order Rate Estimates in Toluene Activity Tests.

Test Location	Quantities	Toluene	o-Cresol	DO	NO_3^--N	Br^-
Biostimulation LC191-P1	Injected mass (mmol)	13.7	0.0	131.2	75.9	125.2
	Extracted mass (mmol)	3.64	0.016	34.5	16.4	41.5
	Mass recovery (%)	26.6	[2]NA	26.3	21.6	33.1
	Rate (µmol/L/hr)	0.83	0.02	--	--	--
Biostimulation[1] LC191-P2	Injected mass (mmol)	12.0	0.0	131.2	79.2	125.5
	Extracted mass (mmol)	3.9	0.034	46.4	21.6	48.86
	Mass recovery (%)	32.4	NA	35.4	27.3	38.9
	Rate (µmol/L/hr)	0.80	0.04	--	--	--
Biostimulation[1] LC192-P1	Injected mass (mmol)	11.6	0.0	131.2	76.7	122.9
	Extracted mass (mmol)	3.85	0.018	53.4	25.2	65.1
	Mass recovery (%)	33.2	NA	40.7	32.9	53.0
	Rate (µmol/L/hr)	1.53	0.01	--	--	--
Biostimulation LC192-P2	Injected mass (mmol)	11.9	0.0	131.2	76.7	126.8
	Extracted mass (mmol)	4.5	0.017	60.2	32.9	85.2
	Mass recovery (%)	37.8	NA	45.9	42.9	67.2
	Rate (µmol/L/hr)	1.79	0.01	--	--	--

[1]Nutrients (modified G4 Minimal Media) were added to ports 1 and 2 at LC192 and LC191, respectively. [2]NA**:** Not applicable

7.3 Push-Pull Activity Tests with Isobutene as a Surrogate Compound

Activity tests with isobutene added as a surrogate compound were then performed. Activity tests were performed by injecting a test solution containing dissolved toluene substrate, isobutene as the surrogate compound, and the bromide tracer to estimate utilization and transformation rates (Table 4). Isobutene was selected as a surrogate compound since laboratory studies by Hicks (2002) indicated that isobutene epoxide would be formed when an ortho monooxgygenase enzyme is expressed. The injected solution of the activity tests was prepared using the same procedures described in the transport and biostimulation tests. Two types of tests were conducted, 20-hr activity tests and natural-drift activity tests. In the push-pull Activity tests, injected groundwater was permitted to reside in the aquifer for 20 hrs before extraction. 200-L of groundwater was then extracted and samples were taken over time. In the natural-drift test, the identical activity test solution was injected into the aquifer. However the solution was left in place and samples taken under natural gradient conditions every 2 hr for a period of 48 hrs. Activity tests involve injecting test solutions containing toluene, isobutene, cis-DCE, bromide, H_2O_2, and nitrate and measuring the concentrations of the original compounds, metabolic products, and CAHs during the injection and extraction phases. In activity tests additional cis-DCE (500ug/L) was added to increase cis-DCE concentrations and to monitor its potential transformation. Solute compositions for activity tests conducted are summarized in Table 4.

Push-Pull Activity Test Results: A summary of measured concentrations and computed masses for toluene, isobutene, DO, nitrate, and bromide for push-pull activity tests are summarized in Table 7. Results of activity tests conducted after biostimulation showed similar bromide mass

Table 7. Summary of Quantities of Injected and Extracted Solutes Mass, Percent Recovery, and Zero-order Rate Estimates in Isobutene Activity Tests.

Test Location	Quantities	Toluene	Isobutene	Isobutene oxide	cis-DCE	DO	NO_3^--N	Br^-
LC191-P1	Injected mass (mmol)	2.52	6.77	0.0	0.62	156.2	82.8	158.1
	Extracted mass (mmol)	0.065	1.42	0.19	0.12	29.5	20.7	48.2
	Mass recovery (%)	2.58	21.0	NA	18.7	18.7	25.0	30.5
	Rate (µmol/L/hr)	0.81	0.73	0.22	0.08	--	--	--
LC191-P2	Injected mass (mmol)	2.40	6.95	0.0	0.58	156.2	86.9	156.2
	Extracted mass (mmol)	0.054	2.09	0.22	0.11	34.3	25.1	63.8
	Mass recovery (%)	2.25	30.0	NA	18.4	21.9	28.9	40.9
	Rate (µmol/L/hr)	0.79	0.63	0.19	0.11	--	--	--
LC192-P1	Injected mass (mmol)	3.04	6.97	0.0	0.62	156.2	77.1	156.2
	Extracted mass (mmol)	0.057	2.37	0.32	0.15	30.2	31.3	79.0
	Mass recovery (%)	1.87	34.0	NA	23.8	19.3	40.7	50.6
	Rate (µmol/L/hr)	1.02	0.80	0.22	0.12	--	--	--
LC192-P2	Injected mass (mmol)	3.47	6.77	0.0	0.62	156.2	80.2	162.0
	Extracted mass (mmol)	0.18	2.55	0.33	0.19	42.0	34	100.4
	Mass recovery (%)	5.19	37.62	NA	32.1	26.9	42.4	62.0
	Rate (µmol/L/hr)	1.11	0.93	0.19	0.10	--	--	--

