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DEVELOPMENT AND EVALUATION OF AN ENRICHMENT CULTURE FOR BIOAUGMENTATION OF THE P-AREA CHLORINATED ETHENE PLUME AT THE SAVANNAH RIVER SITE

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DEVELOPMENT AND EVALUATION OF AN ENRICHMENT CULTURE FOR BIOAUGMENTATION OF THE P-AREA CHLORINATED ETHENE PLUME AT THE SAVANNAH RIVER SITE

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ABSTRACT

The Twin Lakes chlorinated ethene plume at the Department of Energy's Savannah River Site (SRS) emerges in a wetland in which trichloroethene (TCE) is completely reduced to ethene and ethane. Novel strains of *Dehalococcoides* have been detected from the wetland area. The objectives of this study were 1) to develop an enrichment culture capable of completely dechlorinating TCE and tetrachloroethene (PCE) to ethene using samples from the wetland; 2) to evaluate the use of lactate, emulsified vegetable oil and corn syrup as possible electron donors for biostimulation of the P-area chlorinated ethene plume at SRS that is not undergoing natural attenuation; and 3) to evaluate the use of the enrichment culture for bioaugmentation of the P-area chlorinated ethene plume.

The enrichment culture was started with samples from microcosms that were used to confirm reductive dechlorination activity in the SRS Twin Lakes area. Samples were transferred to an anaerobic mineral medium, repeatedly fed with TCE and PCE, and transferred a second time to mineral medium. TCE and PCE concentrations of 35-40 mg/L and 4-8 mg/L, respectively, were completely consumed by the enrichment culture within three to five weeks. Quantitative polymerase chain reaction (PCR) analysis indicated a linear increase in *Dehalococcoides* as increasing amounts of PCE and TCE are reduced to ethene. The *Dehalococcoides* cell density in the enrichment culture has stabilized at approximately 4.5 x 10^8 cells per mL. Lactate was used as the electron donor and carbon source. An electron donor balance indicated that most of the lactate is

fermented to acetate and propionate, with less than 3% used for reductive dechlorination. Methanogenesis in the enrichment culture is insignificant.

A laboratory study was conducted to compare biostimulation and bioaugmentation for removal of PCE and TCE from the SRS P-area plume. Microcosms were prepared with sediment and groundwater from the site, at PCE and TCE concentrations close to the reported maxima of 5 mg/L and 35 mg/L, respectively. The pH of the groundwater was adjusted from 5.7 to 7 with NaOH and resazurin was added as a redox indicator. Lactate, corn syrup and emulsified vegetable oil were used for biostimulation. Bioaugmentation was assessed using varying doses of the SRS enrichment culture (1.0, 0.1, 0.01, and 0.001% v/v), along with one treatment using a commercial bioaugmentation culture (1.0% v/v). After more than eight months of incubation, there was no evidence of PCE or TCE dechlorination in any of the biostimulated treatments, even though the amount of donor added was in considerable excess and redox and pH conditions were favorable. The microcosms were bioaugmented after three months of incubation (to establish favorable redox conditions) and the response was immediate. In the treatment that received the 1.0% dose of SRS culture, reduction of the PCE and TCE to ethene was completed in approximately three weeks. The lower doses of SRS culture were also effective, although up to seven months of incubation was needed before dechlorination was complete. In contrast, dechlorination of PCE and TCE was much slower and incomplete in the treatment that received the commercial bioaugmentation culture, over the same incubation period. The microcosm results indicate that the SRS enrichment culture holds promise for use in

bioaugmentation of the P-area groundwater plume. Efforts are underway to scale up the enrichment culture in preparation for a pilot-scale field test.

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LIST OF ABBREVIATIONS

ACs	Autoclave controls
cDCE	*cis*-dichloroethene
COD	Chemical oxygen demand
DDI	Distilled, deionized
GC	Gas Chromatography
HPLC	High performance liquid chromatography
PBRP	P-Area Burning/Rubble Pit
PCE	Tetrachloroethene
PCR	Polymerase Chain Reaction
SRS	Savannah River Site
TCE	Trichloroethene
TOC	Total organic carbon
VC	Vinyl chloride
WCs	Water controls

CHAPTER 1

INTRODUCTION

Chlorinated ethenes are among the most common groundwater contaminants found at industrial sites throughout the United States. The Department of Energy's SRS located in Aiken, South Carolina, is no exception. The P-Area plume is one of many chlorinated ethene plumes found at SRS. Little is known about the P-Area plume, and work is in progress to characterize it. Field data has shown this site to be contaminated with PCE, TCE, and *cis*-dichloroethene (cDCE). Figures 1.1 through 1.3 show maps of the PCE, TCE and cDCE plume found at the P-Area, respectively. P-Area groundwater discharges to Steel Creek where TCE levels above the current Federal drinking water standard maximum contaminant level set by the United States Environmental Protection Agency have been detected (22). There are two separate source zones for the P-Area plume; the P-Area Burning/Rubble Pit (PBRP) and the P-Area Reactor.

The PBRP was built in 1951 to serve as a burning pit for organic chemicals, waste oils, wood, paper, plastics, and rubber. In 1973, burning was terminated and the pit was covered with soil. From 1973 until it reached its capacity in 1978, the pit was used to dispose of rubble such as brick, tile, concrete, asphalt, rubber, non-returnable empty drums, and waste solvents including chlorinated ethenes (24). After reaching capacity, the pit and debris were covered with soil and no other actions were taken to remediate this site. As a result of these past disposal practices, chlorinated ethenes contaminated both the soil and groundwater beneath the PBRP (24).

Figure 1.1 PCE concentration in the P-Area chlorinated ethene plume.

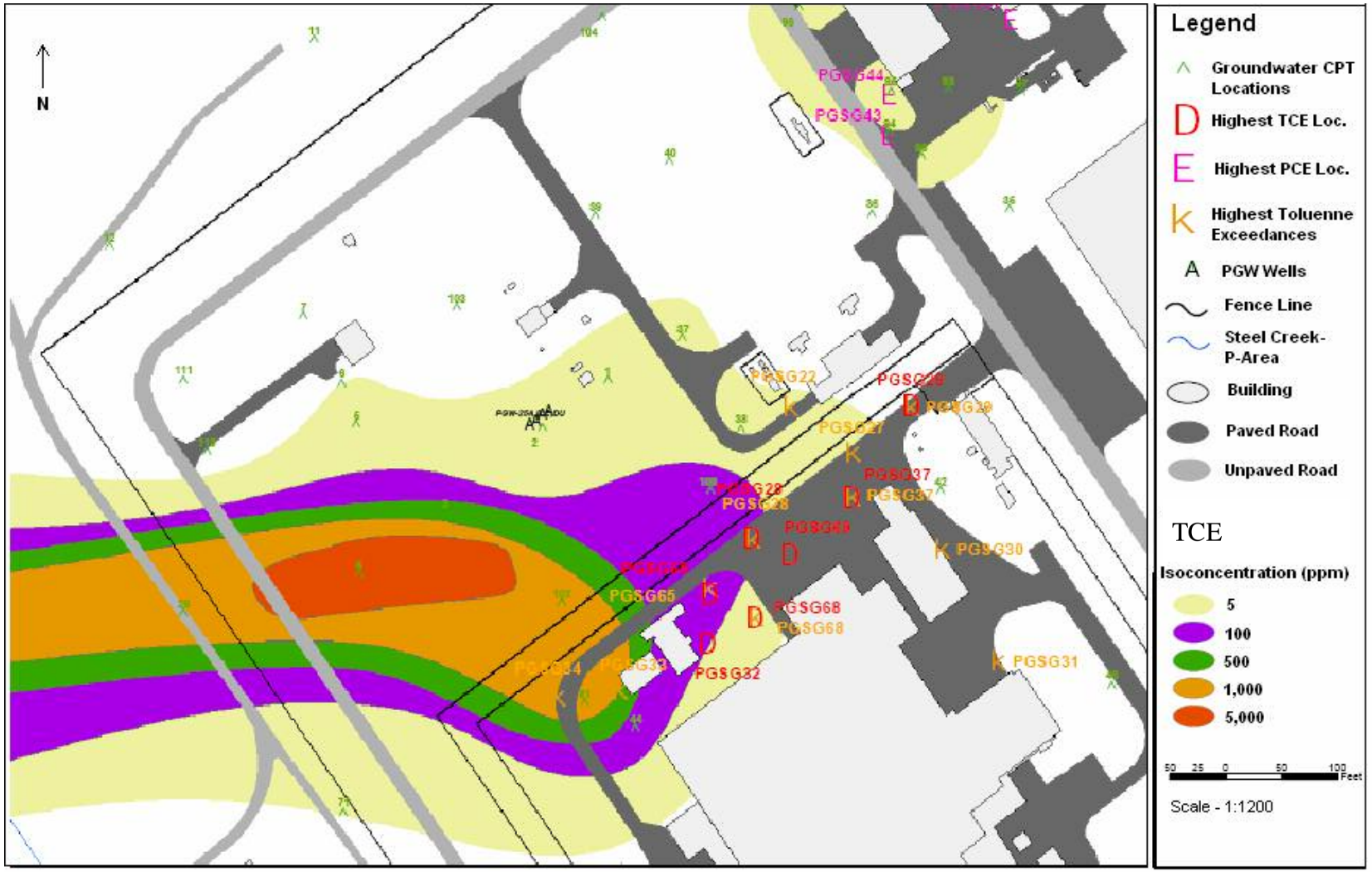

Figure 1.2 TCE concentration in the P-Area chlorinated ethene plume.

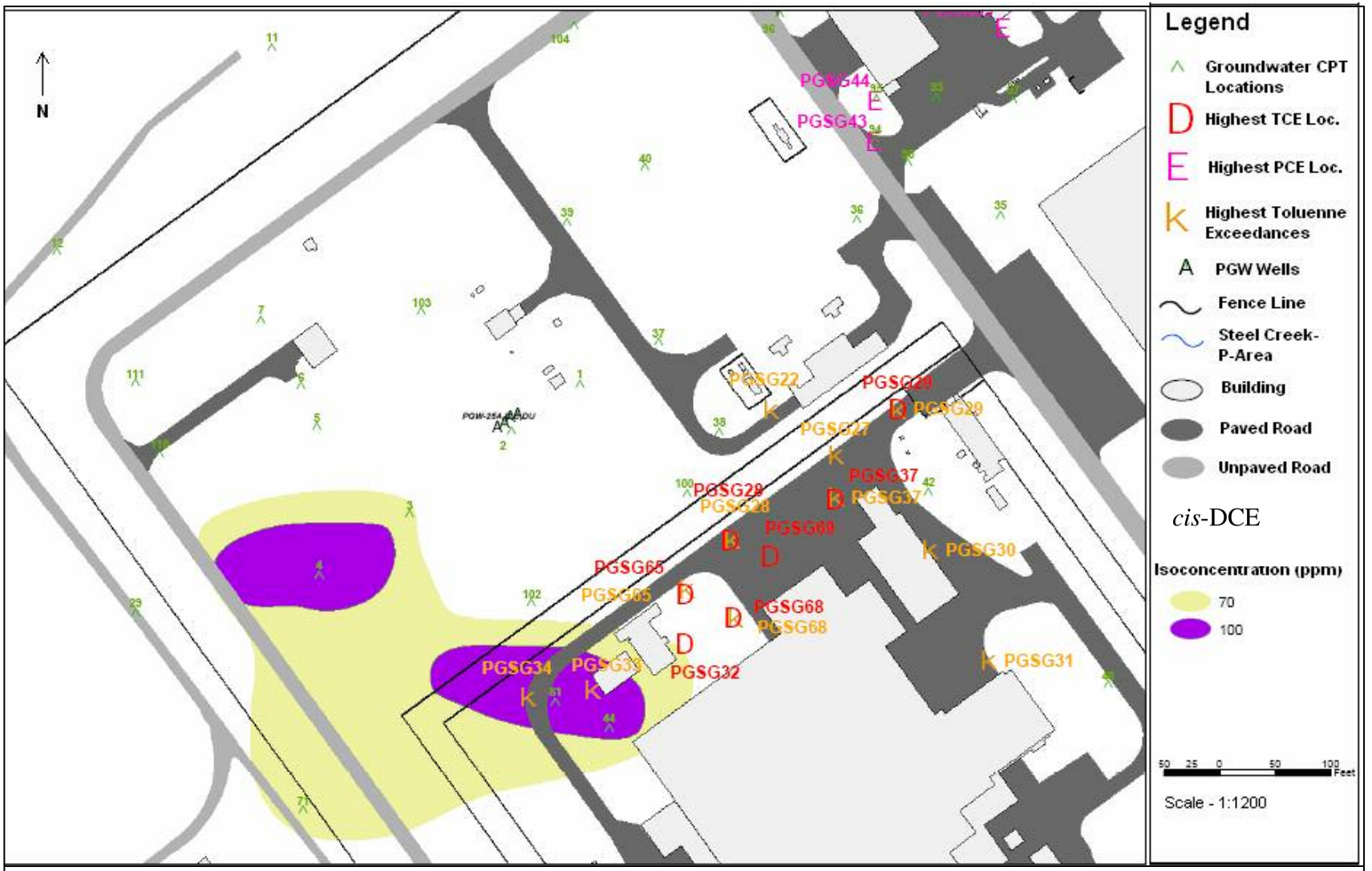

Figure 1.3 *cis*-DCE concentration in the P-Area chlorinated ethene plume.

The second area contributing to the P-Area plume is the P-Area Reactor operable unit. In this area, both TCE and PCE have been detected at levels as high as 35,000 ppb and 2,500 ppb, respectively (25). These compounds have been detected in groundwater at a depth of approximately 15 m. While the exact source of the contamination is unknown, it is believed that dense non-aqueous phase liquid is located near the reactor facility (22).

A similar site at SRS, the C-Area Burning/Rubble Pit, is close to the PBRP and the chlorinated ethene plume is undergoing complete reduction to ethene and ethane. A map of the C-Area Burning/Rubble Pit is given in Figure 1.4. One branch of this plume extends approximately 1220 m to the west and outcrops in the seepline along Twin Lakes and Fourmile Branch. Another branch of the plume extends to the south and outcrops at Castor Creek (23). In collaboration with SRS, the Department of Environmental Engineering & Science at Clemson University conducted a microcosm study using samples from the Twin Lakes seepline in the C-Area to confirm the occurrence of natural attenuation by reductive dechlorination (1). *Dehalococcoides* spp. were detected in sediment samples taken closest to the wetland, but the product signal was especially weak and not considered definitive. None of the sediment samples yielded signature terminal restriction fragments corresponding exactly to *in silico* digest predictions from 16S rRNA genes of *Dehalococcoides ethenogenes* strain 195 or other *Dehalococcoides*-like sequences. However, samples from the microcosms that actively dechlorinated cDCE and vinyl chloride (VC) to ethene and ethane exhibited strong positive signals for *Dehalococcoides* compared to the field samples. Restriction digest analysis indicates strong genotypic similarity between the Bachman Road *Dehalococcoides* 16S rRNA gene

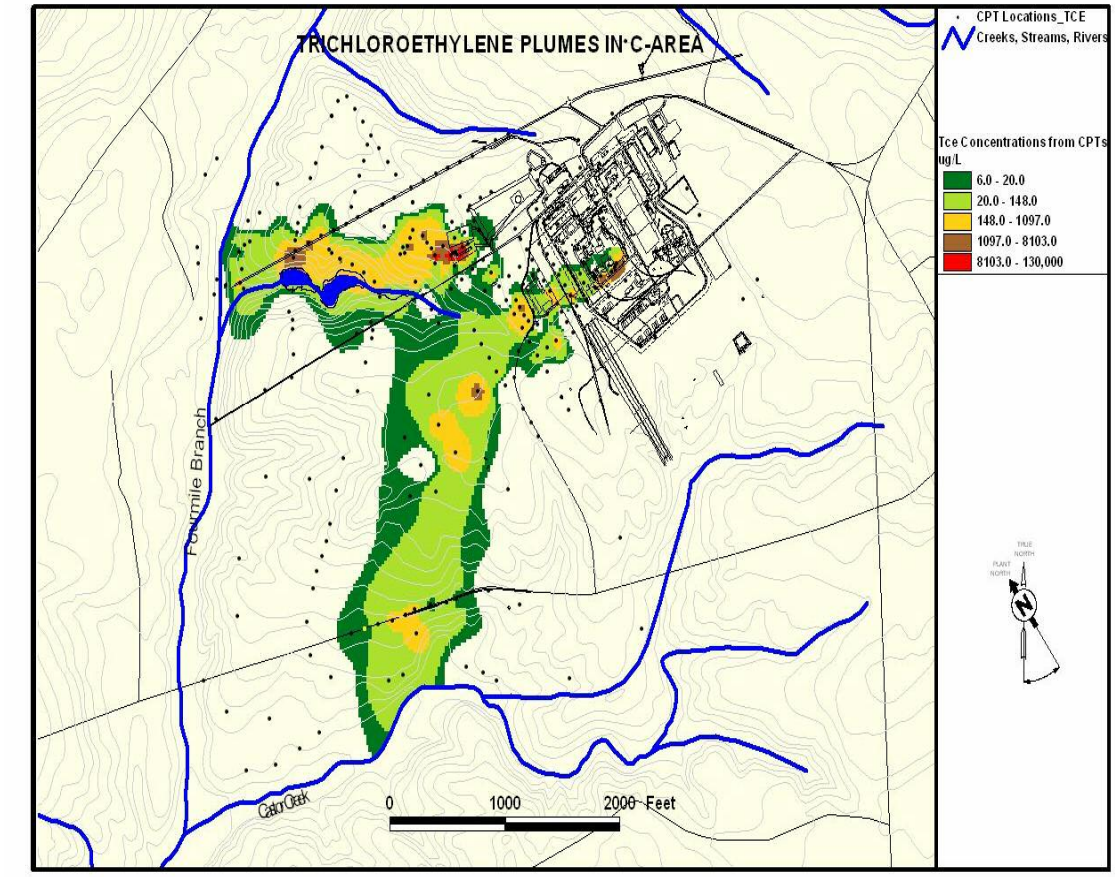

Figure 1.4 TCE plume in the C-Area.

sequence and those from the microcosms prepared with sediment closest to the wetland. Perhaps most significantly, variant genotypes were also recovered, suggesting the presence of novel strains of *Dehalococcoides* (2).

Since the PCE plume in the PBRP is not attenuating naturally beyond cDCE, an active form of in situ remediation is necessary. Both biostimulation (i.e., addition of electron donor) and bioaugmentation (i.e., addition of microorganisms plus electron donor) are possible remediation options. However, biostimulation alone is not likely to be successful. The lack of daughter products beyond cDCE suggests that the required *Dehalococcoides* microorganisms are not present, so even if an excess of electron donor is provided, further dechlorination is unlikely, at least at a reasonable rate. For this reason, bioaugmentation may prove to be the best option for this particular site. Since the microcosms from the C-Area Burning/Rubble Pit have proved able to completely reduce TCE to ethene and ethane, they could be a viable source of culture for use in bioaugmenting the P-Area plume.

In many situations, bioaugmentation has been the most attractive method of enhancing in situ dechlorination, especially at sites at which chlorinated daughter products have accumulated. A comparison of biostimulation versus bioaugmentation was done at the Bachman Road Residential Wells Site near Lake Huron, a site contaminated with PCE. This site used two plots, 4.6 m by 5.5 m, which were separated by a control plot in order to simultaneously compare biostimulation and bioaugmentation. The sediment was composed mostly of sand (7.3 m) above low conductivity clay. The groundwater lies approximately 2.5 m below the surface (16). Two sets of inocula were used, a pure culture of *Desulfurmonas michiganensis* strain BRS1 and a mixed culture

containing at least one *Dehalococcoides* sp. A total of 200 L (0.056% v/v) of inoculum was added to the test portion of the aquifer (355 m^3) (16). The bioaugmentation plot was amended with lactate, phosphate, and nitrate after inoculation. The biostimulation plot was only given lactate (16). The comparison showed that bioaugmentation was not only more complete but occurred in less than half the time of biostimulation. The bioaugmentation plot showed almost complete reduction of the initial PCE, TCE, and cDCE to ethene in 43 days. In the biostimulation plot, there was only 76% reduction to ethene over 121 days (16).

Bioaugmentation has also proven to be effective in pilot scale studies at several other sites contaminated with chlorinated ethenes. One such study was done at Dover Air Force Base, where reduction of TCE had not proceeded past cDCE. An enrichment culture known to dechlorinate both TCE and cDCE to ethene was used in bioaugmentation, and complete reduction to ethene was demonstrated (10). Of a 1.5 million square meter contaminated area, the study site covered approximately 900 square meters. The wells were at a depth of 12 to 15 m below the surface. The soil was composed of sand and silt on top of a Miocene Calvert Clay aquitard. The total organic carbon in the soil was low (less than 1%) and the site was initially aerobic (10). Therefore, in order to obtain reduced conditions lactate was added as an electron donor prior to bioaugmentation. An enrichment culture from the Department of Energy's Pinellas site in Largo, Florida, was used and grown in a chloride-free minimal salts medium. Enrichments were developed through 10% (v/v) transfers until enough culture was obtained. TCE-saturated solution was used to spike the culture to a concentration of 5 mg/L. A total of 351 L of culture was injected (10). However, it is unclear how this

compares with the total liquid volume in the test area. During the bioaugmentation process, lactate was added continuously at a concentration of 200 mg/L. The first signs of success were seen 91 days after bioaugmentation, when VC was first detected. Complete reduction took approximately 200 days and almost 75% of the molar concentration of TCE was recovered as ethane (10).

Another bioaugmentation study was done at Kelly Air Force Base, Texas, where dechlorination of PCE also stopped at cDCE. The size of the test area was 10 m by 7.6 m and 3.1 m deep. The soil was made up of a thin layer (0.31-1.2 m) of organic clay followed by a layer of tan silt-like clay and a thick layer of limestone and chert gravel (17). The hydraulic conductivity of the clay-like sediment was not reported. The culture used was obtained from a site in Southern Ontario and is designated KB-1. It was enriched by repeated 10% (v/v) transfers to pre-reduced mineral medium and was fed TCE and methanol. In order to encourage growth, occasionally 20% of the culture was removed and replaced with fresh medium and the pH was adjusted to 7. For the study at Kelly Air Force Base, 13 L of KB-1 culture was injected (17). Based on the size of the test area and assuming a porosity of 0.3, the volume of water treated was 7.1×10^4 L. The 13 L of KB-1 added represents a dose of 0.018% (v/v). Seventy-three days after inoculation with KB-1, ethene was detected at all of the wells. Ethene became the dominant compound in all wells 69 days later. First order reaction rate constants were in the range of 0.1-0.9 h^{-1} for cDCE and VC and 1-3 h^{-1} for PCE and TCE (17). More importantly, the data showed an increase in the rate over time. All of the wells showed greater than 70% molar recovery of PCE as ethene. PCR results from final samples

showed an order of magnitude increase in *Dehalococcoides* cells per mL of groundwater from the original inoculum (17).

The effectiveness of bioaugmentation depends at least in part on the number of *Dehalococcoides* cells that are delivered, which is a function of the cell density in the enrichment culture and the volume added. At Bachman Road, Dover Air Force Base, and the Kelly Air Force Base, the cell densities per mL were 1.12×10^8, 2×10^8, and 10^8, respectively (10, 16, 17).

Commercially available cultures could be used for bioaugmentation of the P-area and other chlorinated ethene plumes at SRS. However, use of a site-developed culture for application at SRS has several potential benefits. First, the likelihood of obtaining regulatory approval for use of a site-developed culture is higher than using a commercial culture developed from a different location. Second, since the site where the microorganisms were discovered and the site that is being investigated are similar in geochemistry, the culture may be better suited to survive and grow in the PBRP than a commercial culture. Third, there is a potential financial advantage in using the site-developed culture as opposed to a commercially available culture.

CHAPTER 2

RESEARCH OBJECTIVES

The main objectives of this thesis are 1) to develop a sediment-free enrichment culture from the SRS C-Area microcosms that is capable of using chlorinated ethenes as terminal electron acceptors and completely dechlorinating the chlorinated ethenes to ethene and/or ethane, and 2) using microcosms, to compare the effectiveness of biostimulation and bioaugmentation using the SRS enrichment culture to dechlorinate chlorinated ethenes in the PBRP plume. The performance of the SRS enrichment culture will be compared to a commercially available bioaugmentation culture. Groundwater and soil samples from the PBRP will be used to prepare the microcosms.

The specific objectives are:

1) To develop a sediment-free enrichment culture capable of using PCE, TCE, cDCE, and VC as terminal electron acceptors and achieving complete reduction to ethene and/or ethane using the C-Area Burning/Rubble Pit microcosms as the inoculum source.

2) To evaluate biostimulation as a remediation technique for the PBRP plume, using lactate, corn syrup, and emulsified vegetable oil as electron donors. Treatments with no electron donor will be used as controls; and

3) To evaluate bioaugmentation as a remediation technique for the PBRP plume, comparing the SRS enrichment culture developed in objective 1 with a

commercially available culture known to reduce chlorinated ethenes completely to ethene.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

VC (99.5%) was obtained from Fluka. Ethene (polymer grade, 99.9%), ethane (Matheson purity grade, 99.95%), and methane (C.P. grade, 99%) were obtained from Matheson. PCE (99.9%) was obtained from Sigma-Aldrich, TCE (99.5%) from Fisher, cDCE (99%) from TCI America, and 1,1 DCE (99.5%) from Chem Services. PCE, TCE, and cDCE were added to microcosms as saturated water solutions (approximately 0.90 mM PCE, 8.4 mM TCE, and 8.2 mM cDCE). Sodium lactate syrup (containing 58.8-61.2 % sodium lactate; specific gravity = 3.1) was obtained from EM Science. Newman Zone Nonionic emulsified vegetable oil was obtained from Remediation & Natural Attenuation Services, Inc. (Brooklyn Center, MN) and regular type 42/43 corn syrup was obtained from Sweetener Products Company (Vernon, CA). It has a density of 1.418 g/mL and contains 80.3% solids (all sugars), corresponding to approximately 1.2 g/mL of chemical oxygen demand (COD). This was close to the experimentally determined value of 1.6 g/mL (see section 3.4). All other chemicals used were reagent grade quality.

3.2 Enrichment Culture

The starting point for the enrichment culture was a set of microcosms prepared by Bratt (1) that actively dechlorinated TCE, cDCE, and VC to ethene and ethane, using lactate as the electron donor. After his research was completed, Bratt added chlorinated ethenes and electron donor to the microcosms and stored them at 4^{o}C. This thesis

research was initiated by warming Bratt's nine lactate-fed microcosms (three each from sites CRP-44, CRP-48, and CRP-50, as shown in Figure 1.4) to room temperature and adding lactate and TCE and cDCE. These microcosms were selected because when combined, they offered the best opportunity to accomplish complete dechlorination of TCE to ethene and ethane. The CRP-44 microcosms exhibited a high level of dechlorination activity with TCE, although complete dechlorination was sluggish. The CRP-48 and CRP-50 microcosms exhibited a high level of activity with cDCE and VC, hence the decision to use a combination of samples from these microcosms to develop an enrichment culture capable of completely reducing TCE to ethene and ethane.

The enrichment culture was developed in two phases, each representing a significant dilution of the culture that was used for inoculation. Phase I was prepared by combining 3 mL from each of Bratt's nine microcosms, distributing this inoculum to triplicate 160 mL serum bottles and adding 91 mL of anaerobic mineral medium per bottle, for a total liquid volume of 100 mL per bottle. This was done in an anaerobic chamber containing an atmosphere of approximately 98% N_2 and 2% H_2. The medium is described by Edwards and Grbić-Galić (9), with the following modifications: the phosphate buffer was made using 52.5 g K_2HPO_4 per liter instead of 27.2 g KH_2PO_4 and 34.8 g K_2HPO_4; 4.7 g $CaCl_2 \cdot 2H_2O$ and 1.8 g $FeCl_2 \cdot H_2O$ were used instead of 7.0 g $CaCl_2 \cdot 6H_2O$ and 2.0 g $FeCl_2 \cdot 2H_2O$ for the salt solution; 0.2 g $ZnSO_4 \cdot 7H_2O$ was used instead of 0.1 g $ZnCl_2$ for the trace metal solution; the bicarbonate solution was made with 16 g $NaHCO_3$ per liter instead of 260 g/L; 50 mL of the bicarbonate solution was added to the medium instead of 10 mL; and a 5 g/L solution of yeast extract was used

instead of a vitamin solution (Appendix A). These changes were made based on the availability of chemicals and the solubility of sodium bicarbonate in water.

The Phase I serum bottles were sealed with Teflon-faced red rubber septa and aluminum crimp caps. The headspace of the three Phase I enrichment bottles was sparged with a 30% CO_2-70% N_2 gas instead of the 20% CO_2-80% N_2 used by Edwards and Grbić-Galić (9). TCE (25 μL saturated water, providing 0.21 μmol/bottle, or an aqueous phase concentration of 230 μg/L) and cDCE (50 μL saturated water, providing 0.41 μmol/bottle, or an aqueous phase concentration of 1,600 μg/L) were added to each bottle. Lactate was added to meet the electron donor demand. Over approximately 80 days the bottles were given two additional doses of cDCE (50 μL saturated water, providing 0.41 μmol/bottle, or an aqueous phase concentration of 1,600 μg/L), TCE (100-300 μL saturated water, providing 0.83-2.5 μmol/bottle, or an aqueous phase concentration of 910-2,700 μg/L) and one dose of PCE (500 μL saturated water, providing 0.45 μmol/bottle, or an aqueous phase concentration of 540 μg/L).

After the Phase I enrichments repeatedly reduced PCE, TCE, and cDCE to ethene, the Phase II enrichments were started by combining the contents of the three Phase I bottles and transferring 100 mL of the mixture to three 2.6 L glass reagent bottles. Mineral medium was added (1.6 L) so that these larger bottles had the same ratio of liquid volume to headspace volume as in the Phase I serum bottles. The bottles were covered with aluminum foil to exclude light. A Teflon-faced rubber septum (35 mm) was placed inside the cap, which was modified by drilling 24-33 holes (3mm) to provide access for a syringe for sampling. The bottles were stored in the anaerobic chamber horizontally to keep liquid in contact with the septa. These bottles received neat PCE and

TCE (rather than water-saturated solutions) along with lactate as an electron donor. The amount of PCE and TCE added was determined gravimetrically by weighing the syringe with PCE or TCE present, then immediately after adding the PCE or TCE to the bottles. Sodium hydroxide (8 M) was added each time chlorinated ethenes were added to neutralize the HCl produced from dechlorination and as needed to maintain the pH between 6.7 and 7.2. The Phase II enrichment culture received increasing amounts of PCE and TCE until the concentrations added were comparable to the maximum concentrations present in PBRP groundwater (2.5 and 35 mg/L, respectively).

Several aspects of developing the Phase II enrichment culture are noteworthy. First, yeast extract was added on day 23 (1.636 mg/bottle, or 1 mL of a solution containing 1.636 g/L), based on concerns that the initially slow activity on PCE and TCE was due to a micronutrient limitation. Second, to prevent the accumulation of inhibitory compounds (e.g., salts and sulfides), operation of the bottles beginning on day 87 included settling of particulates (taking approximately 3 h), removing the cap (inside the anaerobic chamber) and decanting of 150 mL of the clarified liquid. The 150 mL was replaced with fresh medium, new septa were placed in the caps, the bottles were resealed and more chlorinated ethenes and lactate were added. The amount of liquid removed was gradually increased to 300 mL on day 311. Addition of fresh media provided nutrients and avoided accumulation of salt (NaCl from neutralization) and sulfide from reduction of sulfate in the medium. Approximately 200 mg/L of lactate was added each time the bottles were monitored on the gas chromatograph (GC) for chlorinated ethenes. This dose was doubled when PCE and TCE were added. Periodically, 100-500 mL of completely mixed liquid was removed for addition to the bioaugmentation microcosms

and for measurement of the *Dehalococcoides* spp. concentration by Dr. Christopher Bagwell at the Savannah River National Laboratory . The Phase II enrichment has been maintained for more than 411 days. It will be used in the near future to grow a large enough amount of culture for field-testing in the P-Area.

3.3 Microcosm Experimental Design

Microcosms were prepared with soil and sediment from the P-Area to address the options of biostimulation and bioaugmentation. All treatments were prepared in triplicate. There were two sets of microcosms.

Set I consisted of four live anaerobic treatments to investigate the effect of biostimulation:

- sediment + groundwater only ("as-is");
- sediment + groundwater amended with lactate;
- sediment + groundwater amended with corn syrup;
- sediment + groundwater amended with emulsified vegetable oil;

Set II investigated the effectiveness of bioaugmentation, using five live anaerobic treatments:

- sediment + groundwater amended with lactate + bioaugmented with 1.0% (v/v) of the SRS enrichment culture developed in the laboratory;
- sediment + groundwater amended with lactate + bioaugmented with 0.1% (v/v) of the SRS enrichment culture developed in the laboratory;
- sediment + groundwater amended with lactate + bioaugmented with 0.01% (v/v) of the SRS enrichment culture developed in the laboratory;

- sediment + groundwater amended with lactate + bioaugmented with 0.001% (v/v) of the SRS enrichment culture developed in the laboratory; and
- sediment + groundwater amended with lactate + bioaugmented with 1.0% (v/v) of a commercially available bioaugmentation culture.

Lactate was used as the electron donor for set II because lactate was the electron donor used for the enrichment culture.

In addition to the live treatments, two sets of autoclaved controls (ACs) and two sets of water controls (WCs) were prepared to evaluate the extent of abiotic losses. One set of ACs was prepared containing sediment + groundwater + PCE + TCE + cDCE + VC + ethene. PCE and TCE were added at concentrations comparable to the maximum concentration measured in PBRP groundwater. cDCE, VC, and ethene were added in half the molar amount per bottle of TCE. The other set of ACs contained ethane and methane, which were added in the same amount as VC and ethene. A separate set of ACs for methane and ethane was prepared to avoid interference with ethene, since these three compounds elute close together on the GC column used (i.e., in less than 1 min; see section 3.6). Two sets of WCs were prepared identically to the ACs, although only distilled, deionized (DDI) water was used in place of groundwater and no sediment was added.

3.4 Microcosms

The microcosms consisted of 160 mL serum bottles. The live and AC treatments received 20 g of sediment (wet weight; 16 g dry weight, occupying approximately 11 mL) plus 50 mL of groundwater. The groundwater received resazurin (1 mg/L), which

served as a redox indicator. The groundwater was then adjusted from pH 5.65 to pH 7.4 with sodium hydroxide (8 M), prior to addition to the microcosms. pH adjustment was considered necessary to provide a more favorable environment for chlororespiration.

The sediment and groundwater samples were taken by SRS from the PBRP area. Groundwater samples were taken at a depth of 15.2 m from well PDT-2, an uncontaminated background well. Soil samples were taken at site PGCPT94 (Figures 1.1-1.3) from depths of 3.0-13.7 m. Samples were transported on ice to Clemson University's Department of Environmental Engineering and Science. They were received on August 16, 2005.

All of the live treatments and ACs were prepared in an anaerobic chamber with an atmosphere of approximately 98% N_2 and 2% H_2. Aseptic techniques were used during preparation of the live treatments. A scale was placed in the anaerobic chamber to measure out the sediment. The killed controls were prepared by autoclaving the microcosms for 60 minutes at 121^{o}C for three consecutive days before adding the chlorinated compounds. The WCs were prepared and incubated on the bench top, with room air present in the headspace.

Because the groundwater was taken from an uncontaminated well, no PCE or TCE were present. It was therefore necessary to add PCE and TCE in sufficient amounts to yield aqueous phase concentrations of approximately 35 mg/L TCE and 2.5 mg/L PCE. PCE and TCE were added using saturated water solutions; the approximate concentrations are given in see section 3.1. PCE, TCE, and cDCE were added to the controls also using saturated water solutions. VC, methane, ethene, and ethane were added to the controls using a 1.0 mL gas tight syringe (Pressure-Lok®). All bottles were

sealed with Teflon-faced red rubber septa and aluminum crimp caps, and were incubated in an inverted position (liquid and/or sediment in contact with the septa), at room temperature (21-24 ^{o}C) and in boxes (to exclude light). The live bottles were incubated in the anaerobic chamber, except during sampling.

The amount of electron donor added to the live treatments was based on the amount of PCE and TCE present, along with competing electron acceptors (i.e., nitrate and sulfate). Anion analysis of the groundwater gave a nitrate concentration of approximately 10 mg/L and a sulfate concentration of approximately 20 mg/L (see section 4.3). This required 0.32 mg COD per bottle to remove the nitrate and 0.67 mg COD per bottle to remove the sulfate. To convert 2.5 mg/L of PCE (1.7 μmol/bottle) and 35 mg/L of TCE (22.5 μmol/bottle) to ethene required 1.2 mg COD per bottle. Donor was added on days 9 and 16 to all of the microcosms (16.8 and 42.2 mg COD/bottle, respectively), for a total of 59 mg COD per bottle. This provided approximately a 50-fold excess based on the stoichiometric electron donor needs for chlorinated ethene reduction. The amount of lactate, corn syrup, and vegetable oil needed was calculated based on COD values of 0.669 g COD/mL for sodium lactate syrup, 0.927 g COD/g for corn syrup, and 1.389 g COD/mL for emulsified vegetable oil. The value for lactate syrup was calculated based on its composition (60% by weight) and density (1.31 g/mL) while the values for the vegetable oil and corn syrup were determined experimentally.

For the biostimulation only treatments donor was added two more times, on days 105 and 237. This brought the total COD added to 152.8 mg COD per bottle, providing 127 times more electron donor than needed for stiochiometric reductive dechlorination. Additional donor was also added to the bioaugmentation microcosms to insure the

electron donor was not a limiting factor. These amounts are reported in the Results section.

3.5 Bioaugmentation Procedures

For the bioaugmentation experiments, 1.0, 0.1, 0.01, and 0.001% (v/v) of SRS culture and 1% (v/v) of a commercially available bioaugmentation culture were added to the Set II microcosms following a period of incubation with only electron donor added. A delay between electron donor addition and bioaugmentation was necessary to allow enough time for establishment of the low redox conditions required for chlororespirators in the enrichment culture. Shortly before bioaugmentation, the mass of volatile compounds present was measured by headspace analysis on a gas chromatograph (see section 3.6).

Preliminary data on the *Dehalococcoides* cell concentration in the Phase II enrichment ranged from 1.6 $x10^4$-$4.6x10^4$ cells/mL (C.E. Bagwell, personal communication). Previous work has shown that a *Dehalococcoides* cell concentration of 10^6 to 10^8 cells/mL is preferable for bioaugmentation in field studies (10, 16, 17). In order to achieve a fair comparison between the SRS culture and the commercially available culture, the enrichment culture was concentrated 100 fold. It was decided that instead of adding 5 μL of the concentrate for the 0.01% addition and 0.5 μL of the concentrate for the 0.001% addition, it would be simpler to add 500 μL and 50 μL of the unconcentrated inoculum, respectively. Table 3.1 shows the inoculum percent and the volume of concentrated or unconcentrated culture used to achieve this percent.

The enrichment culture was concentrated aseptically in the anaerobic chamber as follows: Samples from the Phase II enrichment bottles (240 mL total) were added to two

Table 3.1 Volume and type of Phase II SRS enrichment culture used for bioaugmentation.

Percent Inoculum (v/v)	Volume of Culture Added (μL/bottle)	
	100x Concentrate	Unconcentrated
1.0	500	-
0.1	50	-
0.01	-	500
0.001	-	50

250 mL Sepcor centrifuge bottles (120 mL per bottle) with screw top caps containing gaskets. The bottles were centrifuged for 25 min at 10,000 rpm and 23°C on a Sorvall® Evolution RC centrifuge equipped with a SLA-1500 Super-Lite® 6 hole, 250 mL rotor. In the anaerobic chamber, 108 mL was pumped out of the bottles without disturbing the centrifuged solids. Pumping was accomplished using a Cole Palmer model #2016 peristaltic pump with Master-flex 3 mm inner diameter tygon tubing. The remaining 12 mL was agitated to resuspend the solids, combined and transferred to two 12 mL glass conical centrifuge tubes, which were then sealed with Teflon-faced rubber septa and aluminum crimp caps. These were centrifuged for 10 min at 3,000 rpm and 23°C on a Fisher Centrific™ Centrifuge with a 12 hole 15 mL rotor. Centrate (10.8 mL) was pumped from each tube, leaving 1.2 mL. The contents of both tubes were combined. This provided 2.4 mL of 100x concentrated enrichment culture, to inoculate the 1% and 0.1% (v/v) bioaugmentation treatments. The amount remaining was shipped to C.E. Bagwell at the Savannah River National Laboratory for measurement of the *Dehalococcoides* concentration.

3.6 Volatile Organic Compound Analysis

PCE, TCE, cDCE, VC, ethene, ethane, and methane were monitored by headspace analysis using a Hewlett Packard Series II 5890 GC. The mass of each compound present in a bottle was determined by analysis of a 0.5 mL headspace sample, using a flame ionization detector in conjunction with a column packed with 1% SP-1000 on 60/80 Carbopack-B (Supelco, Inc.). The carrier gas used was nitrogen (12).

Standards for the enrichment cultures (Phases I and II) were prepared by adding known amounts of each compound to 160 mL serum bottles containing 100 mL DDI water. Standards for the microcosms were prepared in the same manner except the serum bottles contained 20 g of sediment and 50 mL DDI water. The number of moles of gases added was calculated using the ideal gas law, based on the volume added at room temperature and atmospheric pressure. A stock solution of PCE, TCE, cDCE, and 1,1-DCE was prepared gravimetrically in methanol. After adding known amounts of the gases and stock solution to the serum bottles, they were incubated for 1-4 h. This allowed sufficient time for the compounds to equilibrate, but not enough time for significant biotic or abiotic losses to occur. Peak areas obtained from headspace analysis were used to determine response factors for each compound, in terms of the total mass per bottle per peak area unit from a 0.5 mL headspace sample (12). Since the standards were prepared in 160 mL serum bottles, response factors for the larger Phase II bottles were determined by multiplying the response factors for the 160 mL bottles times the ratios of the total volumes (2650 mL/160 mL). This was possible because the ratio of the headspace and liquid volumes was kept constant (i.e. V_g/V_l = 0.6). Representative response factors are provided in Appendix B.

The GC response to a headspace sample was calibrated to give the total mass of the compound (M) in that bottle. Assuming that the headspace and aqueous phases were in equilibrium, the total mass present was converted to an aqueous phase concentration:

$$C_l = \frac{M}{V_l + H_c V_g} \quad (1)$$

where C_l = concentration in the aqueous phase (μM); M = total mass present (μmol/bottle); V_l = volume of the liquid in the bottle (50 mL for the microcosms, 100 mL for the Phase I enrichments, and 1625 mL for the Phase II enrichment culture); V_g = volume of the headspace in the bottle (99 mL for the microcosms, 60 mL for the Phase I enrichments and 975 mL for the Phase II enrichments); and H_c = Henry's constant (dimensionless) at 23°C (calculated from Gossett (12)). V_g for the microcosms was calculated by subtracting V_l and the measured volume of 20 g of sediment (11 mL) from the total volume (160 mL).

3.7 Hydrogen Analysis

Hydrogen was monitored by headspace analysis of 0.5 mL samples using a Hewlett Packard Series II 5890 GC and a thermal conductivity detector with a 3.2-mm x 3.2-m stainless-steel column packed with 100/120 Carbosieve S-II (Supelco, Inc.). The carrier gas used was nitrogen with the column and reference flow rates set at 46.1 mL/min. The column temperature was isothermal at 105°C and the injector and detector temperatures were 200°C. Thermal conductivity detector sensitivity was set at high, resulting in a minimum detection level of 1.7 μmol H_2 per 2.6 L bottle. This is equivalent to 4.3×10^{-5} atm in a bottle with 60 mL of headspace and assuming a pressure

of 1 atm. The sample run time was 12 min. Representative response curves are shown in Appendix B.

3.8 Anion Analysis

A Dionex DX-100 Ion Chromatograph was used to determine the concentrations of nitrate and sulfate. A sodium bicarbonate (3.9 mM) and sodium carbonate (3.1 mM) eluant was pumped at 1.0 mL/min through a Dionex IonPac AS5A-5µ column (4 mm x 150 mm). Samples were filtered (0.20 µm, Pall) and injected onto the IonPac column, with a sample loop size of 25 µL. Response factors were determined using KNO_3 and K_2SO_4 standards. Representative response curves are given in Appendix B.

3.9 Soil Analysis

The amount of organic matter, carbon, nitrogen, nitrate, phosphorous, potassium, calcium, magnesium, sodium, sulfur, boron, zinc, manganese, copper, iron, and aluminum in P-Area soil used to prepare the microcosms was determined by the Clemson University Agricultural Services Laboratory. A 300 g sample of dried soil was used. The moisture content of the soil was determined by drying triplicate samples at 102^{o}C to a constant weight. The volume of 20 g of wet soil (the amount added to the microcosms) was determined by water displacement.

3.10 Soluble COD Analysis

Soluble COD was measured using the Bioscience Mid Range (20-900 mg/L) accu-TEST kit. Samples were run in duplicate. Preparation of groundwater samples consisted of filtration through triple rinsed 0.45 µm filters (Nalgene; Teflon with PTFE membrane). The soluble COD of the groundwater gave an indication of the background level of electron donor available for reductive dechlorination.

3.11 TOC Analysis

Total organic carbon (TOC) was measured using a TOC-V CSH Total Organic Carbon Analyzer (Schimadzu). Measurements were made in accordance with Standard Method 5310B, a combustion method using infrared detection (8). Samples were filtered through a 0.2 μm Nalgene Teflon filter with a PTFE membrane and diluted to within the range of 0.1 to 30 mg/L. Standards were prepared using a 1000 mg TOC/L stock solution made using potassium hydrogen phthalate. The stock solution was diluted to concentrations of 0.1, 0.5, 1, 5, 10 and 30 mg TOC/L for standards. A representative response curve is shown in Appendix B.

3.12 pH Analysis

pH of the Phase II enrichments was measured in 2 mL samples using a Corning pH meter 345. The pH meter was calibrated before samples were analyzed using pH 4, 7 and 10 buffer solutions. pH of the microcosms was analyzed using test strips. Baker-pHIX strips were used for the pH range of 6.0 to 7.7, in 0.3 pH unit increments. If the pH was below this range, EMD colorpHast indicator strips were used, which have a range of 4.0 to 7.0 in 0.3 pH unit increments. A 50 μL drop was placed on the test strip and the color was read approximately 3 sec after contact.

3.13 Analysis of Organic Acids

Analysis of organic acids was performed by high performance liquid chromatography (HPLC) on a Waters (Milford, MA) instrument equipped with a 600E System Controller, a 490E Programmable Multiwavelength Detector (420 nm) and a 717 plus Autosampler. An Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm, BioRad, Hercules, CA) was used with 0.01 N H_2SO_4 as the eluant, delivered at 0.6

mL/min. Calibration curves for lactate, acetate and propionate were used for quantification of these fatty acids in samples from the Phase II enrichment culture. Representative calibration curves are shown in Appendix B. One milliliter samples were filtered through a 0.2 μm PVDF filter (Pall Life Sciences, East Hills, NY) before analysis. A 200 μL sample loop was used to deliver 50 μL samples. The method run time was 60 min in order to detect any possible longer-chain organic acids such as butyrate, valerate and caproate.

CHAPTER 4

RESULTS

The results for the original microcosms obtained from Bratt are shown first, followed by results for the Phase I and Phase II enrichments. Results for the characteristics of the P-Area soil and groundwater are presented next, followed by the biostimulation and bioaugmentation microcosm study.

4.1 Original Microcosms

Triplicate microcosms from the CRP-44, CRP-48 and CRP-50 sampling locations in the C-Area (Figure 1.4) were obtained from Bratt (1) after being refrigerated for 12 months. The microcosms were allowed to acclimate to room temperature for five days. Approximately 0.3 μmol of TCE per bottle (an aqueous phase concentration of 0.4 mg/L, based on equation 1) was added to each of the three CRP-44 microcosms. An initial dose of 1.4 mg of lactate per bottle was also delivered. Because of the high concentration of methane initially present in the CRP-48 and CRP-50 microcosms, these bottles were opened inside the anaerobic chamber and allowed to equilibrate for 5 min before 4.2 μmol of cDCE per bottle (aqueous phase concentration of 6.4 mg/L) and 0.6 mg of lactate per bottle were added.

All of the microcosms proved to be viable, even after 12 months of refrigeration. Figures 4.1, 4.2 and 4.3 show representative results from the triplicate CRP-44, CRP-48 and CRP-50 microcosms, respectively. On day 42, the nine bottles were opened in the anaerobic chamber and 3 mL of mixed liquid was removed from each one. This explains

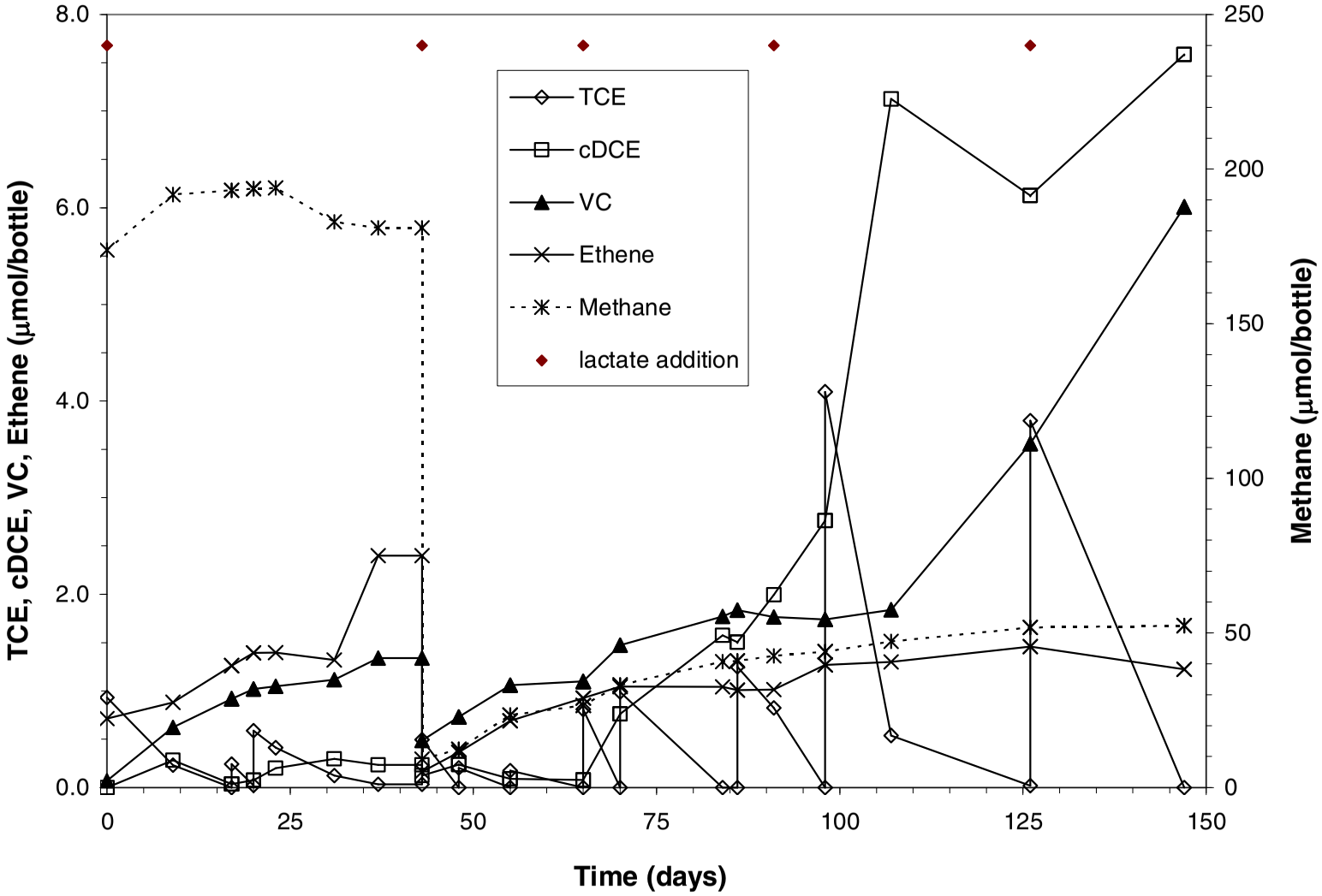

Figure 4.1 Results for one of the CRP-44 microcosms (CRP-44-Lac1); replicate bottles are shown in Appendix C. Solid diamonds indicate when lactate was added (1.4 mg/bottle).