NA, not applicable

recoveries between transport and activity tests (Tables 5 and 7). Results from push-pull activity tests showed that concentrations of toluene, isobutene, DO, and nitrate were reduced during the extraction phase. The injected toluene concentrations was deceased from approximately 20 mg/L in the previous toluene activity test to 2 mg/L for these tests. This was done to observe a greater fraction of toluene removal, and to limit inhibition of isobutene transformation. The decrease of normalized concentrations of isobutene, toluene, cis-DCE, DO, and nitrate in P1 in LC192 after 22.5 hrs of residence in the aquifer are shown in Figure 21A. Essentially complete toluene utilization was observed at the lower injection concentration. Normalized cis-DCE concentrations were also greatly reduced and isobutene was also reduced compared to bromide. The dilution-normalized concentrations are also shown in Figure 21B. The results showed concentrations less than unity for all solutes, indicating biological transformations occurred. A reduction of approximately 50% in isobutene concentration was observed during initial 50-60 L of extraction phase, which coincided with maximum reduction in DO concentrations (Figure 21B). Dilution-normalized concentrations of DO decreased to 0.6 immediately during the initial extraction phase, and gradually increased to the background oxygen concentration (Figure 21B). Significant reductions in cis-DCE concentrations were observed during the initial extraction phase, however, the dilution-adjusted cis-DCE concentration increased as extraction proceeded, which is due to the background cis-DCE in the native groundwater (Figure 21B). TCE removal was minimal in the toluene and surrogate compound activity tests, while results indicated transformation of cis-DCE in biostimulation and activity tests as shown in background adjusted plots of 1-C* in Figure 21C. Transformation of cis-DCE and TCE proved more difficult to assess, since they were present in the injected groundwater at concentrations lower than were

present in the aquifer. However, normalization with respect to the background concentrations indicated that cis-DCE was transformed. The results indicate that the toluene-utilizers stimulated would have the ability to cometabolize cis-DCE, however, TCE transformation was not clearly demonstrated. In previous field studies using toluene as a cometabolic substrate, cis-DCE was transformed more rapidly than TCE (Semprini et al. 1994; Hopkins and McCarty, 1995). Results from the push-pull tests are consistent with these past well-to-well field tests. The reaction time of about 24 hrs may not have been long enough for TCE transformation to be observed. It is also possible that the presence of isobutene may have inhibited TCE transformation. These observations indicate that assessing TCE cometabolic transformation potential, when background TCE is already present, may prove difficult using the push-pull method described here.