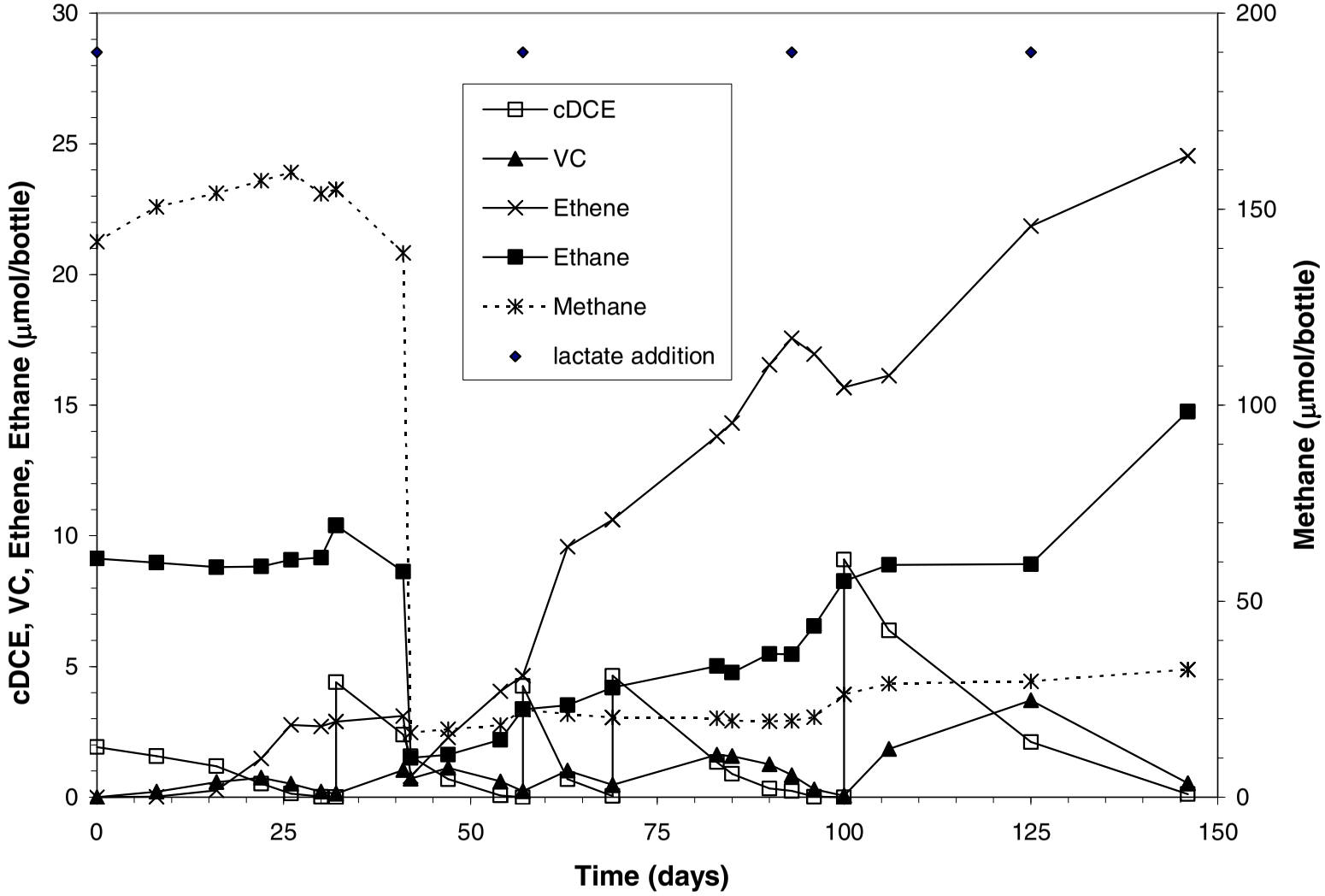

Figure 4.2 Results for one of the CRP-48 microcosms (CRP-48-Lac1); replicate bottles are shown in Appendix C. Solid diamonds indicate when lactate was added (0.94 mg/bottle).

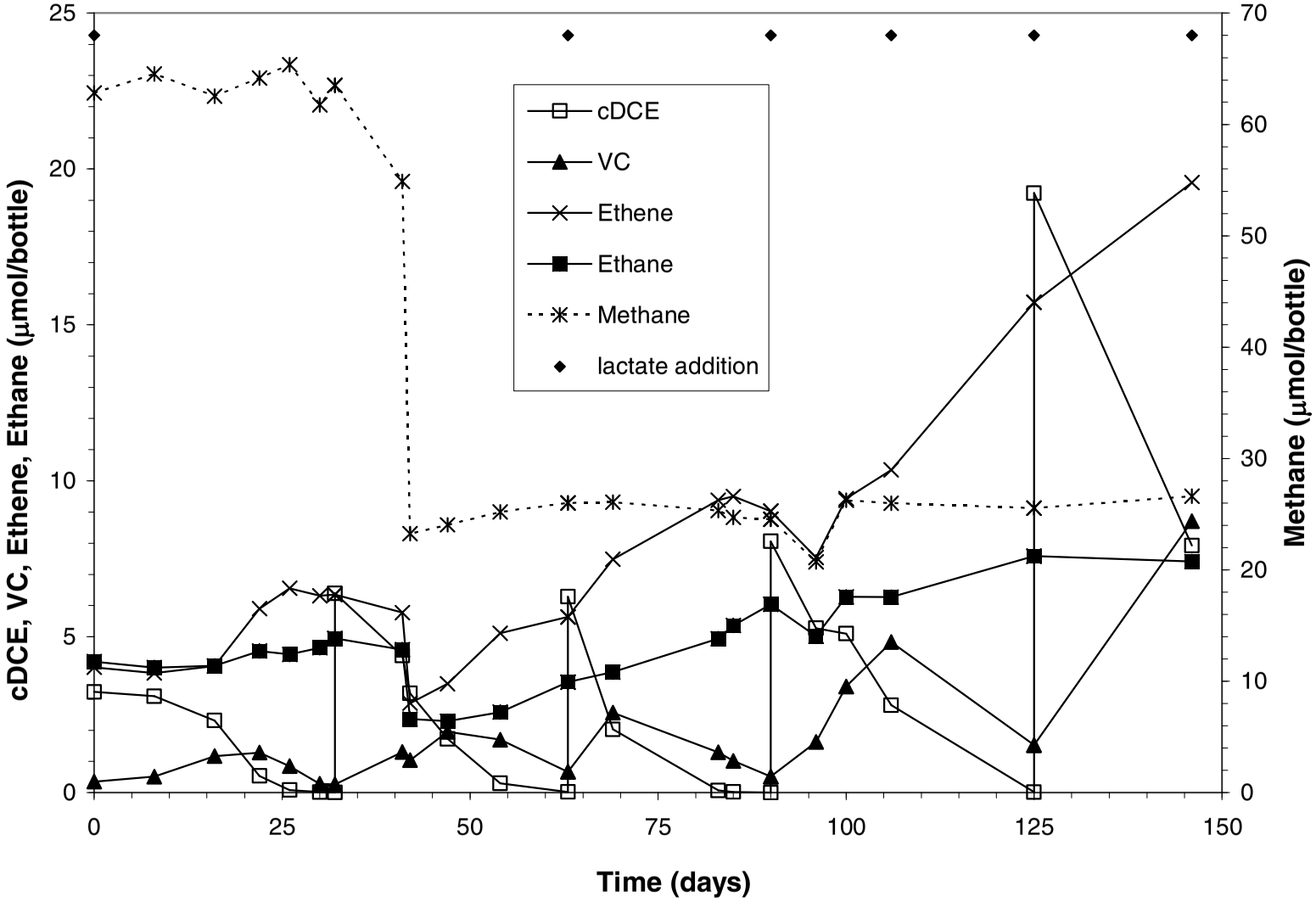

Figure 4.3 Results for one of the CRP-50 microcosms (CRP-50-1); replicate bottles are shown in Appendix C. Solid diamonds indicate when lactate was added (0.94 mg/bottle for the first four additions and 1.4 mg for the final two).

the drop in cDCE, VC, ethene, ethane and methane on that day. The 27 mL of samples were combined and added to mineral medium in order to start the Phase I enrichments (see section 4.2). Since it was unclear if the microcosm culture would continue to dechlorinate when added to mineral medium, the microcosms were maintained beyond day 42 until the results for the Phase I enrichments were definitive. The microcosms proved able to degrade up to 4.0 μmol of TCE per bottle for the CRP-44 microcosms (Figure 4.1), 8.0 μmol of cDCE per bottle for the CRP-48 microcosms (Figure 4.2) and 19.5 μmol of cDCE per bottle for the CRP-50 microcosms (Figure 4.3). These amounts are equivalent to aqueous phase concentration of 6.2, 12.1 and 29.6 mg/L, respectively.

Continual respiking of the CRP-44 bottles with TCE led to an accumulation of cDCE and VC. The microcosms from this upgradient location did not completely reduce TCE to ethene. By contrast, the CRP-48 and CRP-50 microcosms did completely reduce cDCE and VC. This is why microcosms from these three locations were combined, i.e., to develop an enrichment culture capable of completely dechlorinating TCE.

4.2 Development and Characterization of an SRS Enrichment Culture

4.2.1 Phase I Enrichment Culture

The purpose of Phase I was to acclimate the microcosm culture to growth in mineral medium, to develop a tolerance for higher levels of TCE than what was added to Bratt's microcosms, and to determine if the microcosm culture could dechlorinate PCE, which was not added to Bratt's microcosms (1). In order to accomplish this, the concentrations of TCE and PCE were gradually increased. Representative results from the triplicate Phase I enrichment bottles are shown in Figure 4.4. The initial cDCE and TCE amounts (2.9-4.0 and 0.2-0.3 μmol/bottle, respectively) are equivalent to aqueous

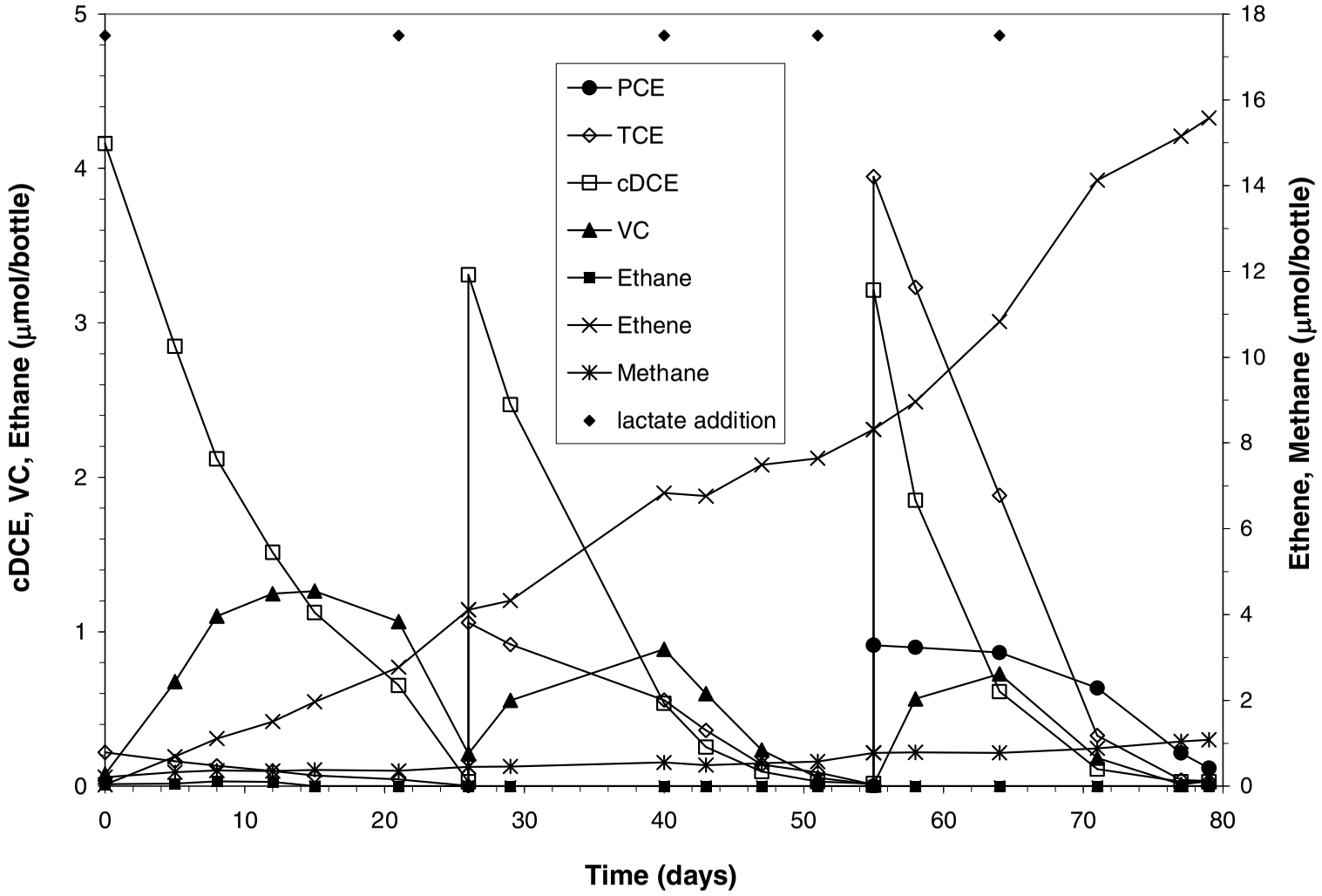

Figure 4.4 Results for one of the Phase I enrichments (E-1A); replicate bottles are shown in Appendix C. Solid diamonds indicate when lactate was added (1.4 mg/bottle for the first addition and 1.1 mg for the remaining additions).

phase concentrations of 2.6-3.6 and 0.2-0.3 mg/L, respectively, based on equation 1. The rate of reduction of TCE increased with each addition. One interesting difference between the Phase I enrichment bottles and the microcosms is the lack of ethane accumulation in the enrichment, whereas ethane was a significant end-product in the microcosms. It is not completely clear what caused the loss of ethene reduction to ethane, but it is likely that the organisms responsible were no longer present in sufficient numbers.

Another difference between the behavior of the nine original microcosms and the Phase I enrichment was observed. Methane production was robust in the microcosms but was almost completely inhibited in the Phase I enrichment bottles, most likely due to the increased levels of TCE and PCE added. In spite of the fact that the microcosms were never exposed to PCE, PCE was dechlorinated by the Phase I enrichments. However, PCE reduction did not start until most of the TCE was consumed, even though most cultures dechlorinate PCE before switching to TCE. The rate of PCE reduction significantly increased when the TCE concentration dropped below 0.5 μmol per bottle. The average molar recovery of ethene from the PCE, TCE and cDCE consumed over 79 days of incubation in the Phase I enrichment bottles was 112% (± 3.4%).

4.2.2 Phase II Enrichment Culture

When the final addition of PCE, TCE and cDCE to the Phase I enrichment was reduced to ethene on day 79, the complete contents of these bottles were used to start the three Phase II enrichment bottles (E-2A, E-2B, and E-2C), as described in section 3.2. After diluting the entire contents of the Phase I enrichment bottles into fresh mineral medium (0.3 L culture added to 4.9 L medium), increasing amounts of PCE and TCE

were added until the concentrations were comparable to the maximum expected at the PBRP site. It can be seen from results for one of the triplicate Phase II enrichment bottles shown in Figure 4.5 that the increasing concentrations of PCE and TCE contributed to an increase in the reduction rate of the chlorinated ethenes. The highest amounts of PCE and TCE added (185 and 633 μmol/bottle, respectively) are equivalent to aqueous phase concentrations of 13.4 and 41.5 mg/L, based on equation 1.

The Phase II enrichment bottles were approximately 15 times larger than the Phase I serum bottles, which explains the larger y-axis scales used in Figure 4.5 in comparison to Figure 4.4 (expressed as μmoles per bottle). The drop in ethene that occurred with each new addition of PCE and TCE was due to opening the bottles in the anaerobic chamber to remove some of the settled liquid and replacing it with fresh media, as described in section 3.2. On day 196 samples of the enrichment were removed to bioaugment the P-Area microcosms.

Table 4.1 is a summary of the stoichiometry of PCE and TCE dechlorination for each of the Phase II enrichment bottles for a time period when the highest amounts of TCE and PCE were being added (days 145 to 353). An overall average of 88.5% (±11.4%) of the chlorinated ethenes present at the start of a cycle (i.e., when PCE and TCE were added) was accounted for as daughter products at the end of each cycle (i.e., after complete degradation of the PCE and TCE). This is a reasonable accounting for the initial chlorinated ethenes when taking into account losses of the volatiles, variability in GC response factors, etc. Ethene was the predominant daughter product; only low amounts of cDCE and VC remained prior to respiking the bottles with PCE and TCE. The overall average of ethene as the final daughter product was 97.6% (±3.8%).

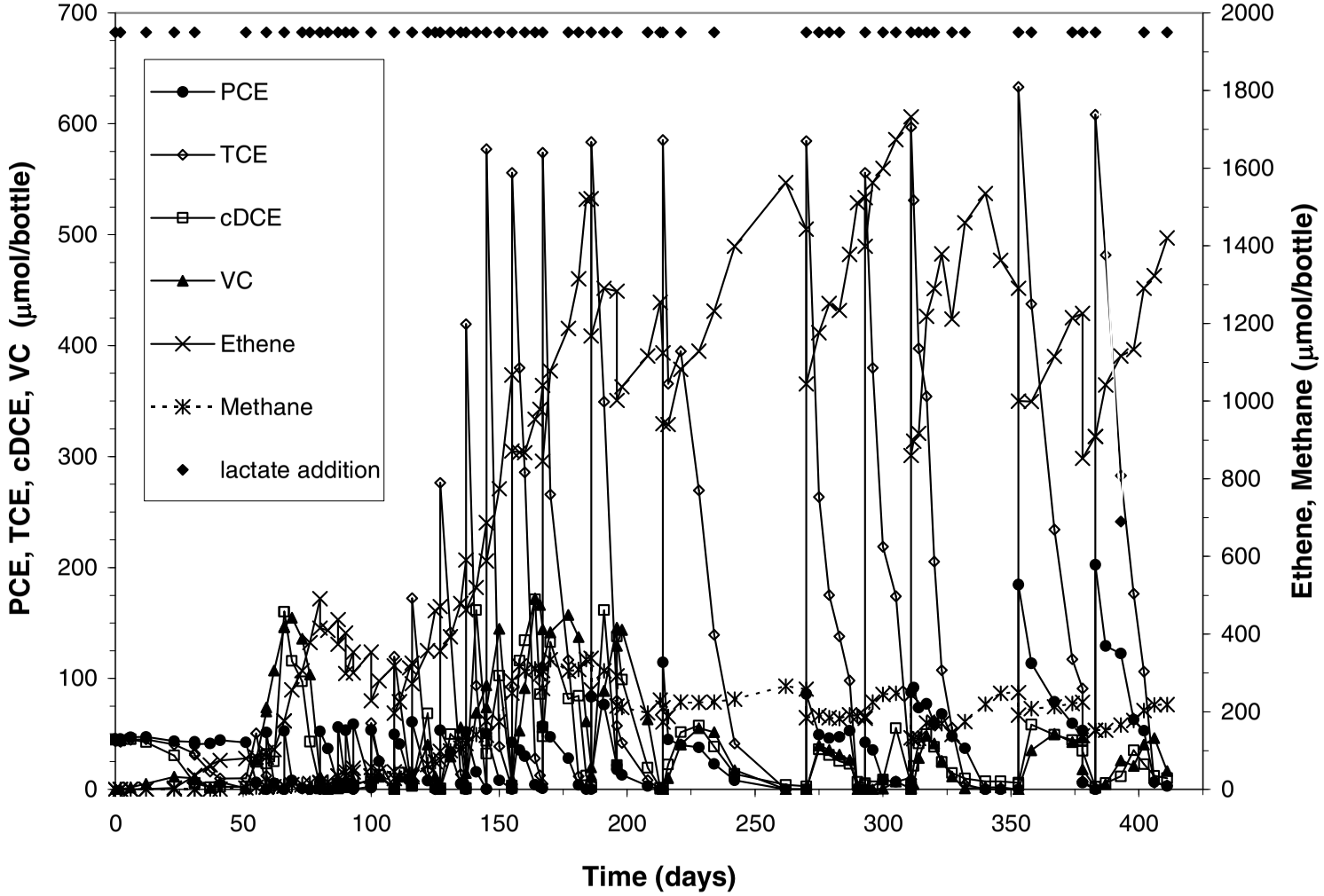

Figure 4.5 Results for one of the Phase II enrichments (E-2A); replicate bottles are shown in Appendix C. Solid diamonds indicate when lactate was added.

Table 4.1 Summary of stoichiometry of PCE and TCE dechlorination for the Phase II enrichment bottles.

Cycle[a]	Initial Chlorinated Ethenes[b] and Ethene (μmol/bottle)			Final Chlorinated Ethenes[b] and Ethene (μmol/bottle)			% Recovery[c]			% Ethene[d]		
	E-2A	E-2B	E-2C	E-2A	E-2B	E-2C	E-2A	E-2B	E-2C	E-2A	E-2B	E-2C
1	1,322	1,428	1,424	1,081	1,214	1,280	81.7%	85.0%	90.1%	98.7%	97.8%	99.6%
2	1,474	1,517	1,670	1,246	1,521	1,563	84.6%	100.3%	93.6%	83.5%	92.2%	94.3%
3	1,636	1,669	1,750	1,545	1,203	1,947	94.4%	72.1%	111.2%	98.5%	97.7%	99.5%
4	1,849	1,659	2,147	1,126	1,528	1,911	60.9%	92.1%	89.0%	99.9%	98.4%	99.2%
5	1,643	1,747	1,686	1,446	1,528	1,870	88.0%	87.5%	110.9%	99.8%	98.4%	96.6%
6	1,714	2,084	1,686	1,530	1,647	1,632	89.3%	79.1%	96.8%	99.6%	99.4%	99.3%
7	2,001	1,585	-	1,758	1,276	-	87.8%	80.5%	-	98.6%	98.9%	-
8	1,545	-	-	1,278	-	-	83.9%	-	-	99.5%	-	-
Averages	-	-	-	-	-	-	83.8%	85.2%	98.6%	97.3%	97.6%	98.1%

[a] Cycle refers to the time from spiking with PCE and TCE and addition of fresh medium and lactate to complete reduction of PCE and TCE to daughter products

[b] Chlorinated ethenes = ∑(PCE + TCE + cDCE + VC)

[c] % Recovery = (μmoles of final chlorinated ethenes + ethene)/(μmoles of initial chlorinated ethenes + ethene) x 100

[d] % Ethene = (μmoles ethene)/(μmoles of final chlorinated ethenes + ethene) x 100

Complete data for calculation of the stoichiometry shown in Table 4.1 is presented in Appendix D.

Methane production in the Phase II enrichment bottles was minimal compared to the amount produced in the microcosms. This continued the trend observed in the Phase I enrichment, i.e. the high levels of chlorinated ethenes and ethene most likely inhibited methanogenesis. Over the period from day 145 to 353, a total of 8.8-9.2 g of sodium lactate was added per bottle, which is equivalent to 7.5-8.3 g of COD. Over the same period, cumulative methane production was 0.17 to 0.74 mmol per bottle, which is equivalent to 10.9-47.4 mg of COD, or less than 1.0% of the lactate COD added. Calculations for lactate and methane were started on day 145 because that is the time when the addition of TCE reached the highest expected concentration of 35 mg/L.

For one cycle of PCE and TCE addition and dechlorination (days 311-356), a COD balance was performed to determine the disposition of the electron donor added (in the form of lactate), including the percent COD used in the reduction of PCE and TCE to ethene. Tables 4.2, 4.3 and 4.4 show the complete electron donor balance for the three Phase II enrichment bottles (E-2A, E-2B, and E-2C, respectively). Initial and final concentrations of lactate, acetate and propionate were measured by HPLC. Only peaks for lactate, acetate and propionate were quantified from the 60 min chromatogram, which is a sufficiently long run time to detect longer chain organic acids such as butyrate, caproate and valerate, if they were present. The initial sulfate concentration was calculated using the known concentration of sulfate in the media (0.87 mM) and the amount of fresh media added at the start of each feeding cycle (0.3 L). The COD of the

Table 4.2 Donor Balance for Phase II Enrichment Bottle E-2A (Days 311-356).

		Initial			Final			COD Balance (mg/bottle)[c]
		mM	mmol/bottle[a]	mg COD/bottle	mM	mmol/bottle[a]	mg COD/bottle	
Donor	Lactate	10.2	16.9	1,620	1.38	2.28	219	+ 1,400
Products	Acetate	8.63	14.3	914	11.1	18.4	1,180	- 262
	Propionate	9.24	15.3	2,710	14.0	23.2	2,600	- 886
	SO_4^{2-}	0.260	0.430	27.7	0.0	0.0	0.0	- 27.7
	CH_4	-[b]	0.130	8.51	-[b]	0.249	16.0	- 7.44
	H_2	-[b]	0.0	0.0	-[b]	0.0	0.0	- 0.0
	Ethene from PCE	-[b]	0.0	0.0	-[b]	0.0876	5.61	- 5.61
	Ethene form TCE	-[b]	0.0	0.0	-[b]	0.597	28.6	- 28.6

[a] Volume of liquid in E-2A = 1.656 L.
[b] GC results in terms of mmol/bottle.
[c] Initial COD – Final COD, except for SO_4^{2-}, which was calculated as final COD – Initial COD, since it was consumed rather than produced.

Table 4.3 Donor Balance for Phase II Enrichment Bottle E-2B (Days 311-356).

		Initial			Final			COD Balance (mg/bottle)[c]
		mM	mmol/bottle[a]	mg COD/bottle	mM	mmol/bottle[a]	mg COD/bottle	
Donor	Lactate	10.3	17.0	1,630	1.13	1.87	180	+ 1,450
Products	Acetate	9.00	14.9	954	12.2	20.3	1,290	- 388
	Propionate	9.63	15.9	1,770	13.3	22.0	2,460	- 679
	SO_4^{2-}	0.26	0.430	27.7	0.0	0.0	0.0	- 27.7
	CH_4	-[b]	0.0490	3.11	-[b]	0.111	7.1	- 4.01
	H_2	-[b]	0.0	0.0	-[b]	0.0	0.0	- 0.0
	Ethene from PCE	-[b]	0.0	0.0	-[b]	0.0923	5.91	- 5.91
	Ethene form TCE	-[b]	0.0	0.0	-[b]	0.581	27.9	- 27.9

[a] Volume of liquid in E-2A = 1.656 L.
[b] GC results in terms of mmol/bottle.
[c] Initial COD – Final COD, except for SO_4^{2-}, which was calculated as final COD – Initial COD, since it was consumed rather than produced.

Table 4.4 Donor Balance for Phase II Enrichment Bottle E-2C (Days 311-356).

		Initial			Final			COD Balance (mg/bottle)[c]
		mM	mmol/bottle[a]	mg COD/bottle	mM	mmol/bottle[a]	mg COD/bottle	
Donor	Lactate	10.7	16.8	1,610	0.670	1.05	101	+ 1,510
Products	Acetate	11.8	18.5	1,180	14.4	22.6	1,450	- 264
	Propionate	11.0	16.7	1,870	16.1	25.2	2,820	- 948
	SO_4^{2-}	0.260	0.410	26.1	0.0	0.0	0.0	- 26.1
	CH_4	-[b]	0.238	15.3	-[b]	0.404	25.9	- 10.6
	H_2	-[b]	0.0	0.0	-[b]	0.0	0.0	- 0.0
	Ethene from PCE	-[b]	0.0	0.0	-[b]	0.0971	6.21	- 6.21
	Ethene form TCE	-[b]	0.0	0.0	-[b]	0.554	26.6	- 26.6

[a] Volume of liquid in E-2A = 1.564 L.
[b] GC results in terms of mmol/bottle.
[c] Initial COD – Final COD, except for SO_4^{2-}, which was calculated as final COD – Initial COD, since it was consumed rather than produced.

sulfate was calculated by assuming that the sulfate was completely reduced to sulfide (the decanted liquid always had a sulfide odor). H_2 and CH_4 were measured by headspace analysis at the beginning of the cycle (day 311, just after adding PCE and TCE) and at the end of the cycle (day 356, when all of the PCE and TCE had been dechlorinated).