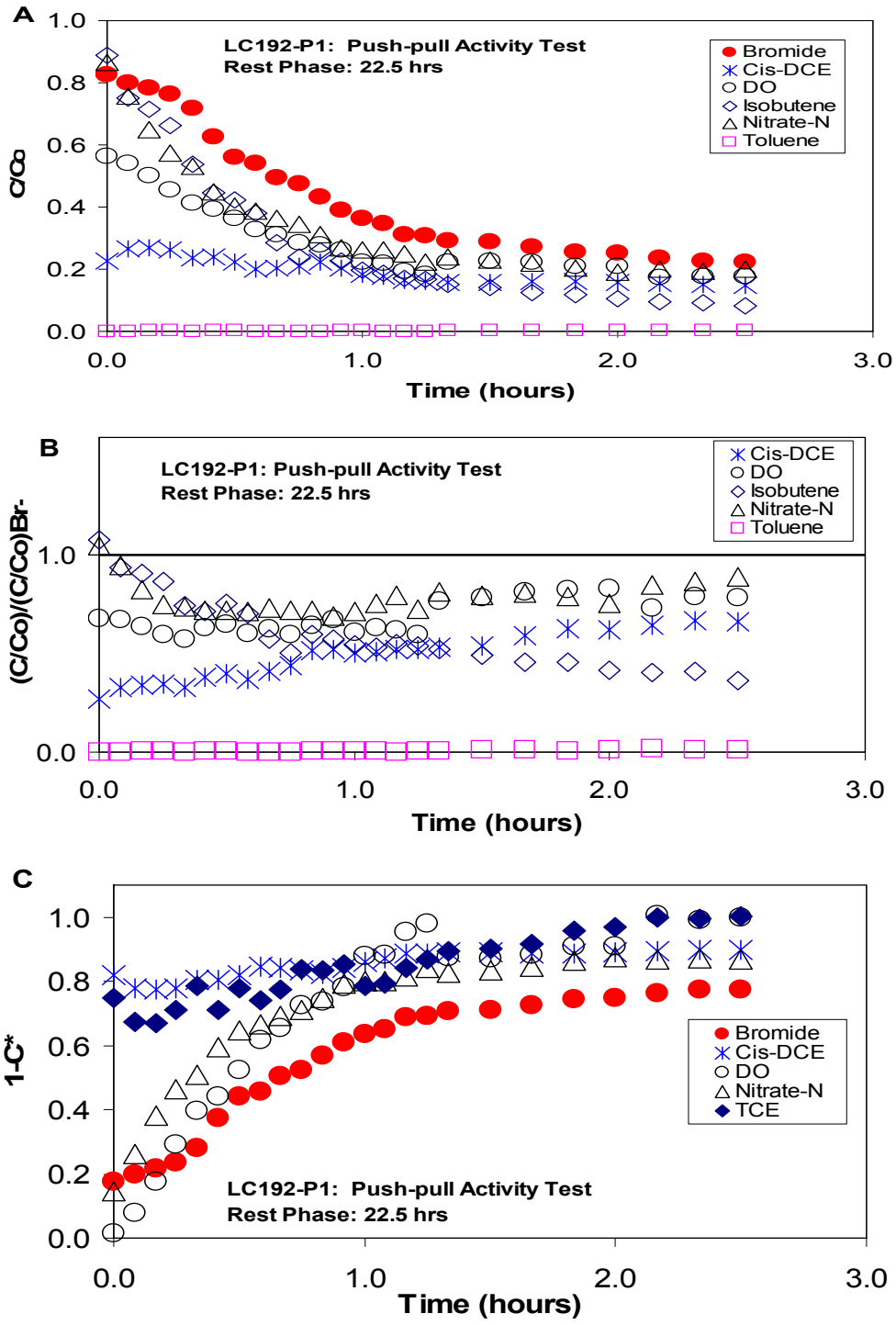

Figure 21. Extraction phase breakthrough curves of isobutene activity tests at Port 1 in LC192 (A). (B) dilution-adjusted concentration and (C) toluene, isobutene, cis-DCE, TCE and DO and (C) background adjusted plots 1-C*.

Toluene mass recoveries in activity tests relative to bromide were 8.5 and 5.5% in P1 and P2 in LC191 and 3.7 and 8.4% in P1 and P2 in LC192 (Table 7). Toluene concentrations were reduced to almost non-detectable after 20 hrs. o-cresol was not detected during the activity tests, likely because the injected toluene concentration was only 2 mg/L. Isobutene mass recoveries ranged from 61 to 73% of the bromide recovery in the activity test (Table 7) compared to 86.3 to 110% in the transport tests (Table 5), indicating transformation occurred. When isobutene was utilized, isobutene oxide was observed as an intermediate oxidation product. Isobutene oxide was identified by retention time comparisons with an authentic isobutene oxide standard. Under the GC operating conditions, as described in Section 5.4, the retention time for isobutene oxide was 12.69 min. Extracted isobutene concentrations and observed isobutene oxide concentrations (uM) in P1 in LC191 and LC192 are plotted Figures 22A and 22B, respectively. The ratios of mass of isobutene oxide produced to the isobutene mass injected were 2.8 and 3.1% in P1 and P2 in LC191 and 4.6 and 4.9% in P1 and P2 in LC192, respectively (Table 7). Reduction in isobutene concentrations and the production of isobutene oxide as an intermediate oxidation product indicated the stimulation of toluene-utilizing microorganisms containing an ortho-monooxygenase enzyme. Similar results for isobutene oxidation by toluene-utilizing microorganisms were observed in laboratory culture studies of Hicks (2002).

Estimated zero-order reaction rates for the injected solutes were calculated using the method of Istok et al., (1997) as discussed in Section 6. The estimated zero-order rates for the injected solutes upon extraction are summarized in Table 7. The estimated zero-order rates for isobutene transformation in the activity tests of P1 and P2 in LC191 were 0.73 and 0.63