Table 4.5 summarizes the average COD recovery and percent of the total COD used in PCE and TCE reduction, based on the data in Tables 4.2-4.4. The average COD recovery was 82.2%, which is reasonable given the uncertainties in measuring all the components in a COD balance. On average 2.3% of the lactate COD added was used for reductive dechlorination of PCE and TCE. This electron donor balance demonstrated that most of the lactate added to the Phase II enrichment culture ended up as acetate and propionate, in almost equal molar concentrations, and relatively little of the COD added was actually used for reduction of the chlorinated ethenes.

Table 4.5 Electron Donor Balance for Phase II Enrichment Bottles (Days 311-356).

Bottle	% COD Recovery[a]	% COD to PCE and TCE Reduction[b]
E-2A	86.9%	2.4%
E-2B	74.6%	2.3%
E-2C	85.0%	2.2%
Average	82.2%	2.3%

[a](COD of products)/(COD of Donor) x 100
[b](COD of ethene from TCE and PCE)/(COD of Donor) x 100

The concentration of *Dehalococcoides* spp. was measured at three time points in order to monitor their growth in the Phase II enrichment culture, during the period when the amount of PCE and TCE added was being increased. Cell densities are shown in Table 4.6 (C.E. Bagwell, personal communication). These numbers were used to evaluate the amount of culture needed to bioaugment the P-Area microcosms (section 3.5). The cell densities were converted to the number of 16S rRNA gene copies per bottle based on

the volume of culture in the bottles (approximately 1.6 L). These values were plotted against the cumulative amount of PCE plus TCE consumed per bottle for the

Table 4.6 *Dehalococcoides* cell concentrations in the Phase II enrichment culture.

Date	Day	DHC (copies/mL)	DHC (copies/bottle)	Cumulative PCE + TCE consumed (μmol/bottle)	Cumulative Cl^- released (μmol/bottle)
8/16/2005	87	$1.6x10^{6}$ [a]	$2.60x10^{9}$	250	927
10/5/2005	137	$2.0x10^{6}$ [b]	$3.25x10^{9}$	1,183	4,053
12/3/2005	196	$4.6x10^{6}$ [a]	$7.48x10^{9}$	3,670	11,748

[a] Data for E-2A only.
[b] Average for E-2A, E-2B and E-2C.

Table 4.7. *Dehalococcoides* cell concentrations in the Phase II enrichment at the time of bioaugmenting the P-Area microcosms.

Culture	Replicate 1 (cells/mL)	Replicate 2 (cells/mL)	Replicate 3 (cells/mL)	Average (cell/mL)	95% Confidence Interval
E-2A	$3.60x10^{8}$	$4.10x10^{8}$	$4.10x10^{8}$	$3.93x10^{8}$	$3.27x10^{7}$
E-2B	$4.60x10^{8}$	$4.30x10^{8}$	$4.40x10^{8}$	$4.43x10^{8}$	$1.73x10^{7}$
E-2C	$5.20x10^{8}$	$3.60x10^{8}$	$4.50x10^{8}$	$4.43x10^{8}$	$9.08x10^{7}$
KB-1	$1.60x10^{8}$	$1.50x10^{8}$	$1.70x10^{8}$	$1.60x10^{8}$	$1.13x10^{7}$
100X Concentration	$1.90x10^{10}$	$1.80x10^{8}$	NR	$1.85x10^{10}$	$9.80x10^{8}$

Table 4.8. Comparison of initial *Dehalococcoides* cell densities for each treatment.

Dose	Cell Density in the Culture or Concentrate Used (cells/mL)	Volume of Culture Added (mL)	Initial Amount of Cells per Microcosm	Initial Density in the Microcosms (cells/mL)
1% SRS	$1.85x10^{10}$	0.5[a]	$9.25x10^{9}$	$1.85x10^{8}$
0.1% SRS	$1.85x10^{10}$	0.05[a]	$9.25x10^{8}$	$1.85x10^{7}$
0.01% SRS	$4.43x10^{8}$	0.5[b]	$2.22x10^{8}$	$4.44x10^{6}$
0.001% SRS	$4.43x10^{8}$	0.05[b]	$2.22x10^{7}$	$4.44x10^{5}$
1% KB-1	$1.60x10^{8}$	0.5[b]	$8.00x10^{7}$	$1.60x10^{6}$

[a] 100X concentrate of the SRS Phase II enrichment culture.
[b] Unconcentrated culture.

corresponding days (Figure 4.6a) and against the cumulative amount of chloride released (calculated based on the PCE and TCE consumed; Figure 4.6b). The strong correlation between the increase in *Dehalococcoides* 16S rRNA gene copies and PCE + TCE consumed, as well as chloride released provides evidence that PCE and TCE dechlorination is a growth-linked process in the enrichment culture. A yield of 4.7E5 copies of *Dehalococcoides* 16 S rRNA gene per µmol Cl^- is apparent from the slope of the best fit line in Figure 4.6b.

Dehalococcoides concentrations in the Phase II enrichment used for bioaugmentation are provided in Table 4.7. Note that these densities are two orders of magnitude higher than the values shown in Table 4.6 for day 196. This is most likely due to a change in the qPCR method used (C.E. Bagwell, personal communication), as will be discussed further in section 5. The inoculum was made up of equal volumes from bottles E-2B and E-2C; at the time E-2A was not dechlorinating as fast and therefore was not included. Table 4.8 provides a comparison of the initial *Dehalococcoides* concentrations for each of the treatments. The microcosms that received the 0.01% (v/v) dose of the SRS culture provide the closest comparison, based on initial number of *Dehalococcoides* cells, to the bottles that received the 1% (v/v) dose of the commercially available bioaugmentation culture.

4.3 P-Area Groundwater and Sediment Characterization

A sample of soil from site PGCPT94 was analyzed by the Clemson University Agriculture Service Laboratory and the results are shown in Table 4.9. The soil is notably low in organic carbon, meaning that it is not likely to serve as a source of electron donor for reductive dechlorination. The soil is also low in most other nutrients,

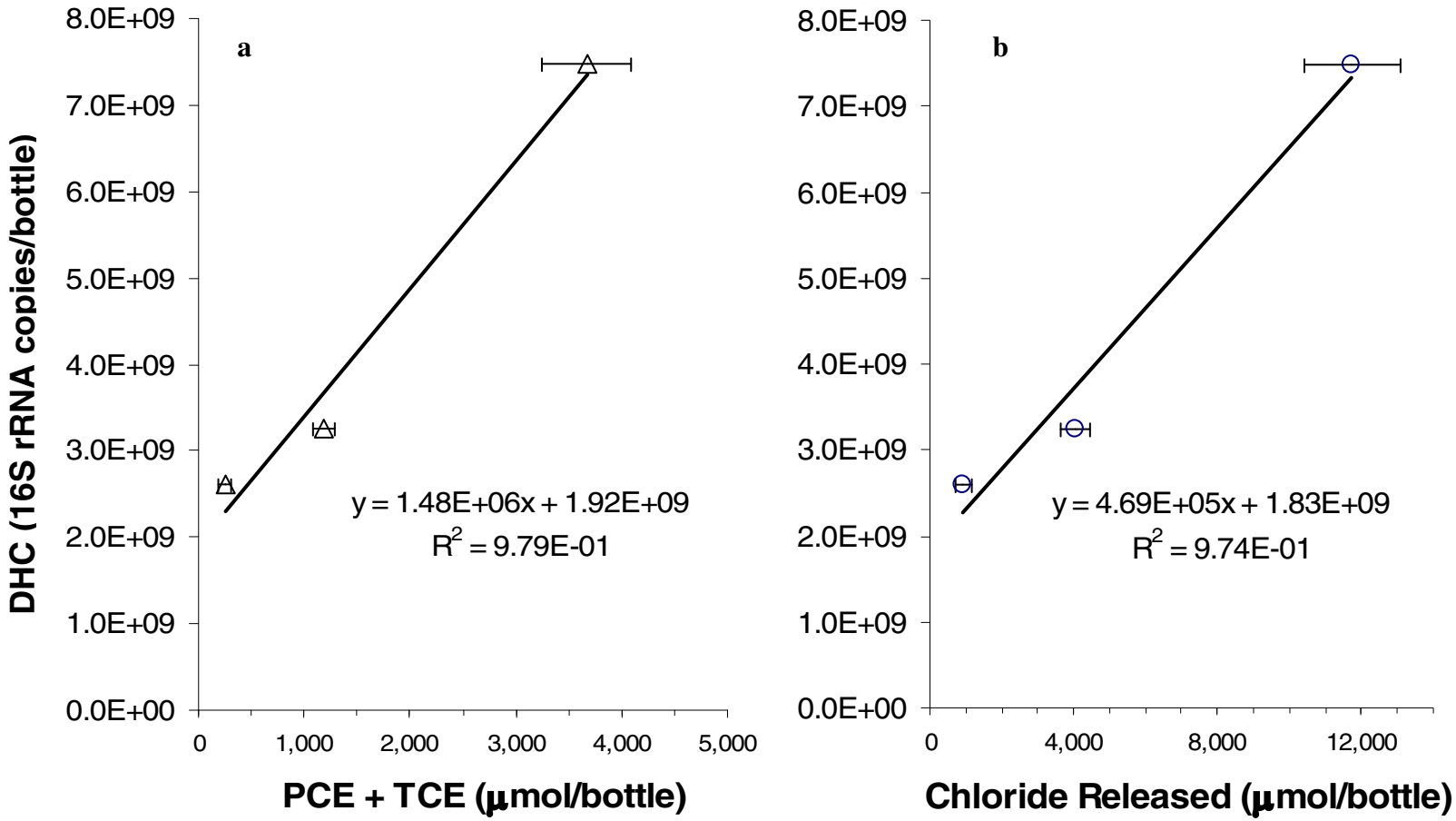

Figure 4.6 Correlation of *Dehalococcoides* gene copies to cumulative PCE + TCE consumed (**a**) and cumulative chloride released (**b**). Error bars represent one standard deviation of triplicate measurements.

Table 4.9 P-Area soil characteristics.

Parameter	Units	Amount
Moisture[a]	%	18.9 ± 0.94
Dry Matter	%	81.10
Organic Matter	%	0.023
Soil pH	-	4.7
Buffer pH	-	7.85
Carbon	%	0.024
Nitrogen	%	0.012
C/N Ratio	-	2.00
Nitrate-N	ppm	2.00
Phosphorous[b]	ppm	1
Potassium[b]	ppm	2
Calcium[b]	ppm	25
Magnesium[b]	ppm	8.5
Sodium[b]	ppm	5.5
Sulfur	ppm	32.51
Boron[b]	ppm	0.05
Zinc[b]	ppm	0.1
Manganese[b]	ppm	0
Copper[b]	ppm	0.1
Iron	ppm	2.92
Aluminum	ppm	50.12

[a] Average based on triplicate sediment samples ± one standard deviation.
[b] Results were provided in lb/acre and converted to ppm by dividing by 2.

causing concern for the ability of the soil to provide the necessary nutrients for cell growth, including the bioaugmentation culture.

Groundwater samples were analyzed for nitrate, sulfate, TOC, and soluble COD. Duplicate samples were used for nitrate, sulfate and TOC. Nitrate concentrations ranged from 10.1-10.2 mg/L; sulfate concentrations ranged from 20.3-20.4 mg/L; and TOC ranged from 0.57-0.61 mg/L. Soluble COD was below the detection limit of 20 mg COD/L (based on the procedure used), which is consistent with the low TOC concentration of the groundwater.

Nitrate inhibits reductive dechlorination and it must be removed before dechlorination can begin. Sulfate in the groundwater can act as a competing electron acceptor for electron donor. Consequently, the electron donor added to the P-area microcosms took into account the amount needed for stiochiometric removal of the nitrate and sulfate (ignoring cell synthesis). The soluble COD in the groundwater was insignificant compared to the electron donor needed for reduction of the nitrate, sulfate, PCE and TCE. Therefore, the background amount of COD in the groundwater was not taken into account when calculating the amount of donor added to the microcosms.

4.4 Biostimulation Results

The biostimulation treatments consisted of triplicate microcosms with no electron donor added (also referred to as the "as-is" treatment), lactate added, corn syrup added and emulsified vegetable oil added. Average results for each treatment are given in Figures 4.7-4.10. The average initial amounts of PCE and TCE present were 1.4 and 22.7 μmol per bottle, respectively, which is equivalent to aqueous phase concentrations of 2.0 and 34.4 mg/L, based on equation 1. No daughter products were detected during the 356 days of incubation. When compared to the control microcosms (Figures 4.11-4.14), there were no significant losses of PCE or TCE in any of the biostimulation microcosms.

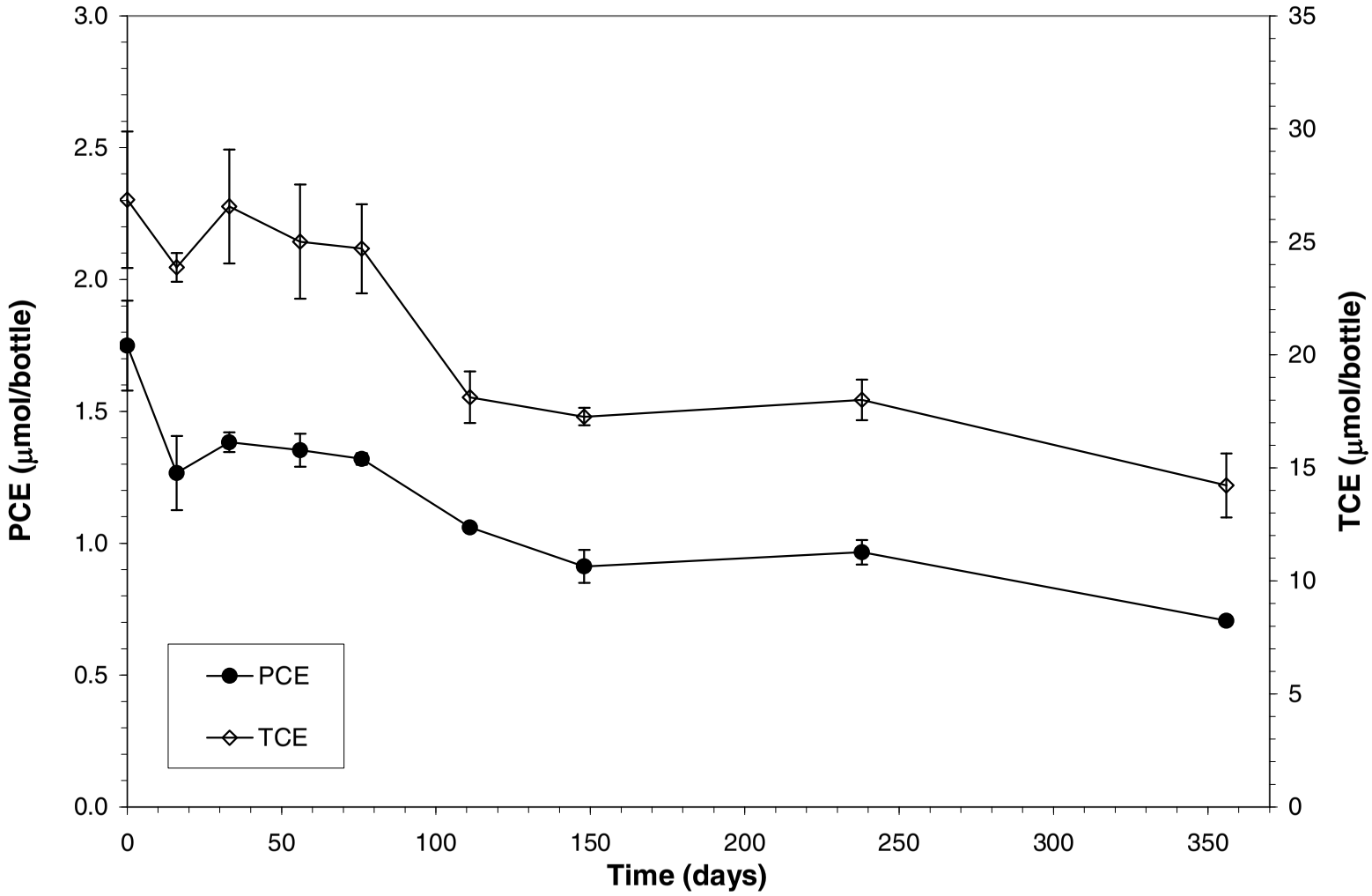

Figure 4.7 As-is microcosms, results averaged from three replicates. Error bars represent ± one standard deviation.

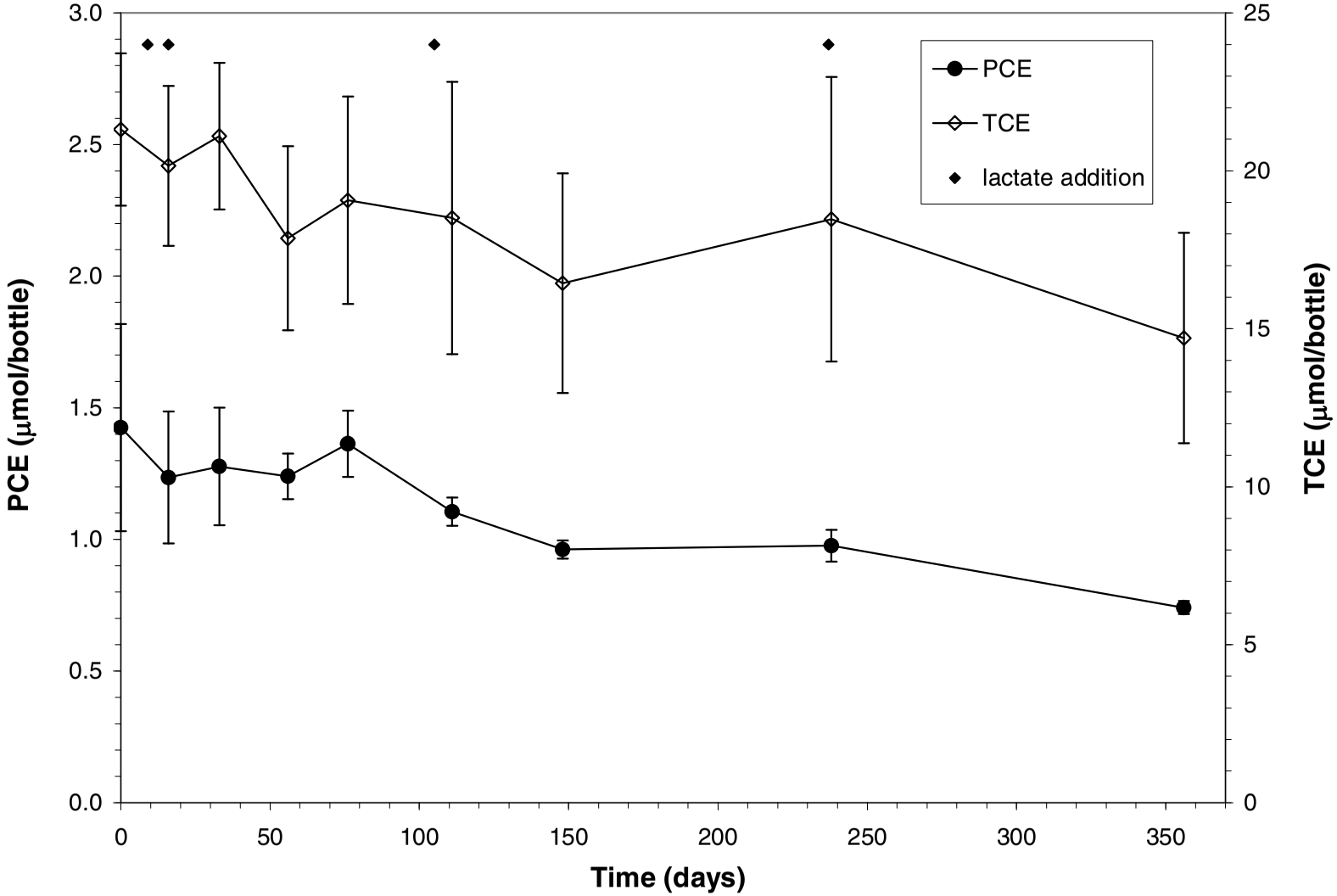

Figure 4.8 Results for lactate-amended microcosms (average of triplicates). Error bars represent ± one standard deviation. Solid diamonds indicate when lactate was added (32.8, 81.9, 95.7 and 87.1 mg/bottle, respectively).

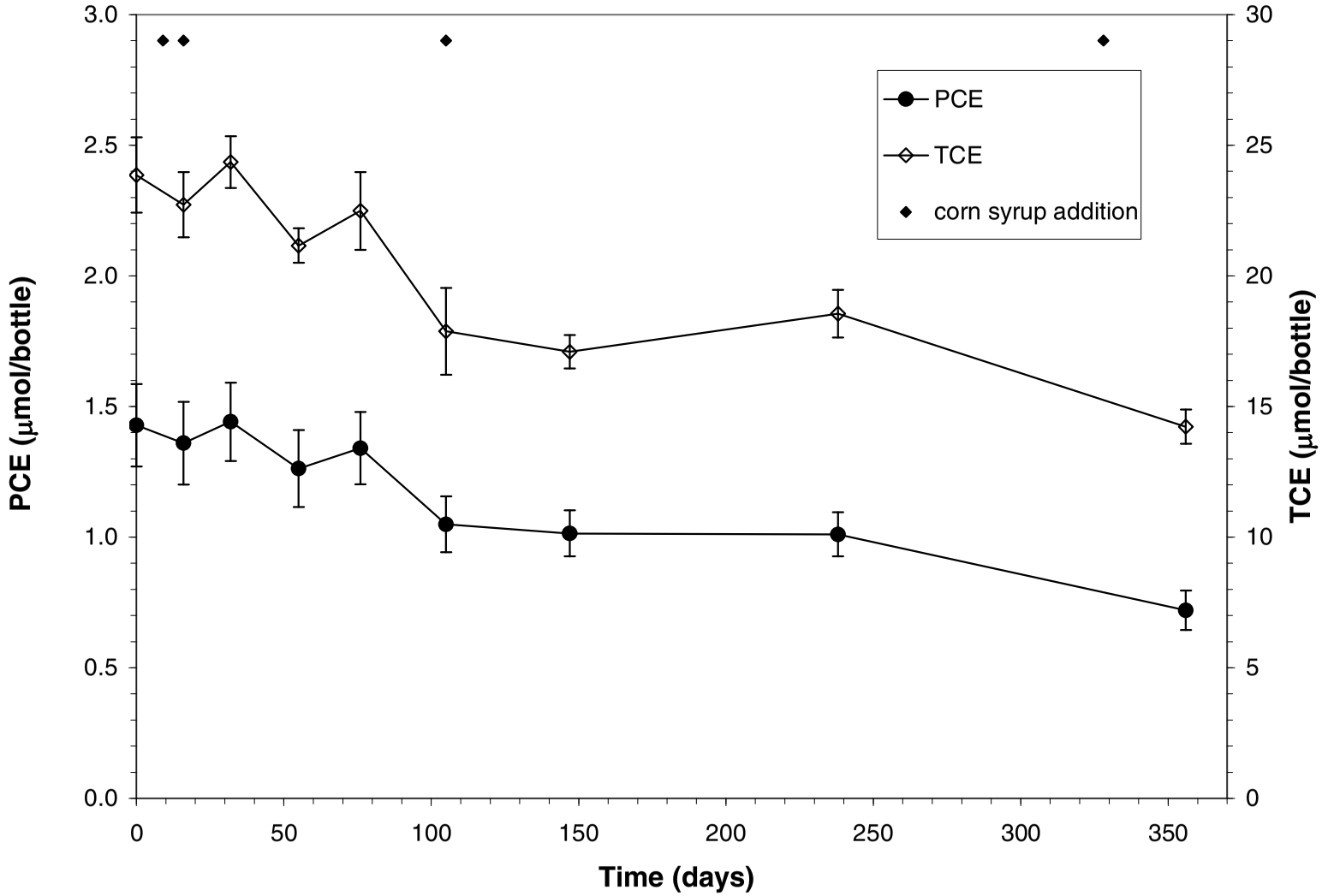

Figure 4.9 Results for corn syrup-amended microcosms (average of triplicates). Error bars represent ± one standard deviation. Solid diamonds indicate when corn syrup was added (19.4, 55.4, 43.8 and 27.1 mg/bottle, respectively).

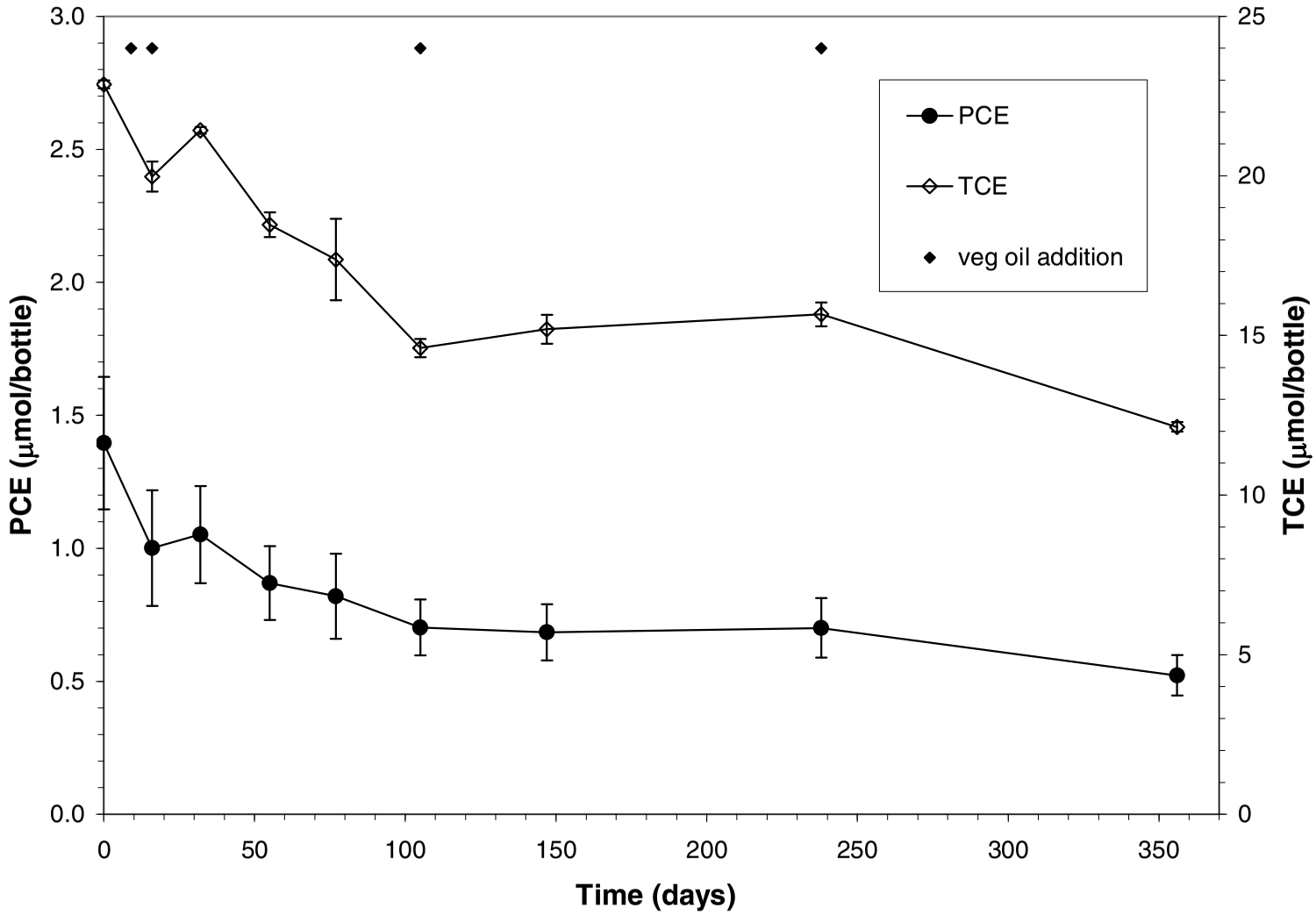

Figure 4.10 Results for emulsified vegetable oil-amended microcosms (average of triplicates). Error bars represent ± one standard deviation. Solid diamonds indicate when vegetable oil was added (12 µL for the initial addition, 36 µL thereafter).