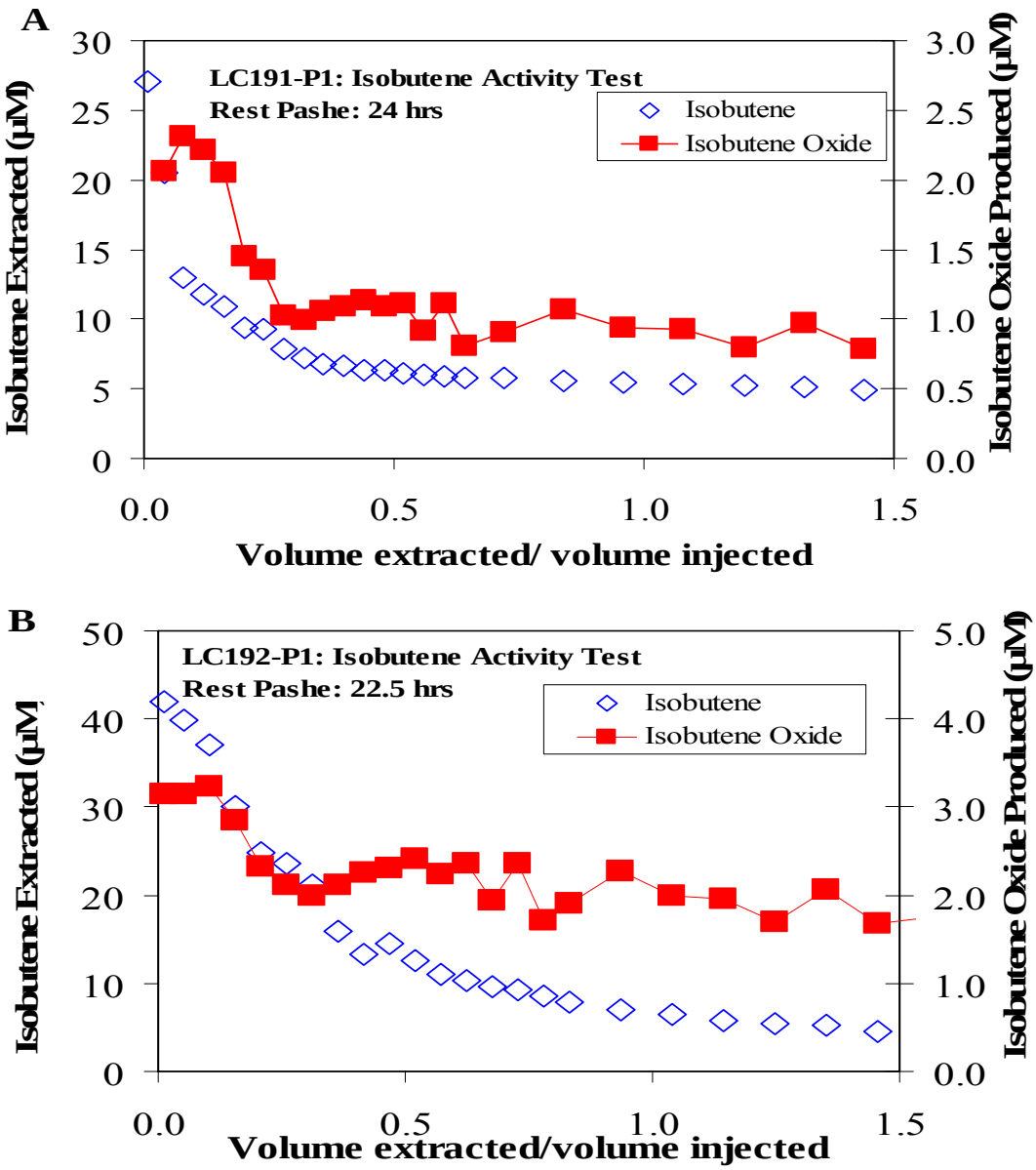

Figure 22. Isobutene transformation and isobutene oxide formation in isobutene activity tests in Port1 in LC191 and LC192.

μmol/L/h and 0.80 and 0.93 μmol/hr/L in P1 and P2 in LC192, respectively (Table 7). Results indicated that about 20% higher transformation activities for isobutene in LC192 compared to LC191.

The estimated zero-order rates for toluene transformation in the isobutene activity tests were 0.81 and 0.79 μmol/L/h in P1 and P2 in LC191 and 1.02 and 1.11 μmol/hr/L in P1 and P2 in LC192 (Table 7). Similar rates for toluene were observed in P1 and P2 in LC191 in the toluene activity tests, but slightly higher rates were observed in P1 and P2 in LC192 (Table 6). These are conservative estimates of toluene utilization rates since essentially all the toluene added was transformed. Thus the higher rates in P1 and P2 in LC192 reflect the greater amount of toluene added. It is also possible that the greater amount of toluene degraded promotes faster rates of isobutene transformation. Higher transformation activities for toluene and isobutene in LC192 compared to LC191 are consistent with results of transport tests, which indicate relatively higher groundwater velocities and lower residence time for microbial Activity in LC191 compared to the LC192 well. The estimated zero-order rates for cis-DCE transformation in the activity tests in P1 and P2 in LC191 were 0.08 and 0.11 μmol/L/hr, which are about the same rates values of 0.12 and 0.1 μmol/L/hr estimated for P1 and P2 in LC192. These are about 10% to 13% of the computed zero-order rates of toluene and isobutene. The results indicated chlorinated ethenes (e.g. cis-DCE) were transformed by toluene-utilizers, but at a slower rate compared to the isobutene surrogate substrate. cis-DCE however was present at a lower concentration than isobutene, which would affect the zero-order rate estimate, and likely the actual rate of transformation.

7.4 Natural Drift Activity Tests with Isobutene as a Surrogate Compound

Natural drift tests were performed similar to activity tests except that no extraction pumping was performed periodically collected samples. Natural drift activity tests involved injecting a test solution containing toluene, isobutene, cis-DCE, trans-DCE, H_2O_2, and nitrate (Table 4). In natural drift tests, trans-DCE, which was not present as a background contaminant, was also added to further confirm the cometabolic transformation. Breakthrough curves for toluene, isobutene, cis-DCE, trans-DCE, and DO during natural drift activity tests were all reduced compared to bromide (Figure 23A). For example, the normalized concentrations of bromide decreased from 1 to 0.2 during the 48 hrs following injection in P1 in LC192, while toluene concentrations were reduced to essentially zero, 8 hrs after the injection (Figure 23A). No o-cresol was detected during natural drift activity tests, likely because the injected toluene concentrations were only 3.3 mg/L. The isobutene concentrations were gradually reduced to zero 48 hrs after injection. When isobutene was utilized, isobutene oxide was observed as an intermediate oxidation product. Isobutene oxide was observed after 10 hrs of residence in the aquifer and then increased and reached to maximum of about 0.4 uM after 24 hrs as shown in Figure 24. Isobutene oxide concentrations gradually reduced to non-detect at the end of 48 hrs of isobutene residence in the aquifer (Figure 24). cis-DCE concentrations were gradually reduced and reached to the background level during the 48 hrs drift tests (Figure 23A). Trans-DCE concentrations also decreased and reached zero after 30 hrs of residence in the aquifer as shown in Figure 23A. The dilution-normalized concentrations of toluene, isobutene, cis-DCE, and trans-DCE and DO were lower than those of bromide, as shown in Figure 23B, indicating that these compounds were cometabolically transformed. An increase of cis-DCE concentrations

after 40 hrs of residence in the aquifer resulted from the presence of the background cis-DCE and DO in the aquifer (Figure 23A). TCE removal was minimal in during natural drift activity as shown in background adjusted plots of 1-C* in Figure 23C. These results indicate that the toluene-utilizers stimulated would have the ability to cometabolize cis-DCE. The results at the other three test locations were essentially the same as those observed at LC192 in P1. It should be noted that increasing the cis-DCE in the injected solution above the background concentration resulted in observable cis-DCE transformation. Addition of TCE was not performed in these tests. Future tests should include the addition of TCE above background concentration to determine if it is transformed.