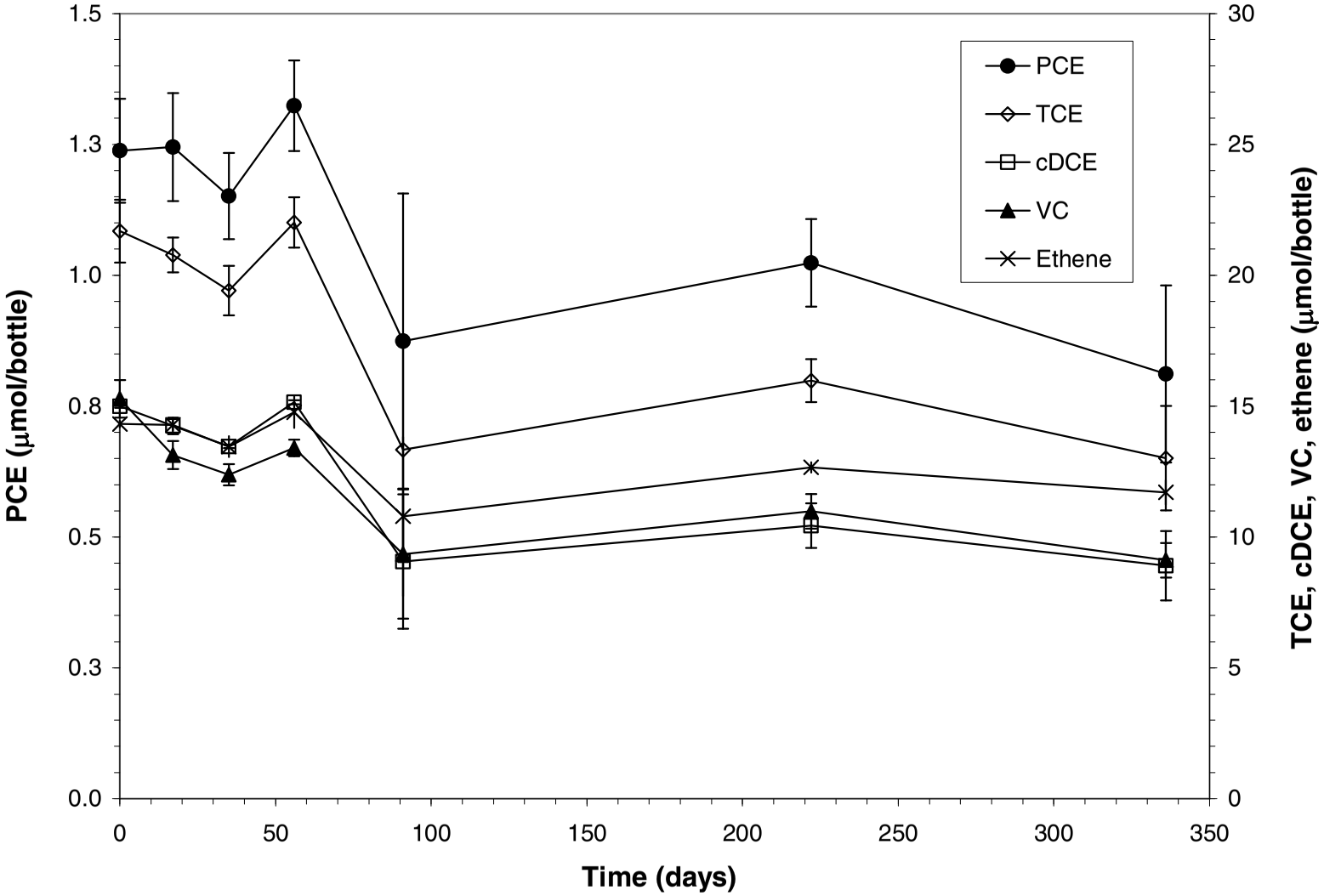

Figure 4.11 Results for autoclaved control microcosms for chlorinated ethenes and ethene (average of triplicates). Error bars represent ± one standard deviation.

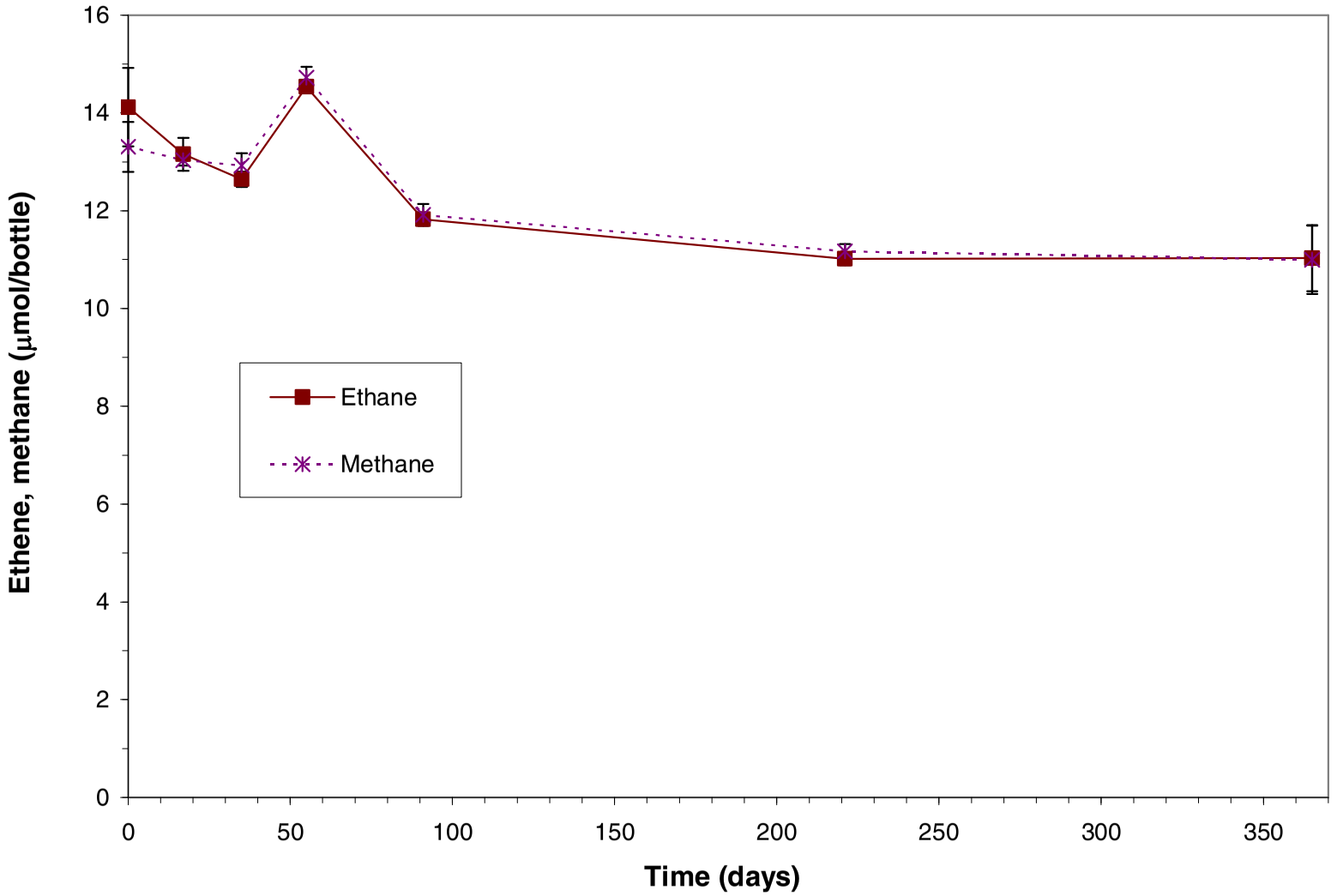

Figure 4.12 Results for autoclaved control microcosms for ethane and methane (average of triplicates). Error bars represent ± one standard deviation.

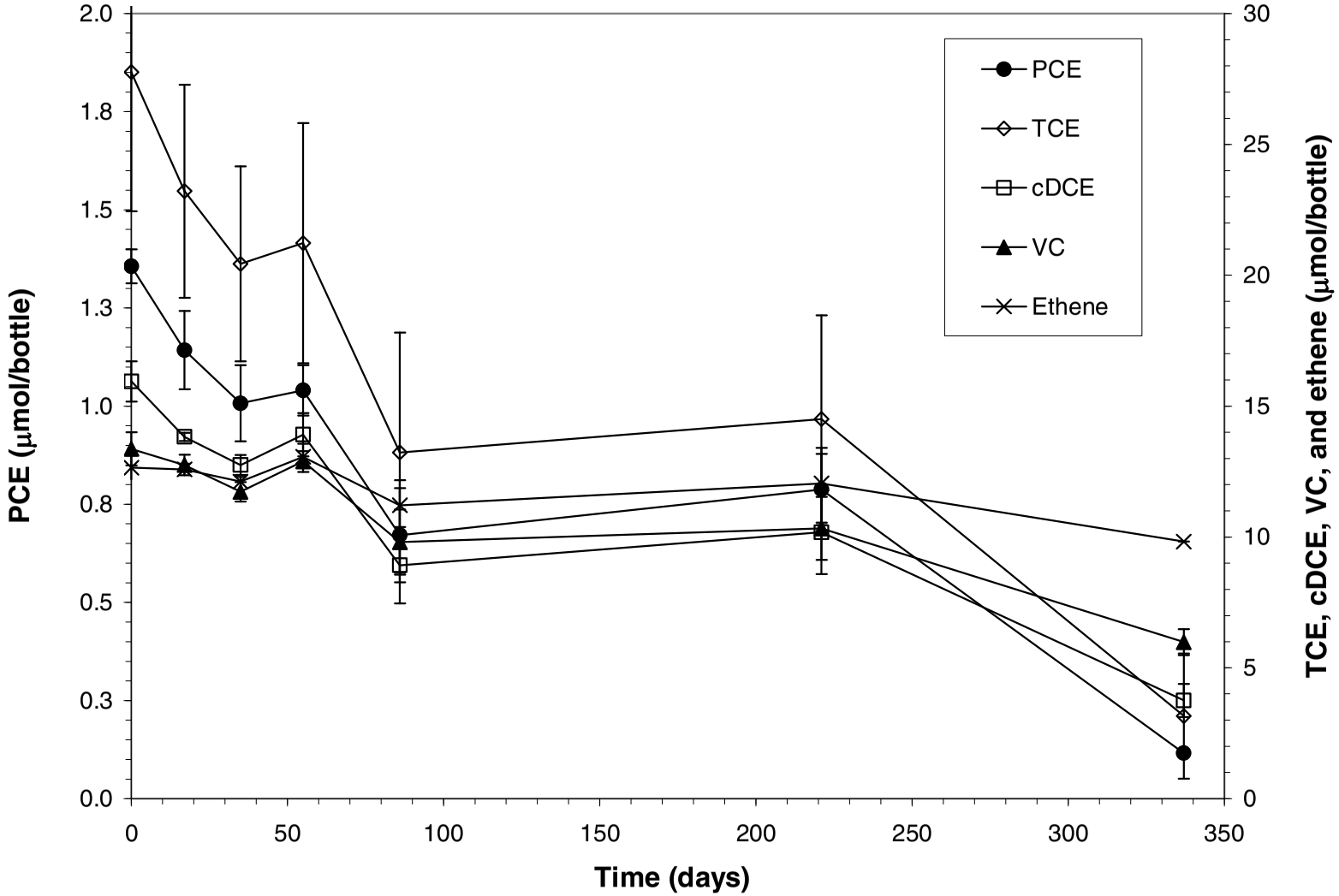

Figure 4.13 Resulst for water control microcosms for chlorinated ethenes and ethene (average of triplicates). Error bars represent ± one standard deviation.

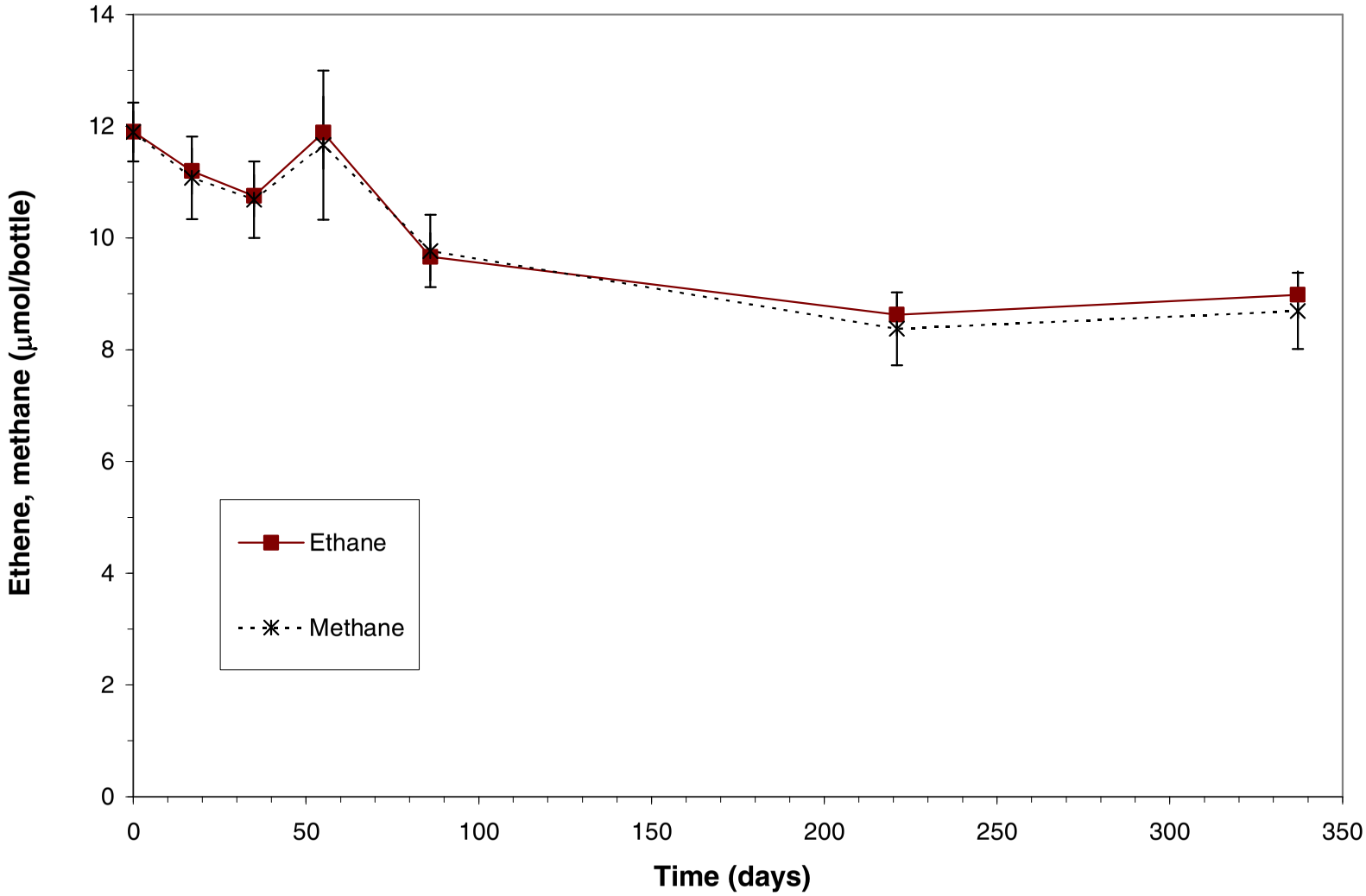

Figure 4.14 Results for water control microcosms for methane and ethane (average of triplicates). Error bars represent ± one standard deviation.

The resazurin that was added to the groundwater turned from pink to clear within 24-40 days for the "as-is" bottles, 16-19 days for the lactate amended bottles, 6-16 days for the corn syrup amended bottles and 24-55 days for the emulsified vegetable oil bottles. The colorless condition indicated that the E_h was below -100 mV and was therefore amenable to reductive dechlorination. pH was checked bimonthly and adjusted if it dropped below 6.7. The pH for the lactate and emulsified vegetable oil amended microcosms stayed within the acceptable range of 6.7-7.3 during the 240 days of incubation. On day 89 and again on day 102 the pH in the corn syrup amended bottles dropped below 6.7 but not below 5.7. Both times the pH was brought up to 7.0 with the addition of sodium hydroxide (8 M). The redox conditions, pH and amount of donor added were sufficient for reductive dechlorination to occur in the biostimulation bottles, yet it did not during 240 days of incubation. This suggests the organisms needed to reduce PCE and TCE to ethene are not present or were unable to grow in the PRBP soil and groundwater.

4.5 Bioaugmentation Results

The microcosms were bioaugmented on day 101. The incubation period prior to bioaugmentation provided time for the redox conditions to become sufficiently low (i.e., < -100 mV) and allowed enough time to build up the population of *Dehalococcoides* in the Phase II enrichment culture. The microcosms were monitored as long as 250 days after bioaugmentation or until complete reduction of the PCE and TCE occurred. pH was checked periodically and stayed between 6.7 and 7.0 for all the bioaugmentation microcosms, which received lactate as an electron donor.

4.5.1 1% Bioaugmentation Dose with Phase II Enrichment Culture

Results for the microcosms that received a 1% dose of the concentrated SRS enrichment culture are presented in Figures 4.15-4.17. PCE and TCE were both reduced to cDCE and VC 12 days after bioaugmentation. PCE decreased at the same time as TCE. Complete reduction of all chlorinated ethenes to ethene occurred 32 days after bioaugmentation. For the period of day 101 to day 125, the average molar recovery of PCE and TCE as ethene was 90% (± 3.4%), with 98.5% (± 0.4%) of the daughter products recovered as ethene. Methane production was very low during this interval. However, after day 125, methane production increased significantly. Overall, methane accounted for an average of 2.9% (± 1.4%) of the COD added to the bottles as lactate and the reduction of PCE and TCE to ethene accounted for 0.31% (± 0.001%).

The drop in ethene and methane on day 147 was due to changing the septa in the anaerobic chamber.

4.5.2 0.1% Bioaugmentation Dose with Phase II Enrichment Culture

Results for the microcosms that received a 0.1% dose of the SRS enrichment culture are presented in Figures 4.18 and 4.19. Results for the third bottle are not shown because a significant leak was detected on day 160 and it was not monitored thereafter. For the period of day 101 to day 356, the molar recovery of PCE and TCE as ethene average 88.9%, with 90-100% of the daughter products recovered as ethene. Unlike the 1% (v/v) microcosms, PCE did not begin to transform until the TCE was almost completely reduced. The rate of TCE reduction slowed after approximately day 120 and slowed even further after day 160. pH was checked and more donor was added, neither of which seemed to restore the initially higher rate of TCE dechlorination.

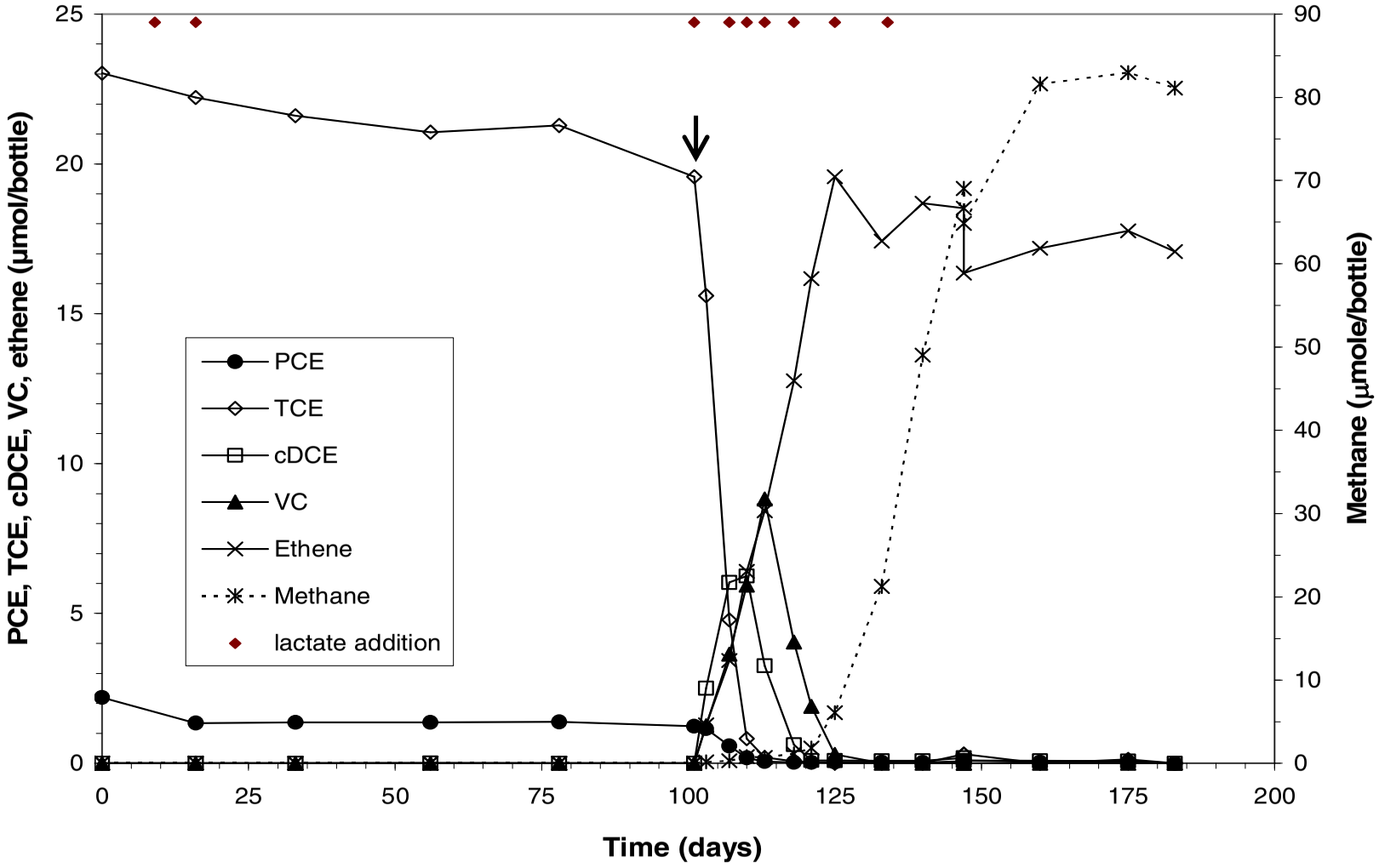

Figure 4.15 Results from a 1% (v/v) SRS culture bioaugmentation microcosm (CE-LA-BA_1%-1). Solid diamonds indicate when lactate was added (32.8 mg/bottle for the first addition, 95.7 mg for the second addition and 81.9 mg for the remaining additions).

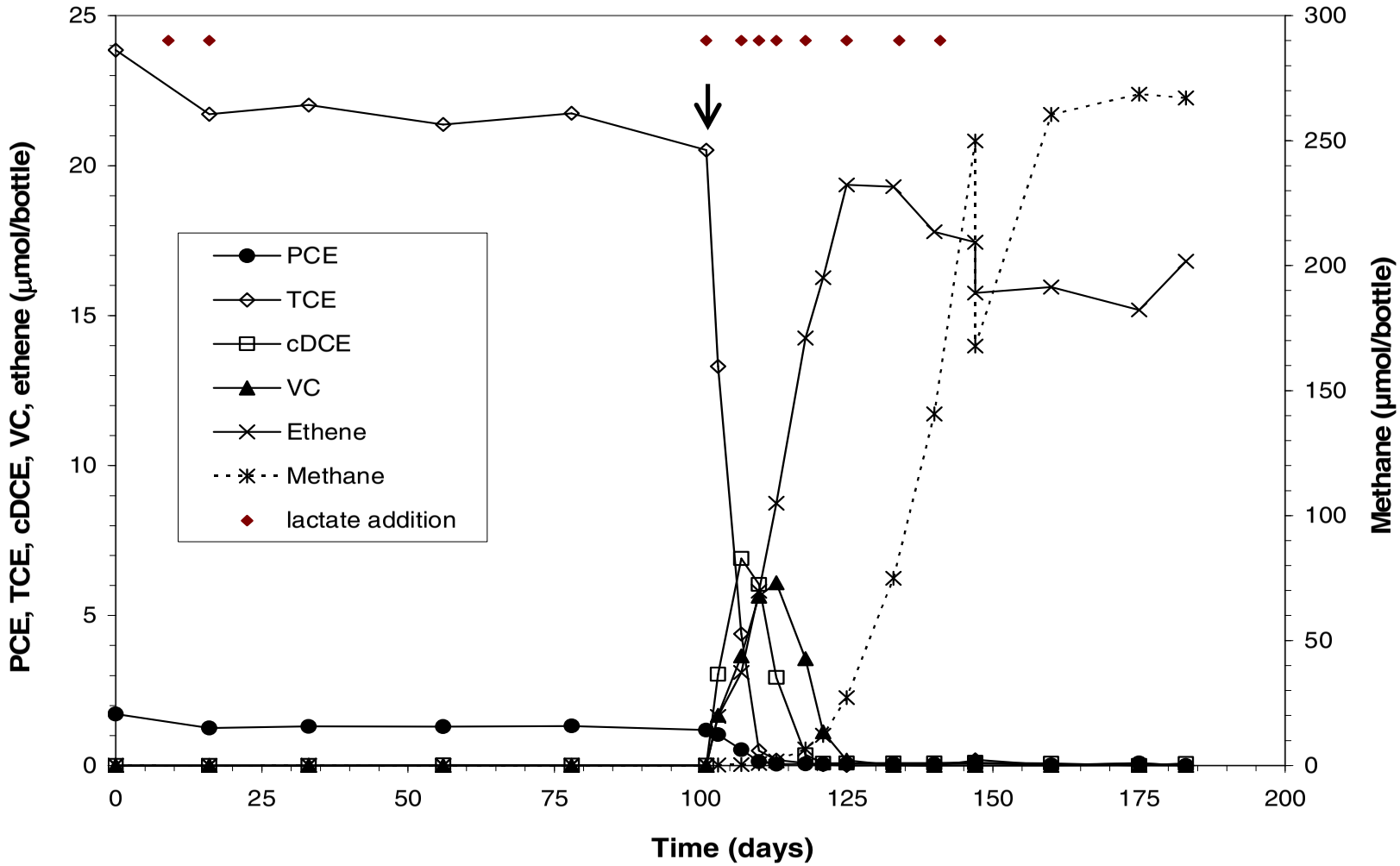

Figure 4.16 Results from a 1% (v/v) SRS culture bioaugmentation microcosm (CE-LA-BA_1%-2). Solid diamonds indicate when lactate was added (32.8 mg/bottle for the first addition, 95.7 mg for the second addition and 81.9 mg for the remaining additions). The arrow indicates when bioaugmentation occurred.

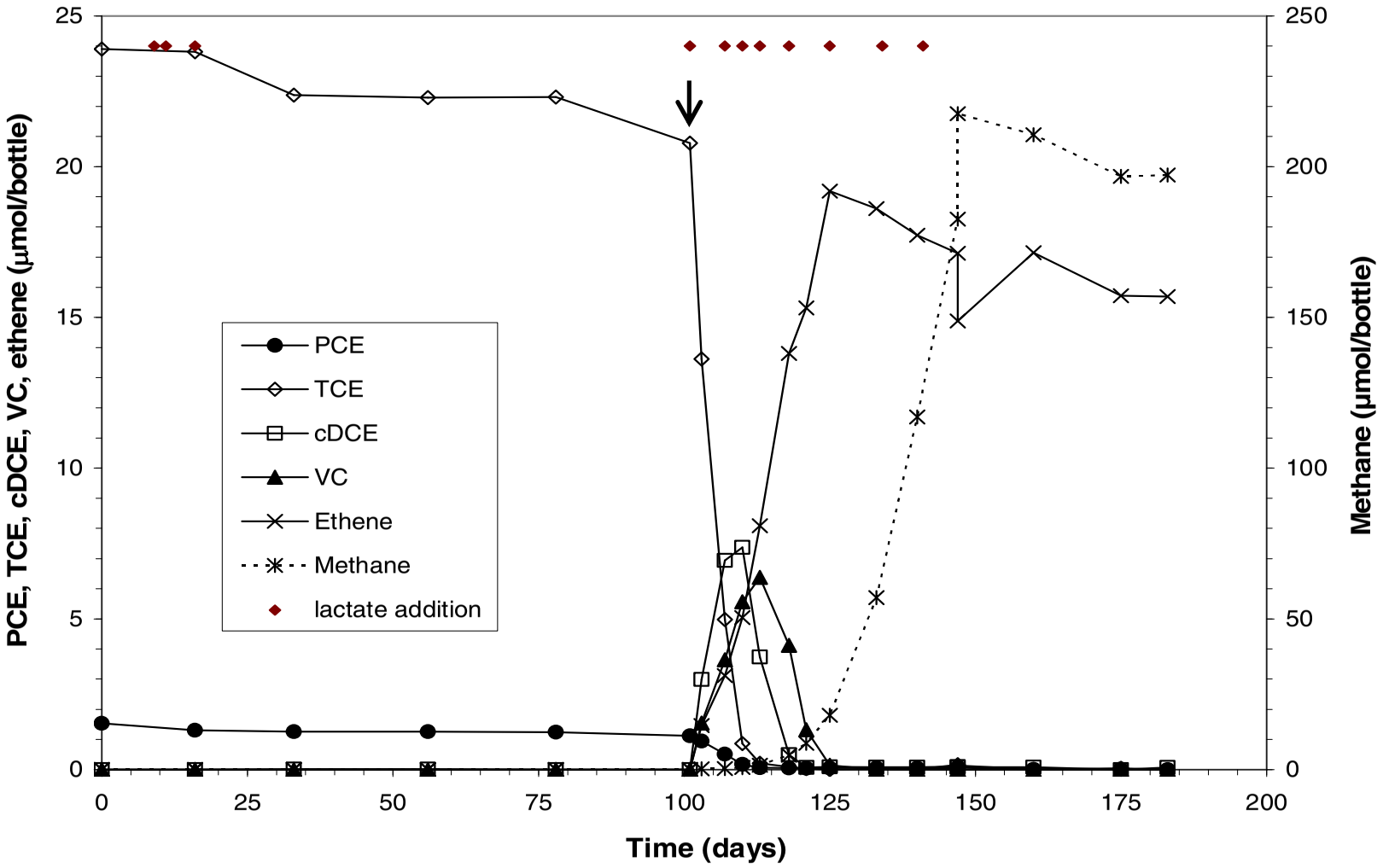

Figure 4.17 Results from a 1% (v/v) SRS culture bioaugmentation microcosm (CE-LA-BA_1%-3). Solid diamonds indicate when lactate was added (32.8 mg/bottle for the first addition, 95.7 mg for the second addition and 81.9 mg for the remaining additions). The arrow indicates when bioaugmentation occurred.

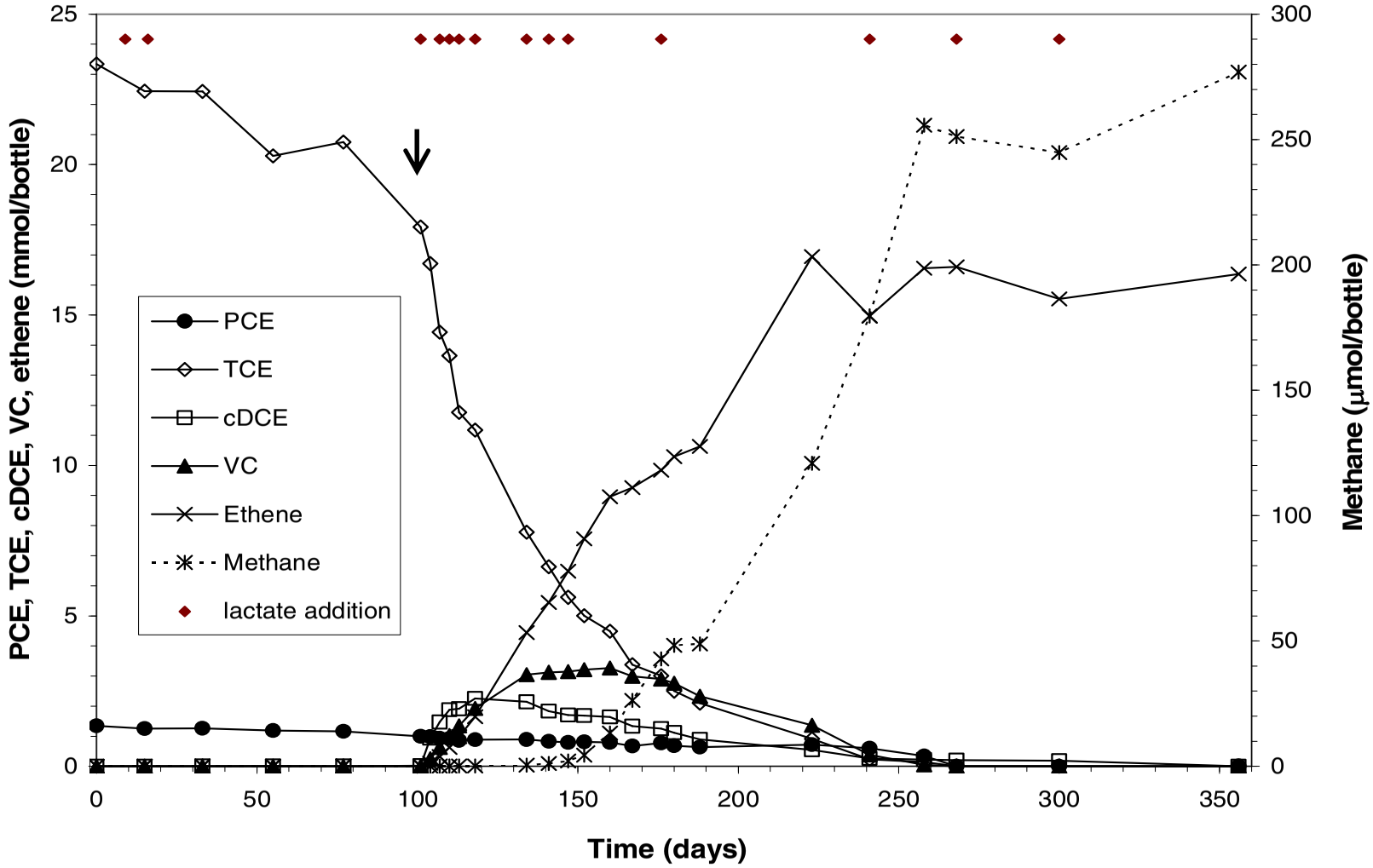

Figure 4.18 Results from a 0.1% (v/v) SRS culture bioaugmentation microcosm (CE-LA-BA_0.1%-1). Solid diamonds indicate when lactate was added (32.8 mg/bottle for the first addition, 95.7 mg for the second addition and 81.9 mg additions for subsequent additions except the last two, which were 80.0 mg). The arrow indicates when bioaugmentation occurred.

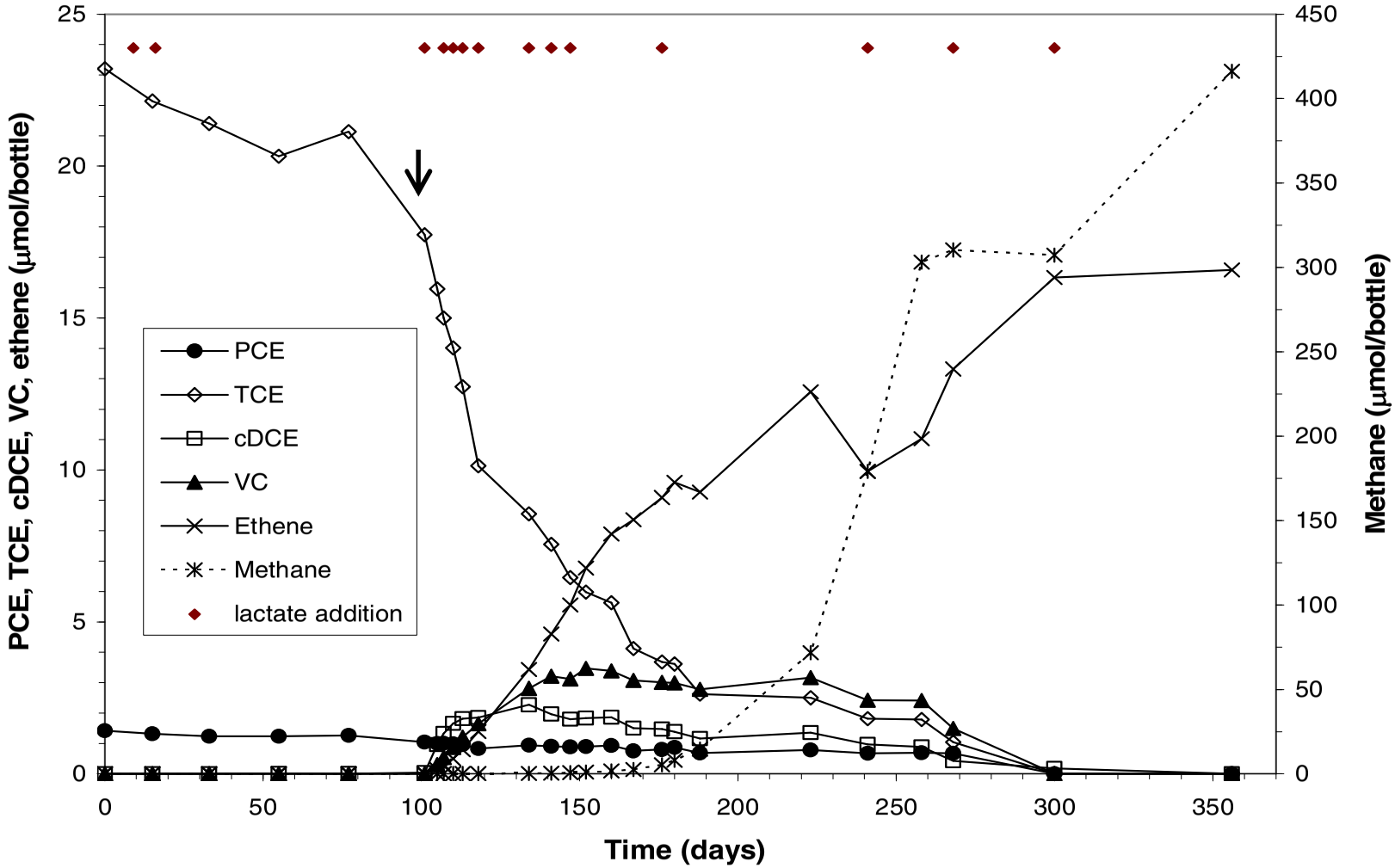

Figure 4.19 Results from a 0.1% (v/v) SRS culture bioaugmentation microcosm (CE-LA-BA_0.1%-3). Solid diamonds indicate when lactate was added (32.8 mg for the first addition, 95.7 mg for the second addition and 81.9 mg for the subsequent additions except the last two, which were 80.0 mg). The arrow indicates when bioaugmentation occurred.

Methane production was slow at first, but as the concentration of TCE and PCE dropped below 5 μmol per bottle the rate of methane production increased. This occurred during the time period when the rate of dechlorination was slowest. Methane output leveled off between days 250 and 300 and then increased again, most likely in response to new additions of lactate. Methane accounted for 3.1-4.7% of the COD added to the bottles as lactate and the reduction of PCE and TCE to ethene accounted for 0.21%.

4.5.3 0.01% Bioaugmentation Dose with Phase II Enrichment Culture

Results for the microcosms that received a 0.01% dose of the SRS enrichment culture are presented in Figures 4.20-4.22. Compared to the microcosms that received a 1% or 0.1% SRS enrichment culture dose, the microcosms receiving a 0.01% dose of the SRS enrichment culture exhibited slow dechlorination rates at first. It was not until the final one to three months of incubation that dechlorination increased to a rate comparable to that of the 1% (v/v) bottles. Dechlorination of PCE and TCE was complete by the final day of analysis (day 355). PCE dechlorination was negligible until all of the TCE was consumed, as often occurs in the enrichment culture (Figure 4.5).

cDCE and VC were present starting 12 days after bioaugmentation. These daughter products stayed at a relatively constant concentration until TCE was completely reduced but before reduction of PCE began. For the period of day 101 to day 356, the average molar recovery of PCE and TCE as ethene was 78.6% (± 0.6%), with 99.6% (± 0.8%) of the daughter products recovered as ethene. Methane production began between day 175 and 200 and rose significantly as the concentration of chlorinated ethenes continued to decrease. The total amount of methane produced ranged between 139 and 394 μmol/bottle. Methane accounted for an average of 3.1% (± 1.8%) of the COD added

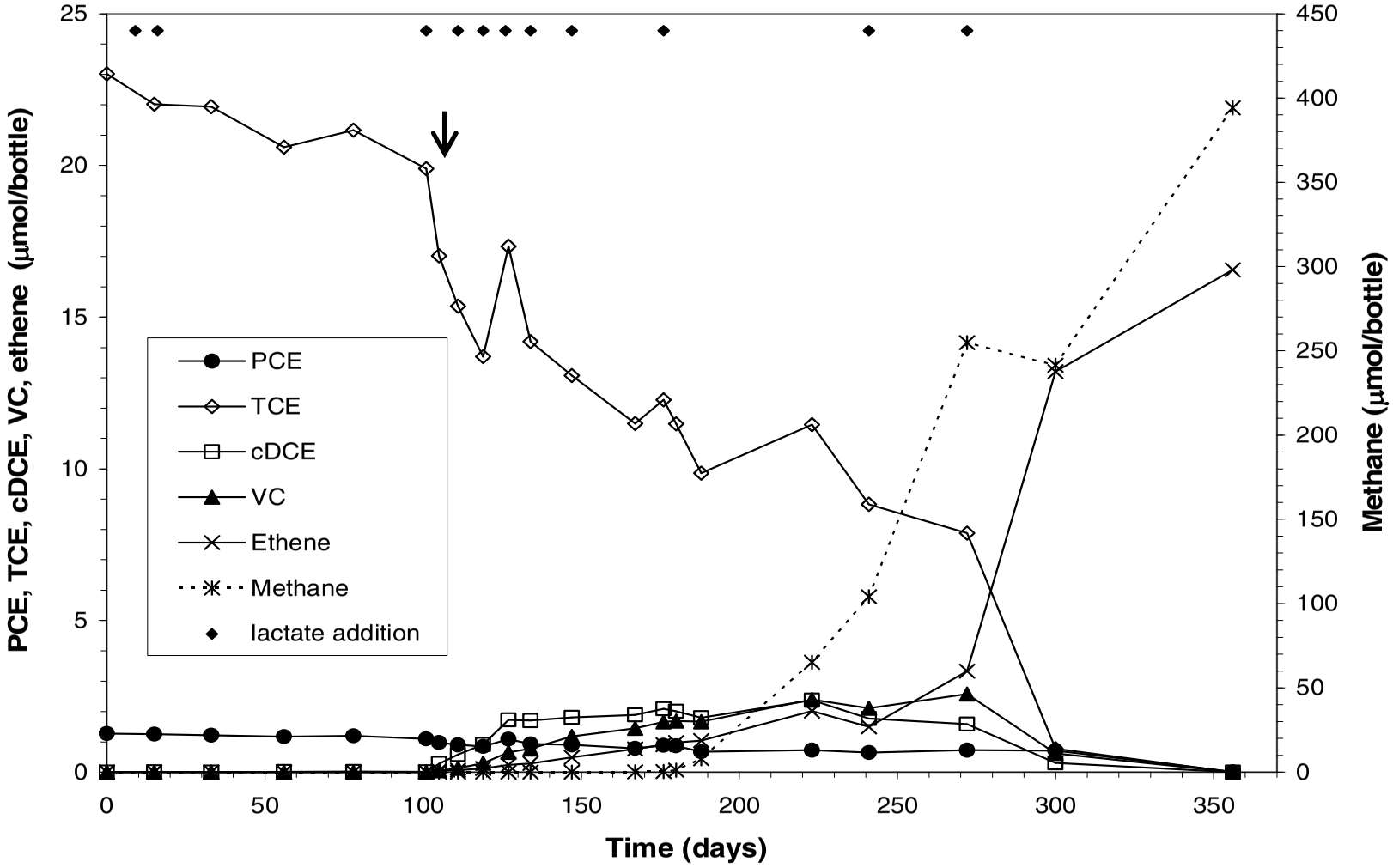

Figure 4.20 Results from a 0.01% (v/v) SRS culture bioaugmentation microcosm (CE-LA-BA_0.01%-1). Solid diamonds indicate when lactate was added (32.8 mg/bottle for the first addition, 95.7 mg for the second addition and 81.9 mg for the subsequent additions except the last two, which were 80.0 mg). The arrow indicates when bioaugmentation occurred.

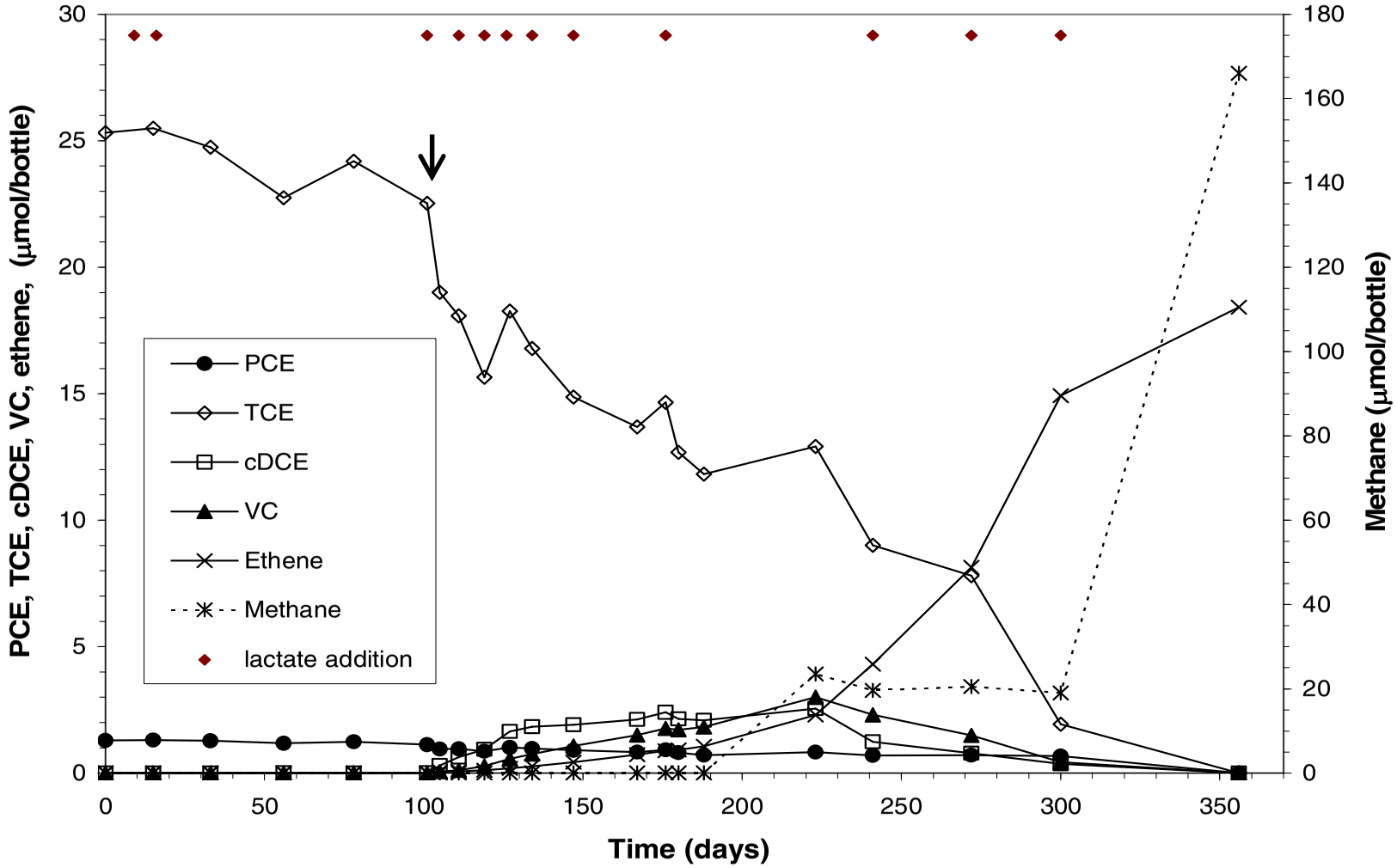

Figure 4.21 Results from a 0.01% (v/v) SRS culture bioaugmentation microcosm (CE-LA-BA_0.01%-2). Solid diamonds indicate when lactate was added (32.8 mg/bottle for the first addition, 95.7 mg for the second addition and 81.9 mg for the subsequent additions except the last two, which were 80.0 mg). The arrow indicates when bioaugmentation occurred.

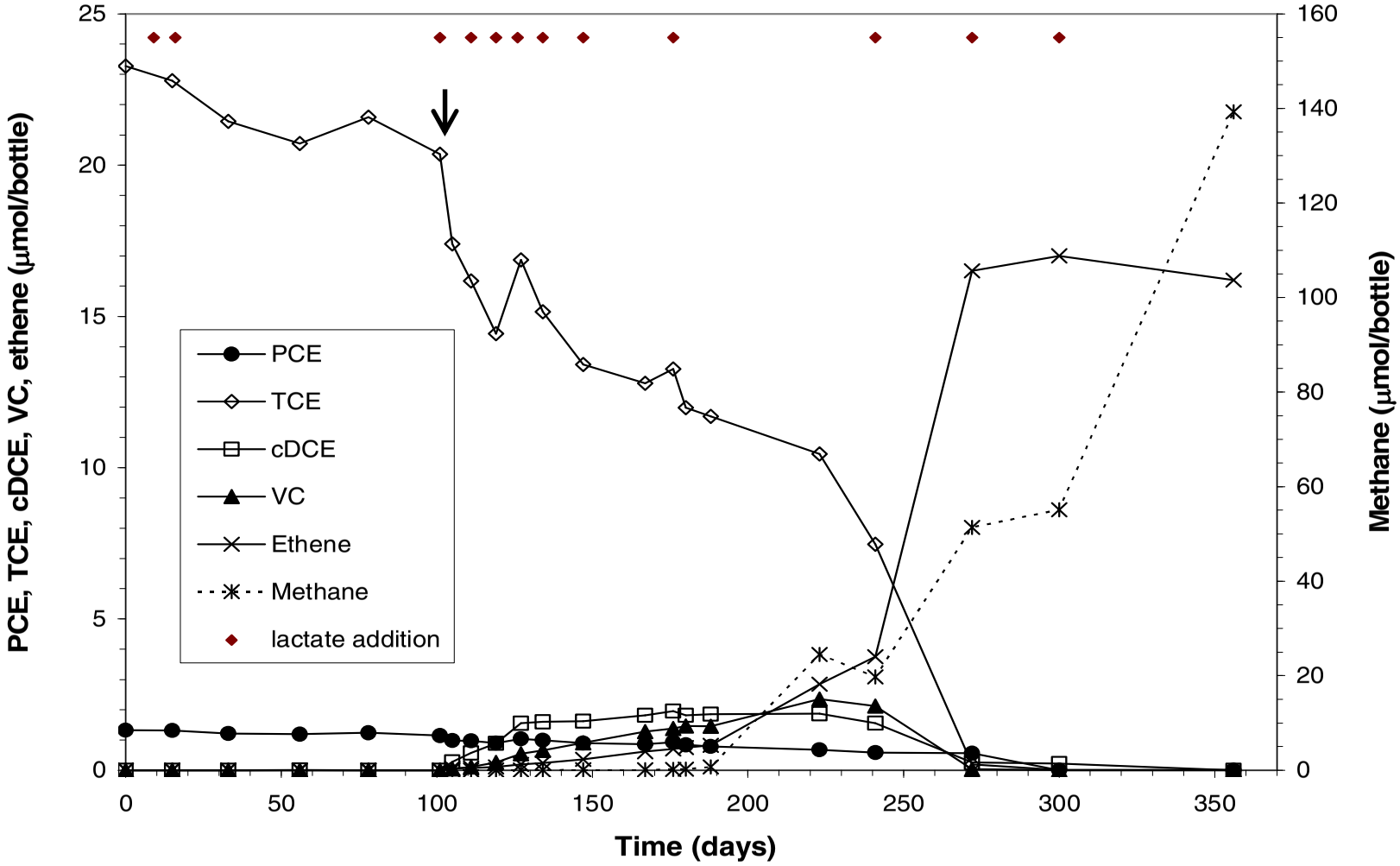

Figure 4.22 Results from a 0.01% (v/v) SRS culture bioaugmentation microcosm (CE-LA-BA_0.01%-3). Solid diamonds indicate when lactate was added (32.8 mg/bottle for the first addition, 95.7 mg for the second addition and 81.9 mg for the subsequent additions except the last two, which were 80.0 mg). The arrow indicates when bioaugmentation occurred.

to the bottles as lactate and the reduction of PCE and TCE to ethene accounted for 0.25% (± 0.01%).