In natural drift activity tests, mass balances of the injected solutes were calculated by integrating the area under the breakthrough curve (C/C_o), as presented in Table 8. This was done since unlike the push-pull activity tests, the extraction phase of the natural drift tests consists of discrete sampling events instead of the continuous extraction phase pumping and sampling used for push-pull activity tests.

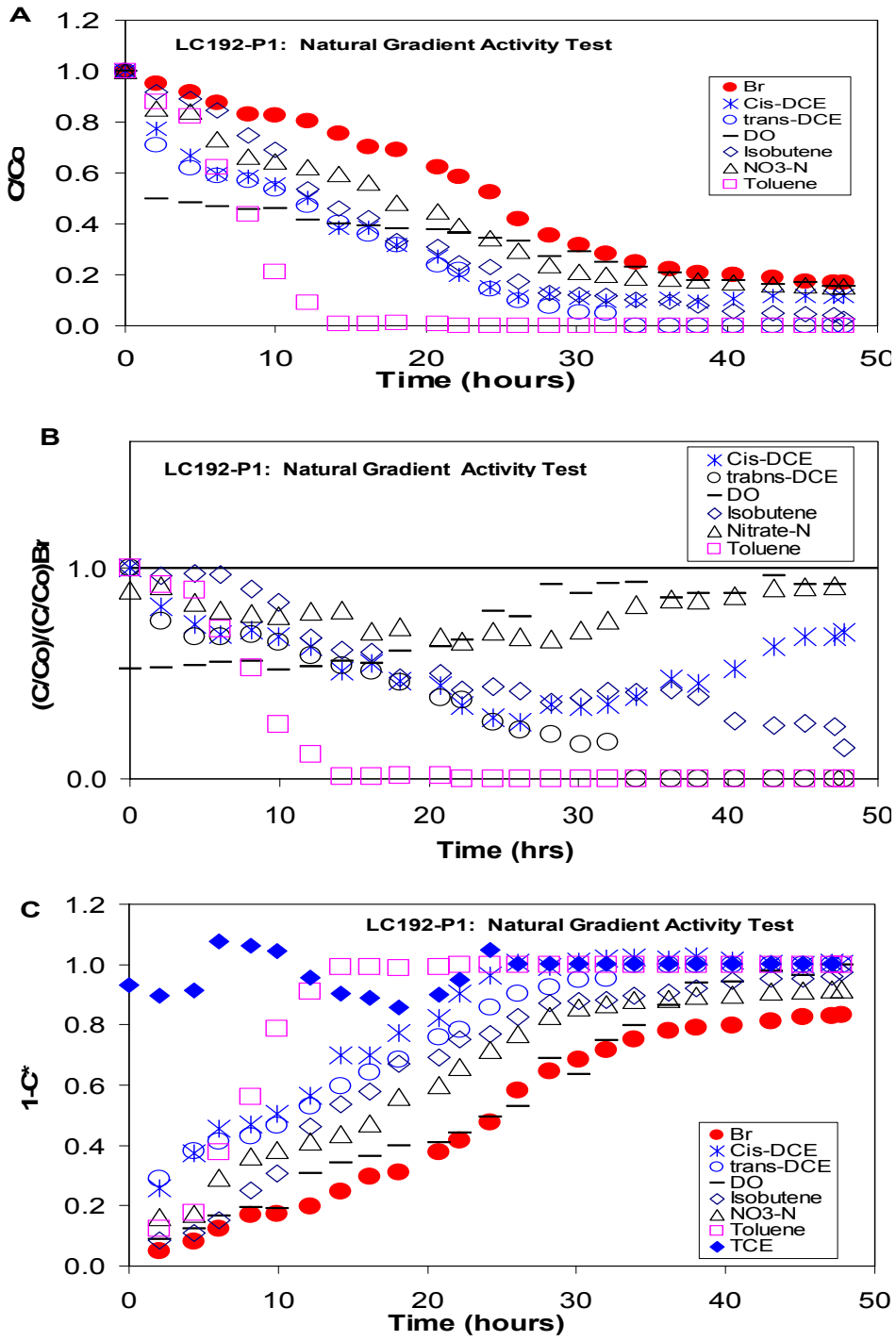

Figure 23. Extraction phase normalized concentrations (A) and dilution-adjusted concentrations of injected solutes (B) in natural drift activity tests and (C) background adjusted plots 1-C*.