4.5.4 0.001% Bioaugmentation Dose with Phase II Enrichment Culture

Results for the microcosms that received a 0.001% dose of the SRS enrichment culture are presented in Figures 4.23-4.25. The 0.001% (v/v) microcosms began dechlorination sooner and had a faster rate of dechlorination than the 0.01% (v/v) microcosms, which was unexpected based on the lower bioaugmentation dose. PCE degradation did not begin until after TCE was reduced, which is consistent with the behavior of the microcosms that received a 0.1% or 0.01% (v/v) dose of the SRS enrichment culture. For the period of day 101 to day 300, the average molar recovery of PCE and TCE as ethene was 79.8% (± 6.1%), with 99.1% (± 0.8%) of the daughter products recovered as ethene. Methane accounted for an average of 2.2% (± 1.3%) of the COD added to the bottles as lactate and the reduction of PCE and TCE to ethene only accounted for 0.30% (± 0.06%).

One of the replicates receiving a 0.001% dose of the SRS enrichment culture exhibited behavior significantly different from the other two bottles in the set (Figure 4.25), i.e., it showed activity much sooner than expected based on the bioaugmentation dose. There is no logical explanation for this behavior other than the possibility that an incorrectly large dose of the SRS enrichment culture was used.

4.5.5 1% Bioaugmentation Dose with Commercially Available KB-1 Culture

Results for the microcosms that received a 1% dose of the commercially available KB-1 culture are presented in Figures 4.26-4.28. A modest reduction in TCE occurred in the first 12 days after bioaugmentation on day 106. Reductive dechlorination ceased

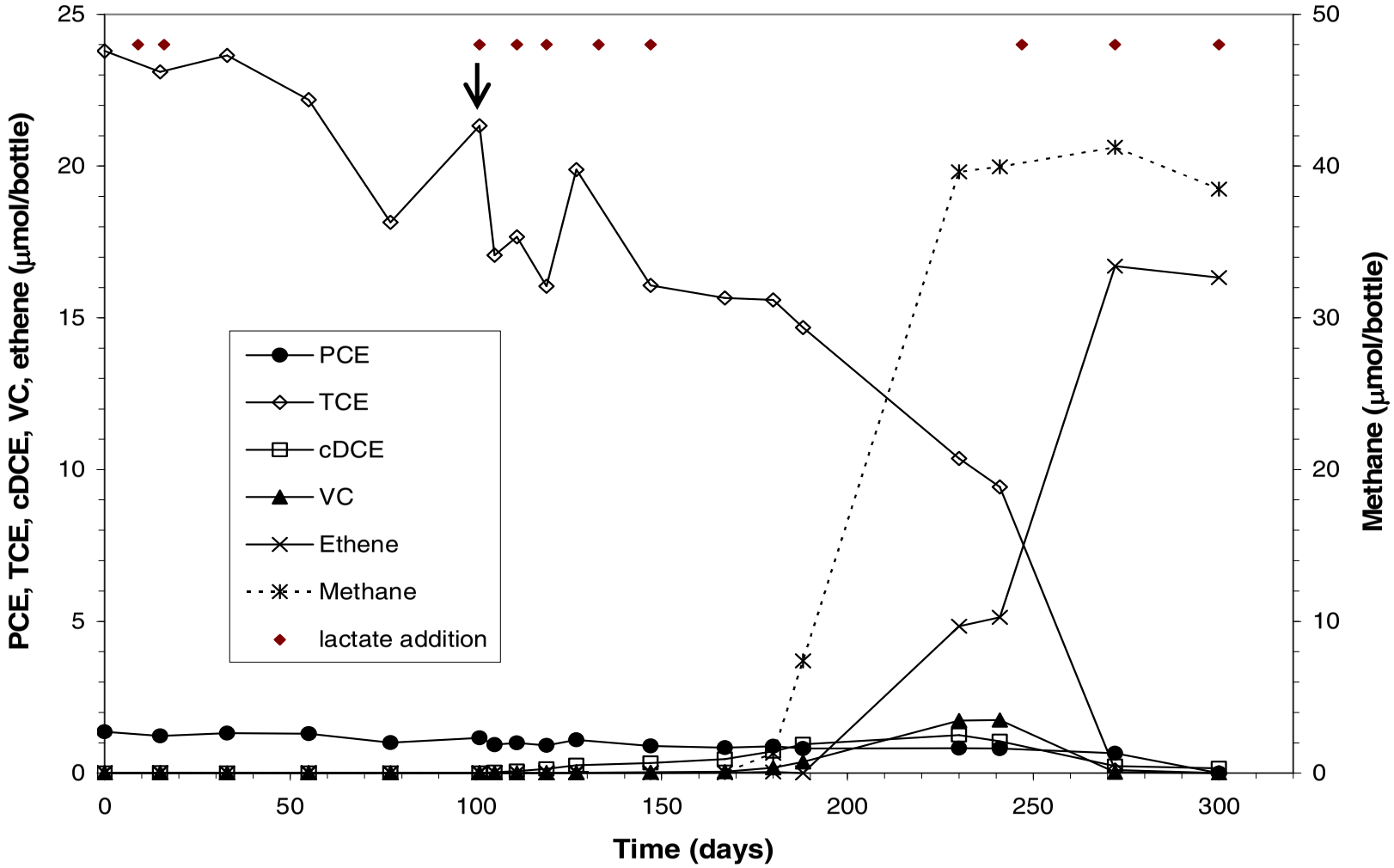

Figure 4.23 Results from a 0.001% (v/v) SRS culture bioaugmentation microcosm (CE-LA-BA_0.001%-1). Solid diamonds indicate when lactate was added (32.8 mg for the first addition, 95.7 mg for the second addition and 81.9 mg for the subsequent additions except for the last two, which were 80.0 mg). The arrow indicates when bioaugmentation occurred.

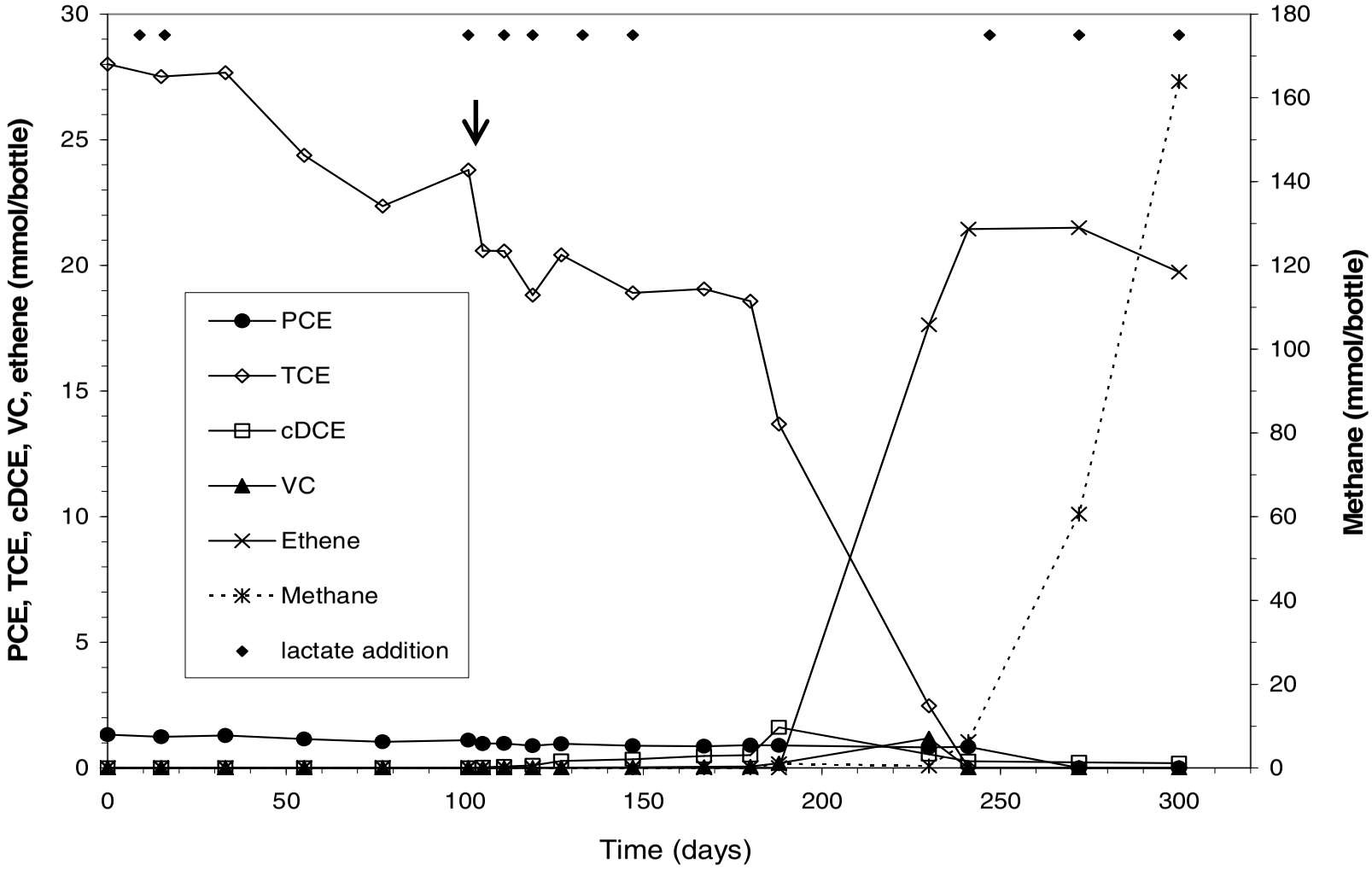

Figure 4.24 Results from a 0.001% (v/v) SRS culture bioaugmentation microcosm (CE-LA-BA_0.001%-2). Solid diamonds indicate when lactate was added (32.8 mg for the first addition, 95.7 mg for the second addition and 81.9 mg for the subsequent additions except for the last two, which were 80.0 mg). The arrow indicates when bioaugmentation occurred.

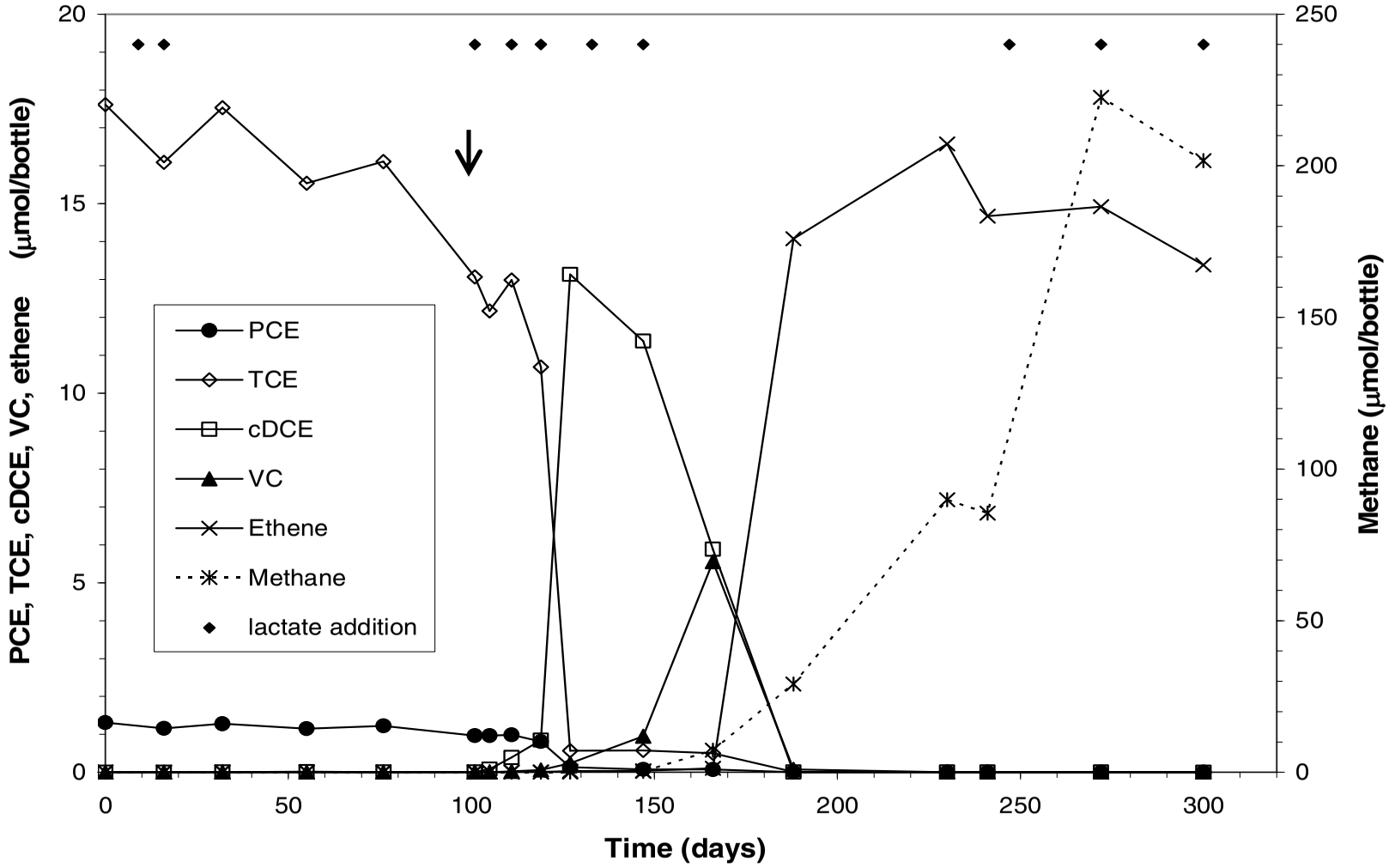

Figure 4.25 Results from a 0.001% (v/v) SRS culture bioaugmentation microcosm (CE-LA-BA_0.001%-3). Solid diamonds indicate when lactate was added (32.8 mg for the first addition, 95.7 mg for the second addition and 81.9 mg for the subsequent additions except for the last two, which were 80.0 mg). The arrow indicates when bioaugmentation occurred.

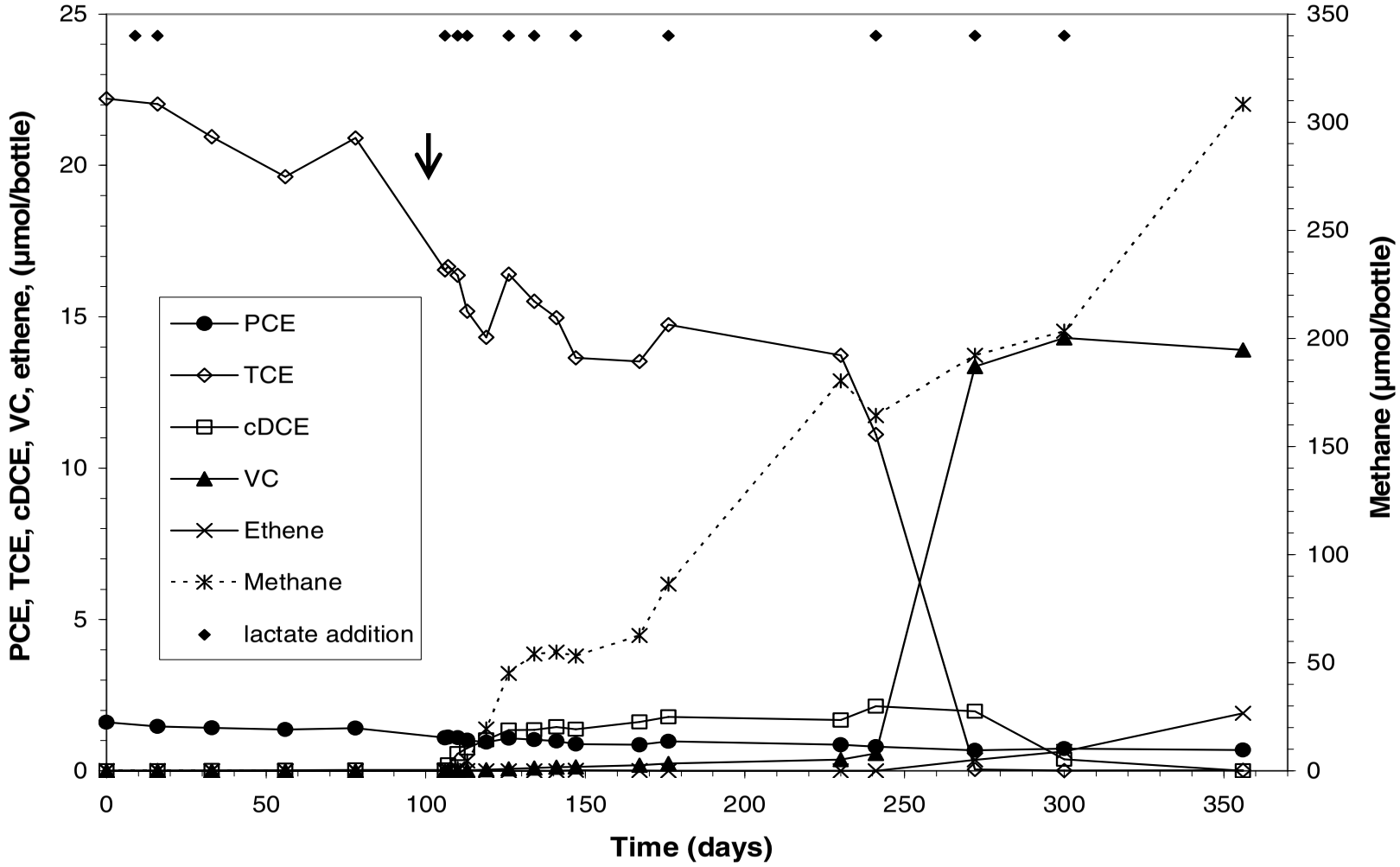

Figure 4.26 Results from a 1% (v/v) KB-1 culture bioaugmentation microcosm (CE-LA-BA_KB1-1). Solid diamonds indicate when lactate was added (32.8 mg for the first addition, 95.7 mg for the second addition and 81.9 mg for subsequent additions except the last two, which were 80.0 mg). The arrow indicates when bioaugmentation occurred.

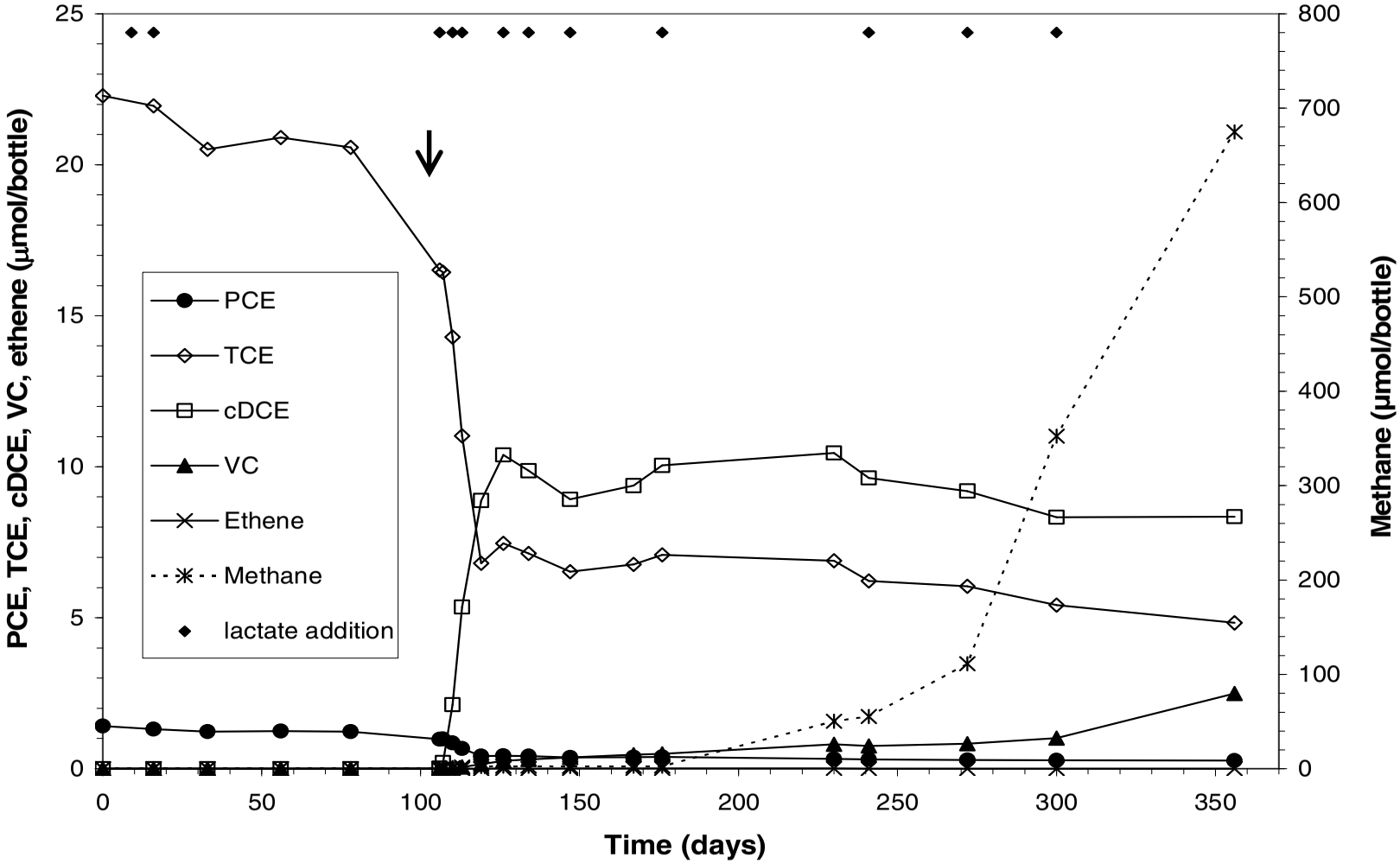

Figure 4.27 Results from a 1% (v/v) KB-1 culture bioaugmentation microcosm (CE-LA-BA_KB1-2). Solid diamonds indicate when lactate was added (32.8 mg for the first addition, 95.7 mg for the second addition and 81.9 mg for subsequent additions except the last two, which were 80.0 mg). The arrow indicates when bioaugmentation occurred.

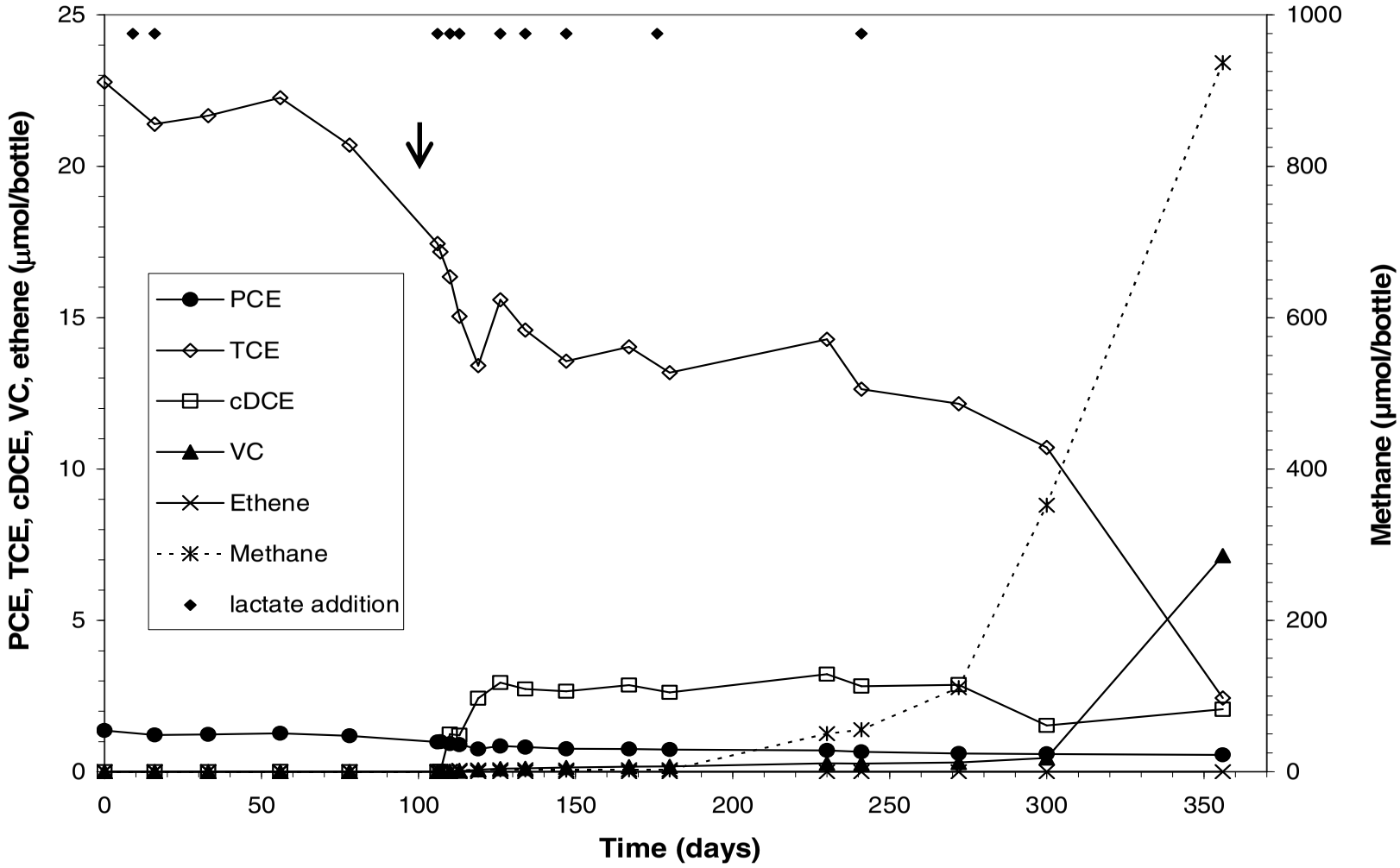

Figure 4.28 Results from a 1% (v/v) KB-1 culture bioaugmentation microcosm (CE-LA-BA_KB1-3). Solid diamonds indicate when lactate was added (32.8 mg for the first addition, 95.7 mg for the second addition and 81.9 mg for subsequent additions except the last two, which were 80.0 mg). The arrow indicates when bioaugmentation occurred.

after day 126 and only resumed in two of the bottles between days 230 and 300. pH was checked regularly along with the addition of electron donor to exclude both as reasons for a lack of activity. Increased production of methane occurred during the interval when reductive dechlorination ceased. For the period of day 106 to day 355, the average molar recovery of PCE and TCE as daughter products was 70.3% (±19.8%); less than 10% of the daughter products were recovered as ethene. Methane accounted for 7.7% (± 3.8%) of the COD added as lactate.