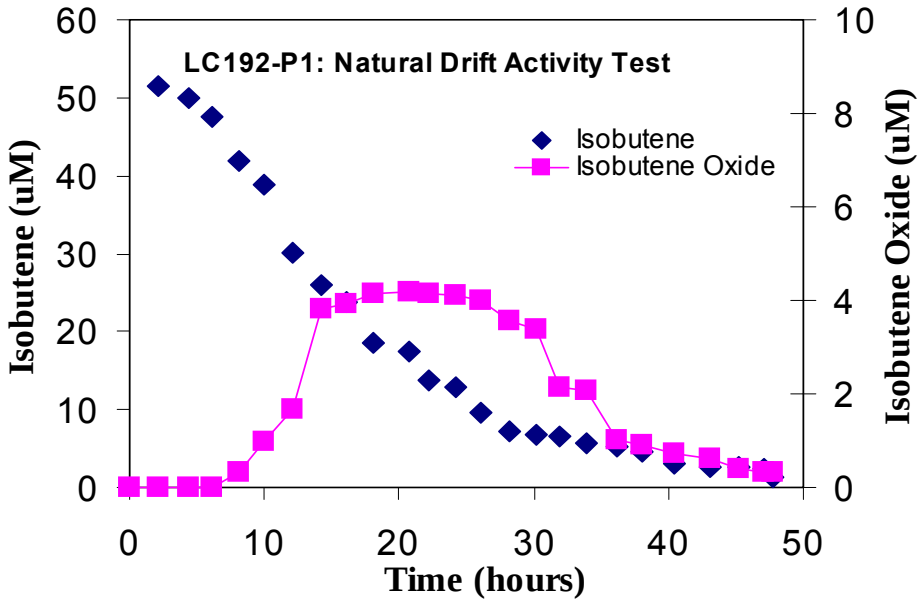

Figure 24. Isobutene transformation and isobutene oxide formation in natural gradient activity tests.

Table 8. Summary of Quantities of Injected and Extracted Solutes Area under Breakthrough Curves in Natural Drift Activity Tests.

Test Location	Quantities	Toluene	Isobutene	Isobutene oxide	cis-DCE	trans-DCE	DO	NO_3^--N	Br^-
Drift Activity LC191-P1	Area under Breakthrough Curve	7.1	18.0	13.3[1]	13.2	9.3	16.2	20.0	24.5
	Rate (µmol/L/hr)	1.27	1.12	0.18	0.12	0.11	--	--	--
Drift Activity LC191-P2	Area under Breakthrough Curve	6.3	15.7	8.2[1]	13.3	11.4	14.1	19.9	23.3
	Rate (µmol/L/hr)	5.16	0.75	0.11	0.15	0.10	--	--	--
Drift Activity LC192-P1	Area under Breakthrough Curve	7.3	16.4	6.75[1]	14.4	12.0	16.1	20.7	25.0
	Rate (µmol/L/hr)	2.14	1.37	0.09	0.09	0.08	--	--	--
Drift Activity LC192-P2	Area under Breakthrough Curve	10.6	17.7	4.98[1]	13.5	11.7	14.9	19.4	24.7
	Rate (µmol/L/hr)	1.07	1.26	0.07	0.10	0.09	--	--	--

[1] Area under isobutene oxide concentration curve (Figure 24)

Similar bromide areas under the breakthrough curve were observed for all four locations (Table 8). These results differ from the push-pull activity tests, which showed lower bromide mass recoveries in P1 and P2 in LC191 compared to P1 and P2 in LC192. This may have been caused by seasonal changes in groundwater velocities, since natural drift tests were conducted in mid-September 2003, compared to push-pull activity tests, which were conducted in early June 2003. The integrated breakthrough areas for isobutene were similar in both ports in LC191 and LC192, showing these recoveries of about 70% of those observed for bromide (Table 8). The integrated areas under isobutene oxide concentration curve (Figure 24) for P1 and P2 in LC191 were greater than those of well LC192. One possible explanation is that abiotic transformation of isobutene oxide may have occurred with longer time for LC192 samples prior to analysis. The integrated breakthrough areas for cis-DCE and trans-DCE are very similar in both ports in LC191 and LC192 (Table 8), indicating that cis-DCE and trans-DCE both were transformed to similar extents.

The estimated zero-order reaction rates for the injected solutes were calculated by multiplying dilution-adjusted concentrations $(C/C_o)/(C/C_o)Br^-$ by the corresponding initial concentration (C_o). Zero-order transformation rates were estimated by the slope of linear regression of decreasing dilution-adjusted concentrations $(C)/(C/C_o)$ Br^- versus time (Figures 25A and 25B). The estimated zero-order rates of the injected solutes in the natural drift tests are summarized in Table 8. Zero-order rates of toluene and isobutene transformation were relatively higher than those observed in the push-pull activity tests (Table 7). Isobutene shows a fairly linear decrease in the normalized concentration. cis-DCE and trans-DCE showed very similar rates of decrease in concentration. cis-DCE rates were determined with data collected during the

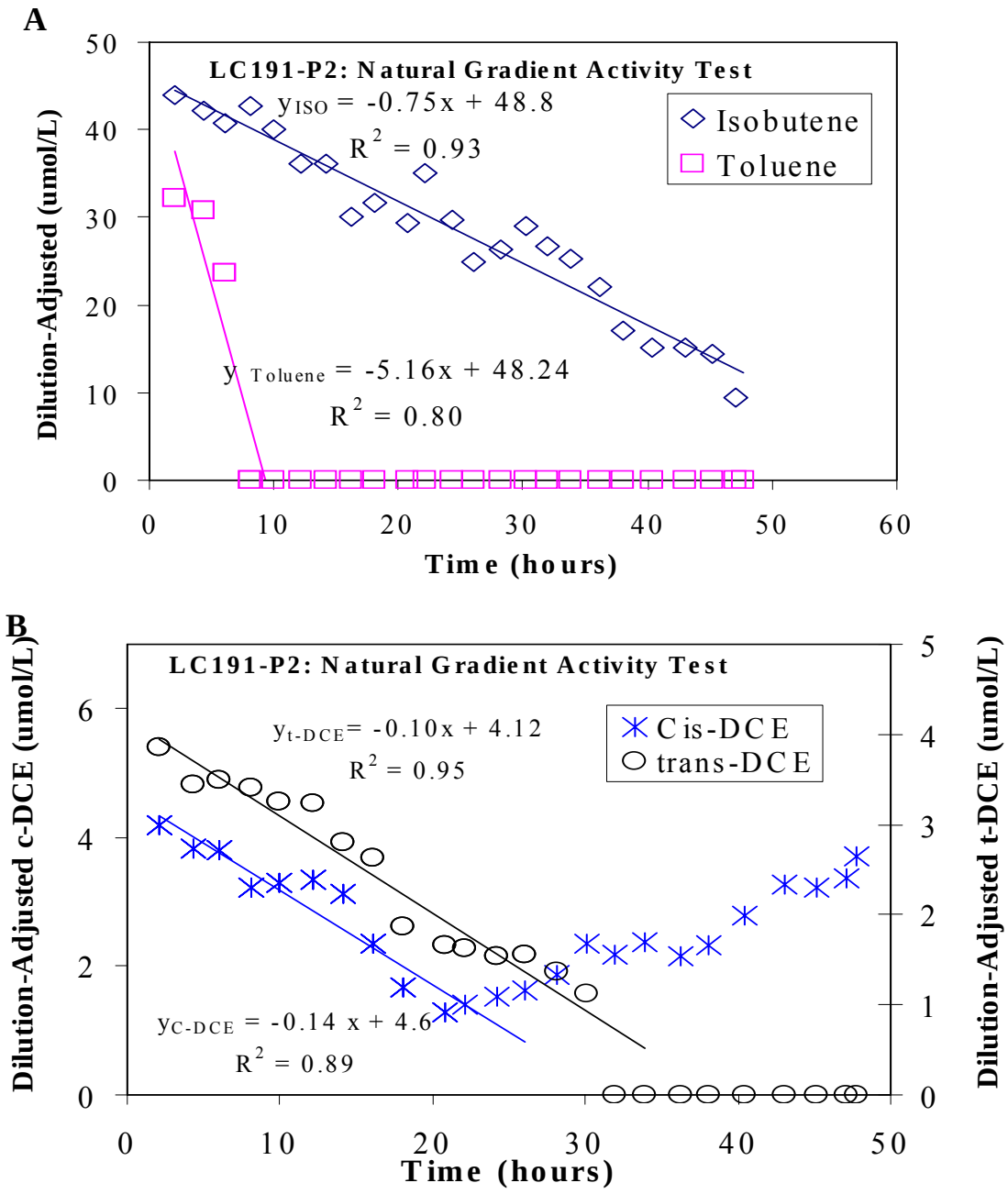

Figure 25. Estimated zero-order rates of injected solutes in natural gradient activity of tests, isobutene and toluene (A), cis-DCE and trans-DCE (B).

first 20 hrs, before the normalized concentrations began to increase as a result of background groundwater. cis-DCE transformation rates were about 0.1μmol/L/h, which were similar to those of the push-pull activity tests (Tables 7 and 8). These results indicate indigenous microorganisms were able cometabolise cis-DCE and trans-DCE after stimulation on toluene.

7.5 Inhibition Tests

Inhibition tests were performed as the final phase of the demonstration. The injected solution included 1-butyne, which acts as a mechanism-based inactivator of the ortho-monooxygenases expressed by toluene-oxidizing bacteria (Yeager, 2002). The inhibition tests were performed under natural gradient flow conditions using the procedures as the activity tests and including the same solutes. The concentration of 1-butyne in the injection solution was 20 mg/L (370 μM). Groundwater (105-L) containing dissolved hydrogen peroxide, toluene, and nitrate was injected into the aquifer to stimulate toluene-utilizers prior to the inhibition tests.

Push-Pull Inhibition Test Results: 1-butyne completely blocked the utilization of the transformation of toluene, isobutene, cis-DCE, and trans-DCE (Figures 26A and 26B). Extraction breakthrough curves for toluene, isobutene, 1-butyne, cis-DCE, trans-DCE, and DO during inhibition test were very similar to the breakthrough curve of the bromide tracer, indicating conservative transport and no transformation of any of the injected solutes (Figure 26A). This is directly in contrast with the results of natural drift activity tests shown in Figure 23A, where transformation was observed. Figure 26B also shows no decrease in the dilution-adjusted concentrations, with all the concentrations centered around unity. These results at the other three test locations were the same as those observed at LC191 in P2. No o-cresol or isobutene oxide was detected during the inhibition tests, and cis-DCE and trans-DCE

transformation was also blocked by 1-butyne, indicating an ortho-monooxygenase enzyme was likely involved in their transformation.

In the natural drift inhibition tests, the integrated areas under the breakthrough curve were determined. The inhibition tests results showed similar areas for each injected solute at all four locations (Table 9). Similar areas under the breakthrough curve of bromide between the natural drift activity and inhibition tests were observed in both ports in LC191 and LC192 (Tables 8, 9). The results clearly show that at all locations microbial utilization of toluene, DO, and nitrate was essentially completely inhibited by 1-butyne, as well as the transformation of toluene and isobutene, cis-DCE, and trans-DCE. The results when compared with those obtained in the natural drift activity tests (Table 8), further demonstrate that the microbial utilization and transformation observed in the activity tests.

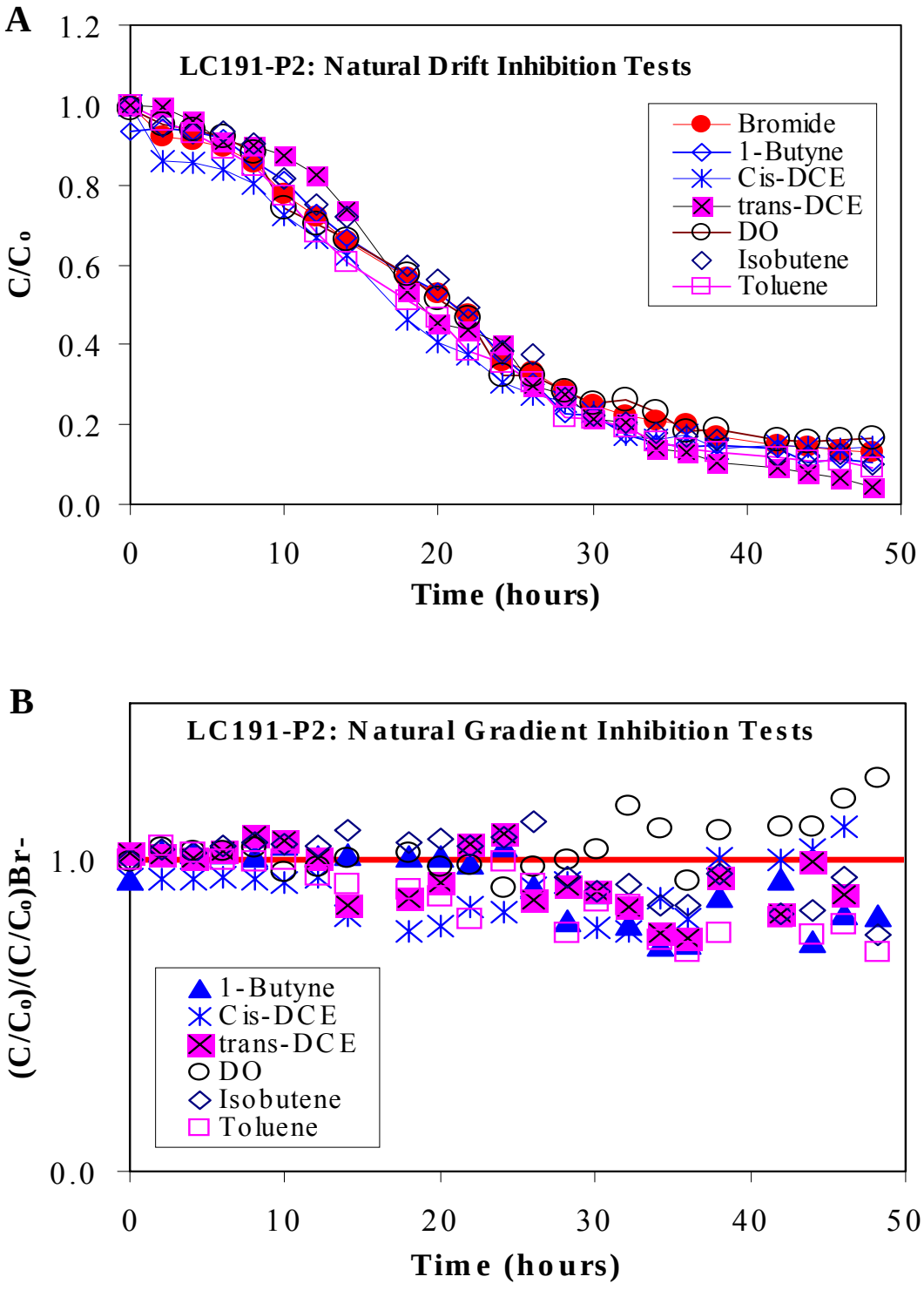

Figure 26. Extraction phase normalized concentrations (A) and dilution-adjusted concentrations of injected solutes in natural gradient inhibition tests.

Table 9. Summary of Quantities of Injected and Extracted Solutes Areas under Breakthrough Curves in Inhibition Tests.

Test Type	Quantities	Toluene	Isobutene	1-Butyne	cis-DCE	trans-DCE	DO	NO_3^--N	Br^-
Inhibition LC191-P1	Area under Breakthrough Curve	26.4	22.3	21.6	22.0	21.9	22.9	22.7	21.4
Inhibition LC191-P2	Area under Breakthrough Curve	20.7	22.7	21.7	21.7	21.7	22.6	23.2	22.2
Inhibition LC192-P1	Area under Breakthrough Curve	22.8	24.2	24.2	21.2	21.4	21.3	24.3	23.8
Inhibition LC192-P2	Area under Breakthrough Curve	21.3	24.5	23.9	23.9	23.3	22.7	24.6	24.4

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