Only one of the microcosms that received a 1% dose of KB-1 underwent complete dechlorination of TCE to VC by the time when monitoring was stopped (Figure 4.26). PCE did not undergo dechlorination in any of the triplicate microcosms.

CHAPTER 5

DISCUSSION

Bioaugmentation has been tested in several pilot scale studies and recommended as a possible remediation technique for many sites contaminated with chlorinated solvents (3, 10, 16, 17, 21). The results of this study suggest that bioaugmentation is a feasible treatment for the PCE and TCE plume in the P-Area at SRS. The dechlorination activity in the bioaugmentation microcosms was significantly higher than the "as-is" or any of the biostimulation microcosms. Although bioaugmentation is a relatively new treatment technique for remediation of sites contaminated with chlorinated ethenes, it has been recommended for use by several studies (3, 10, 16, 17, 21).

A number of previous studies have evaluated the potential for bioaugmentation using microcosms. A study was preformed for Kelly Air Force Base before proceeding to a pilot scale field study. In these microcosms, 3 mL of a KB-1 enrichment culture (2% v/v or an initial cell density of 2 x 10^6 cells per mL after addition of the inoculum) was added to the bioaugmentation treatments (17). Complete conversion of TCE to ethene occurred in all the bioaugmentation treatments with the time required depending on the initial TCE concentration (60 days for an initial TCE concentration of 6 μM or 0.8 mg/L and 150 days for 600 μM or 80 mg/L) (17). The trend of decrease in concentration of TCE followed by an increase in concentration of cDCE, then VC, and finally ethene followed that of the trends seen in the 1% (v/v) SRS enrichment culture bioaugmentation

microcosms. However, the 1% (v/v) microcosms only required 32 days for complete reduction of 31 mg/L TCE and 1.7 mg/L PCE to ethene.

Both a column and microcosm study were done using the Pinellas culture before its use in field studies at the Dover Air Force Base (5, 10, 14). Both studies concluded that bioaugmentation was the best remediation strategy for this site. In the column part of this study, a 4% and a 1% inoculum were evaluated. They showed similar degradation trends as other studies, including first the appearance of cDCE followed by VC and finally ethene. The fact that the column with the 4% inoculum reduced TCE to ethene in about half the time as the column bioaugmented with the 1% inoculum agrees with the results from the SRS enrichment culture bioaugmented microcosms, i.e., the more concentrated the initial cell density the faster reductive dechlorination occurs (14).

This study investigated the effects of biostimulation on the P-Area. Lactate, corn syrup and emulsified vegetable oil were used to biostimulate the P-Area microcosms. All three donors have been successful in acting as an electron donor for cultures containing *Dehalococcoides* (7, 18-21). Data from the PBRP shows the presence of cDCE, indicating the site has undergone some natural attenuation (Figure 1.3). However, neither the "as-is" nor any of the biostimulation treatments showed reduction of PCE or TCE to cDCE. One possible explanation for this is based on the location at which the soil samples were taken. Only one area in the PBRP is showing natural attenuation of TCE to cDCE. The soil samples were taken from an area that is not currently exhibiting signs of reductive dechlorination. It is possible that microcosm studies with sediment taken from the area where cDCE is present would exhibit increased reduction of PCE and TCE to cDCE with the addition of electron donor such as lactate, corn syrup or emulsified

vegetable oil. However, since there is no field evidence for reduction past cDCE, it is unlikely that biostimulation alone would be sufficient to remediate the PBRP chlorinated ethene plume.

Nevertheless, the biostimulation results provide some insight into the preferred electron donor for establishing low enough redox conditions prior to bioaugmentation. The "as-is" microcosms incubated 20 to 40 days before the resazurin turned from pink to clear. Since the soil and groundwater were low in organic matter, it was most likely hydrogen from the anaerobic chamber atmosphere that was used to lower the redox level in the as-is microcosms. This was similar to the microcosms amended with emulsified vegetable oil, which incubated 24 to 55 days before turning clear. Emulsified vegetable oil was apparently not readily biodegraded, since this treatment reached low redox levels no faster than the as-is treatment. Lactate reduced the time needed to reach low redox conditions, requiring 16 to 19 days. Corn syrup was fastest, with the resazurin in the groundwater turning from pink to clear within 16 days. One drawback to using corn syrup as an electron donor is its effect on pH. The biostimulation microcosms amended with corn syrup required pH adjustments twice during the 238 days of incubation. Neither the lactate nor emulsified vegetable oil amended microcosms required a pH adjustment during incubation. The time benefit from using corn syrup to establish low redox conditions is more than offset by the likely need for subsequent pH adjustment or increased addition of a buffer.

Bioaugmentation has been proven in several field studies (10, 16, 17). One important aspect of bioaugmentation is the addition of sufficient *Dehalococcoides* cells. Past research showed densities of *Dehalococcoides* between 1×10^6 and 2×10^8 cells/mL

were successful (10, 16, 17). For this study, microcosms received *Dehalococcoides* densities ranging from 4.4 x 10^3 to 1.9 x 10^6 (Table 4.8). The 1% (v/v) SRS enrichment bioaugmented microcosms completely reduced the PCE and TCE faster than the other treatments, including the microcosms bioaugmented with 1% (v/v) of the commercially available culture. The 0.1% (v/v) SRS enrichment bioaugmented microcosms were the second treatment to show dechlorination activity (with one exception being bottle CE-LA-BA_0.001%-3, Figure 4.25). However, as the PCE and TCE concentration decreased so did the rate of reduction. This was different from the other SRS enrichment bioaugmentation treatment in which the dechlorination rate stayed either constant or increased with time. The 0.01% (v/v) bottles started dechlorinating more slowly than the 1% and 0.1% (v/v) but the rate accelerated after days 240-270. The 1%, 0.1% and 0.01% acted as expected; the higher the initial concentration of *Dehalococcoides* cells (after inoculation), the faster reductive dechlorination proceeded. The 0.001% (v/v) SRS enrichment bioaugmented microcosms behaved similarly to the 0.01% treatments with the exception of the bottle CE-LA-BA_0.001%-3, which was unexpectedly faster, perhaps due to an error in the amount of inoculum added. Harkness et al. (14) demonstrated a faster rate of TCE removal in columns inoculated with 4% culture versus 1% culture, as expected. No other studies were found that evaluated the effect of different inoculum doses.

While KB-1 has proven successful in other laboratory and field studies (7, 17), two out of the three replicates in this study were unable to completely dechlorinate the TCE and PCE. None of the replicate KB-1 treatments showed significant dechlorination

of PCE. The reason for this is not clear. Lactate was provided in excess and the redox and pH conditions remained favorable.

To obtain a yield for the SRS enrichment culture the total *Dehalococcoides* 16S rRNA gene copies per bottle were plotted against the calculated amount of Cl^- released, based on the measured amounts of PCE and TCE that were dechlorinated (Figure 4.6). The results was 4.7 x 10^5 copies of the 16S rRNA gene per µmol Cl^- released. Cupples et al. (4) determined an average yield for *Dehalococcoides* strain VS to be 5.2 x 10^8 (±1.5 x 10^8) copies of the 16S rRNA gene per µmol Cl^-. Other studies have shown that the yield for *Dehalococcoides* spp. ranges from 1.6 x 10^8 to 5.2 x 10^8 gene copies per µmol Cl^- released (4, 15). It is not yet clear why the yield for *Dehalococcoides* in the SRS enrichment culture is three orders of magnitude lower than previously reported values. One possible explanation is a significant difference in the method of quantification. Cupples et al. (4) used a cPCR approach while Bagwell (personal communication) used a qPCR method modeled after Ritalahti et al. (20).

In order for reductive dechlorination to occur an electron donor must be provided. In this study both the SRS enrichment culture and the bioaugmentation bottles were given lactate as the electron donor. Lactate was chosen because it was the donor given to the original microcosms by Bratt (1). The biostimulation microcosms received either lactate, emulsified vegetable oil or corn syrup as an electron donor. Many other electron donors have proven successful for reductive dechlorination, including hydrogen release compound, molasses, formate, and methanol. While hydrogen release compound, molasses, and lactate solutions are most commonly used in field studies, methanol has

proven to be most effective in laboratory studies, based on the percentage of the electron donor used for reductive dechlorination (6, 21).

From the donor balance preformed on the enrichment culture, an average of 2.3% of the lactate added was used in the dechlorination of PCE and TCE. This is much lower than the reported 31% reported for methanol (6). Most of the lactate ended up as acetate or propionate (Tables 4.2-4.4). The ratio of propionate to acetate was approximately 2:1 in E-2A and E-2C, and 1:1.1 in E-2B. The 2:1 ratio is consistent with fermentation of lactate by the acrylate pathway (13):

$$3 \text{ lactate} \rightarrow 2 \text{ propionate} + \text{acetate} + CO_2 \qquad (2)$$

The higher amount of acetate in E-2B suggests that some of the propionate may have been converted to acetate + CO_2. The lack of succinate as an end product (it was detectable with the HPLC method used but did not appear) indicates the fermentation most likely occurs in the SRS enrichment culture via the acrylate pathway rather than the succinate-propionate pathway (13).

Harkness et al. (14) performed an electron donor balance on one of the columns used in their study. The column was amended with lactate and methanol. At low TCE concentrations (30 µM) reductive dechlorination accounted for only 0.25% of the electron donor consumed. The percentage of donor used for reductive dechlorination increased to 6% when the TCE concentration was increased to 1300 µM (14). These percentages are close to the value of 3% of donor used for reductive dechlorination by the SRS enrichment culture, at TCE concentrations of approximately 300 µM. Harkness et

al. (14) also determined that methanogenesis does not play a large role at high concentrations of chlorinated ethenes, but does play a role at low concentrations. This agrees with the results from the SRS enrichment culture and the microcosm study.

This study demonstrated the successful use of the SRS enrichment culture in bioaugmentation of the P-Area microcosms. The lack of evidence for dechlorination in the "as-is" and biostimulation microcosms indicates that the organisms needed are not present in sufficient number or at all in the P-Area. Therefore, neither monitored natural attenuation nor biostimulation would be an acceptable choice for remediation at this site. The bioaugmentation aspect of the study indicates that using the SRS enrichment culture may be a feasible option for bioaugmenting the P-Area chlorinated ethene plume.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

1) A sediment-free enrichment culture was developed using inoculum derived from the TCE groundwater plume in the C-area at SRS. The culture grows in a defined anaerobic mineral medium with PCE and TCE provided as the electron acceptors and lactate as the electron donor. TCE and PCE concentrations of 35-40 mg/L and 4-8 mg/L, respectively, are completely consumed by the enrichment culture within three to five weeks. Quantitative PCR analysis indicated a linear increase in *Dehalococcoides* as increasing amounts of PCE and TCE are reduced to ethene. The *Dehalococcoides* cell density in the enrichment culture stabilized at approximately 4 x 10^6 cells per mL. An electron donor balance indicates that most of the lactate provided is fermented to acetate and propionate, with less than 3% used for reductive dechlorination. Methanogenesis in the enrichment culture is insignificant.

2) Biostimulation using lactate, corn syrup and emulsified vegetable oil was evaluated as a remediation strategy for the P-Area chlorinated ethene plume. After more than eight months of incubation, there was no evidence of PCE or TCE dechlorination in any of the biostimulated treatments, even though the amount of donor added was in considerable excess and redox and pH conditions were favorable. This indicates that the organisms needed for reductive dechlorination or either not present or are in too small of numbers in the PBRP. During this study, it was determined that corn syrup causes the redox conditions in the microcosms to become favorable more quickly

than either lactate or emulsified vegetable oil, but also caused a significant drop in the pH.

3) The microcosm results indicate that the SRS enrichment culture holds promise for use in bioaugmentation of the P-area groundwater plume. In the treatment that received the 1.0% (v/v) dose of SRS enrichment culture, reduction of PCE and TCE to ethene was complete in approximately three weeks. The lower doses of SRS culture were also effective, although up to seven months of incubation was needed before dechlorination was complete. In contrast, dechlorination of PCE and TCE was much slower and incomplete in the treatment that received the commercial bioaugmentation culture, over the same incubation period.

Based on the results of this study the following recommendations are offered:

1) The enrichment culture should be scaled-up in preparation for its use in a field trial in the P-area groundwater. This will require increasing the volume of culture and maintaining or increasing the concentration of *Dehalococcoides* present, by increasing the rate and possibly concentrations of PCE and TCE additions.

2) The lack of nutrients in the P-area soil and groundwater suggest that growth of the enrichment culture in this environment might be improved by addition of nutrients during bioaugmentation. An additional microcosm study should be performed to assess the effect of macronutrients (including nitrogen, phosphorus, iron, calcium and magnesium) and micronutrients (including trace metals and yeast extract) on growth of the SRS enrichment culture in P-area soil and groundwater.

3) Further characterization of the enrichment culture should be performed. This should include an assessment of how well the culture grows on individual chlorinated

ethenes, by providing PCE, TCE, cDCE and VC separately to sub-cultures. In addition, the ability of the culture to utilize other chlorinated aliphatic compounds should be tested, including 1,2-dichloroethane, 1,2-dibromoethane, vinyl bromide, and chlorinated benzenes. Electron donors other than lactate should be evaluated, with the aim of finding a substrate that is more efficiently used for dechlorination.

4) A pilot scale test of the enrichment culture should be conducted in the P-area. This test should include control plots with no addition, biostimulation, and bioaugmentation. This will allow an assessment of bioaugmentation for the P-area as well as a comparison between the field results and the microcosm study, to determine how well the microcosm results are predictive of the field response.

APPENDICES

Appendix A

Preparation of Enrichment Culture Media

Solutions needed for media-

- Phosphate buffer-
  In a 100 mL volumetric flask add 5.25 g K_2HPO_4. Then fill to 100 mL with DDI water.
- Salt solution-
  In a 100 mL volumetric flask add 5.35 g NH_4Cl, 0.46976 g $CaCl_2 \cdot 2H_2O$, and 0.17787 g $FeCl_2 \cdot H_2O$. Then fill to 100 mL with DDI water.
- Trace metals solution
  In a 100 mL volumetric flask add 0.03 g H_3BO_3, 0.0211 g $ZnSO_4 \cdot 7H_2O$, 0.075 g $NiCl_2 \cdot 6H2O$, 0.1 g $MnCl_2 \cdot 4H2O$, 0.01 g $CuCl_2 \cdot 2H_2O$, 0.15 g $CoCl_2 \cdot 6H_2O$, 0.002 g Na_2SeO_3, 0.01 g $Al_2(SO_4)_3 \cdot 16H_2O$, and 1 mL concentrated HCl. Then fill to 100 mL with DDI water.
- Magnesium sulfate solution-
  In a 100 mL volumetric flask add 6.25 g $MgSO_4 \cdot 7H_2O$. Then fill to 100 mL with DDI water.
- Bicarbonate solution-
  In a 500 mL volumetric flask add 8.0 g $NaHCO_3$. Then fill to 500 mL with DDI water.
- Redox solution-
  In a 10 mL volumetric flask add 0.01 g resazurin. Then fill to 10 mL with DDI water.
- Amorphous ferrous sulfide solution-
  In 160 mL serum bottle add 3.9169 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 2.4018 g $Na_2S \cdot 9H_2O$. To this add 100 mL boiling autoclaved water, let solution settle and decant clear water. Wash solution 4 more times the same way. Bring volume to 100 mL with boiling water and cap with a grey rubber septum. Store in an anaerobic chamber (9).
- Yeast extract solution
  In a 100 mL volumetric flask add 0.5 g yeast extract. The fill to 100 mL with DDI water.

Media Preparation

In a 1 L bottle add 10 mL phosphate solution, 10 mL salt solution, 2 mL trace metals solution, 2 mL magnesium sulfate solution, 1 mL redox solution, and 900 mL DDI water. Autoclave this solution and then add 50 mL filter sterilized bicarbonate solution, 10 mL filter sterilized yeast extract, and 10 mL amorphous ferrous sulfide solution.

Appendix B

Response Factors and Standard Curves

Table B.1 GC response factors for enrichment culture.

Compound	GC Retention Time (min)	Response Factor (µmol/bottle)	R^2	Conversion Factor[a] (µmol/btl to µM)	Conversion Factor[a] (µmol/btl to mg/L)
Methane	0.49	2.0574E-06	0.9949	0.00086	0.00001
Ethene	0.70	1.2051E-06	0.9979	0.11515	0.00322
Ethane	0.79	1.0749E-07	0.9873	0.05529	0.00166
VC	2.60	2.8180E-06	0.9894	0.38366	0.02398
cis-DCE	6.84	1.3410E-05	0.9997	0.56737	0.05503
TCE	10.02	6.4240E-06	1.0000	0.50888	0.06687
PCE	14.64	3.9840E-06	1.0000	0.44376	0.07359

[a] Assumes 100 mL liquid, 60 mL headspace, 23°C

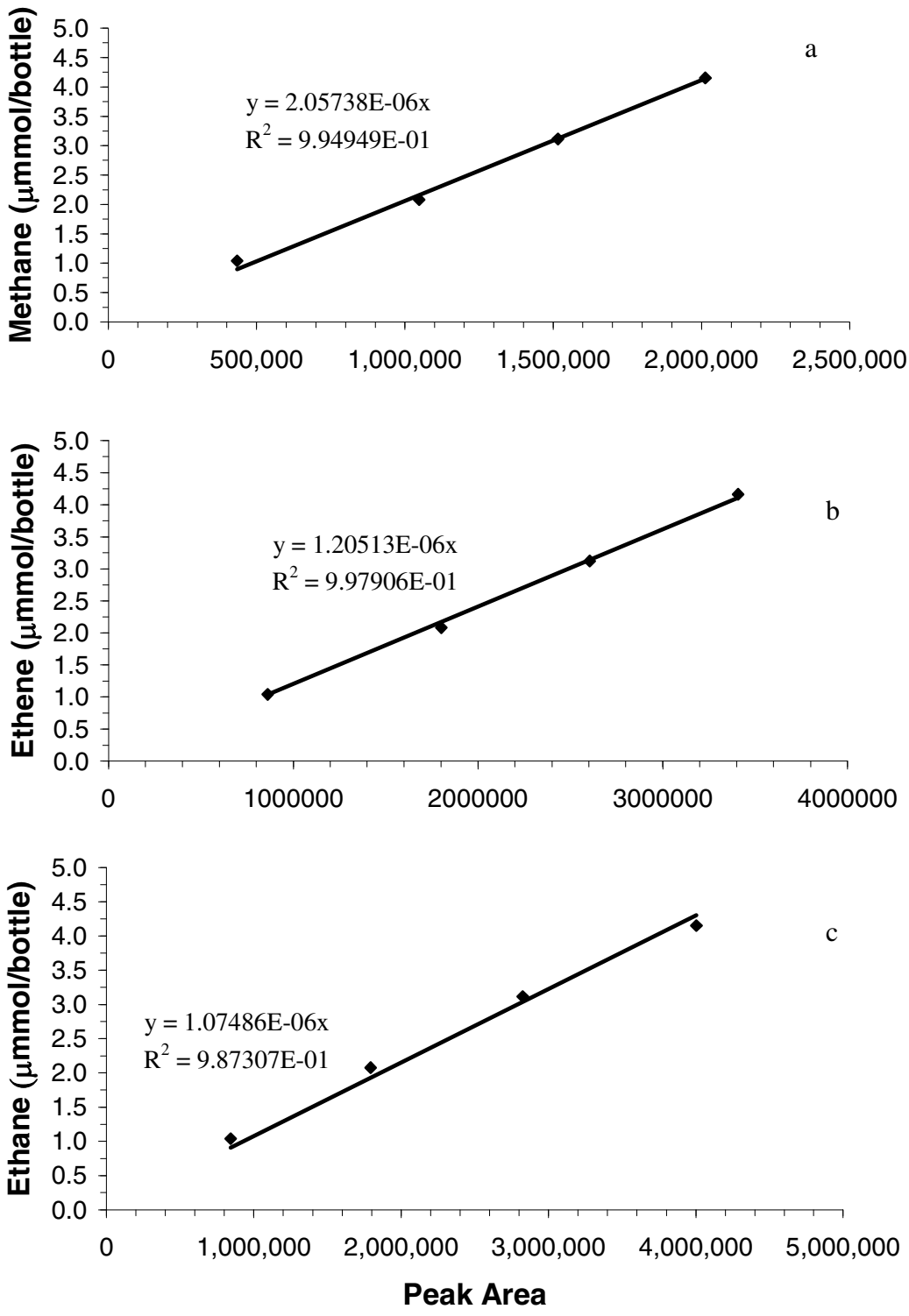

Figure B.1 GC response curves for methane (a), ethene (b), and ethane (c).

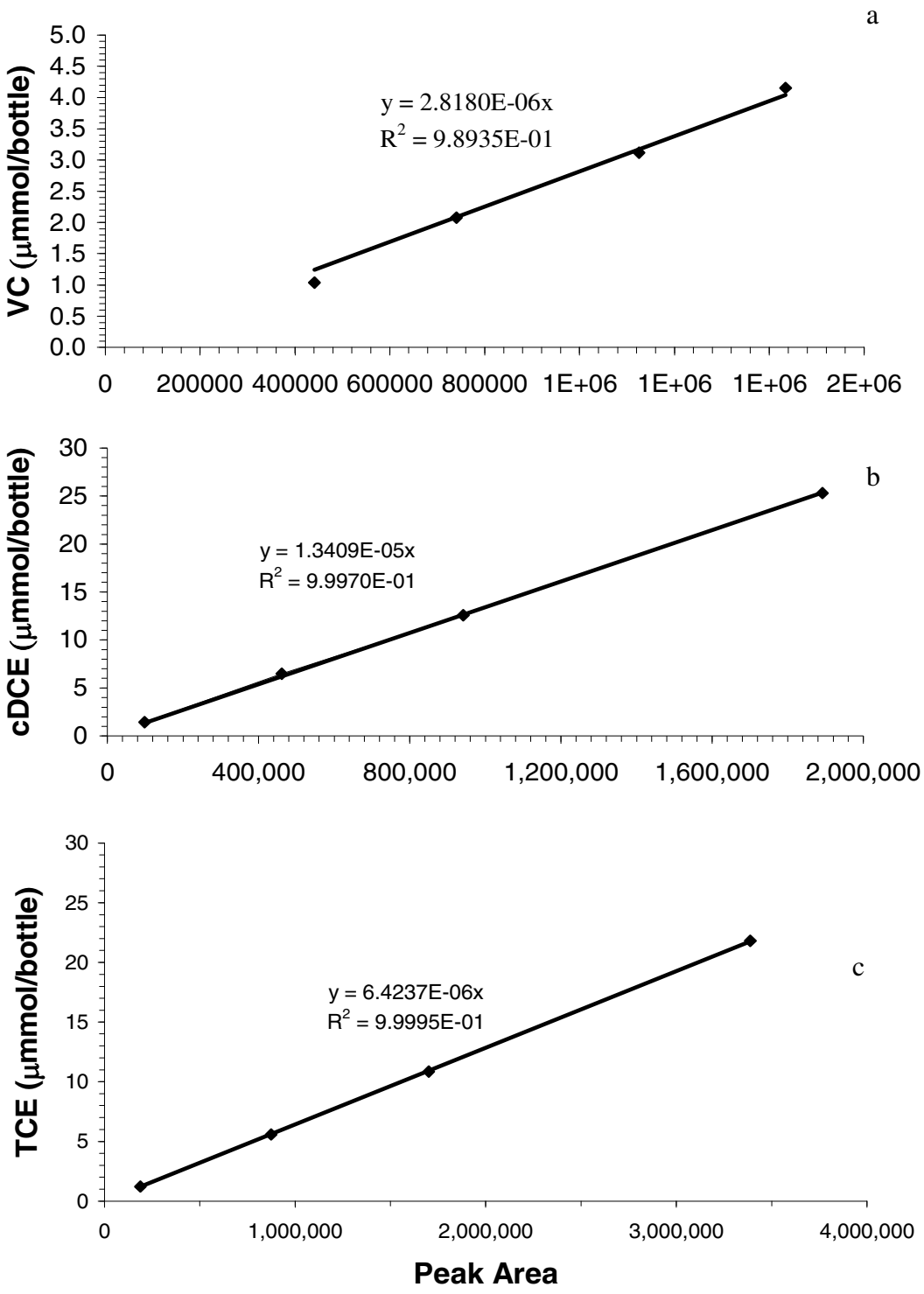

Figure B.2 GC response curves for VC (a), cDCE (b) and TCE (c).

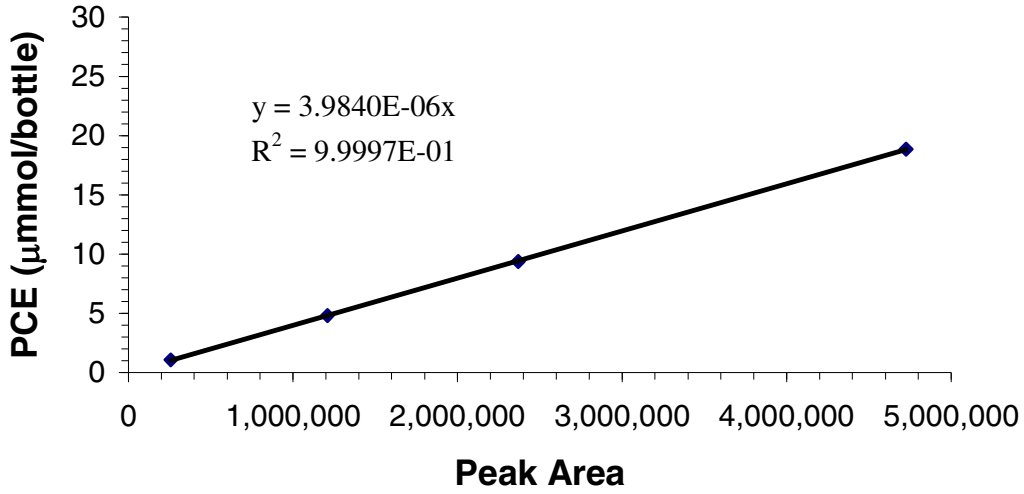

Figure B.3 GC response curve for PCE.

Table B.2. GC response factors for microcosms.

	GC RT	Response Factor		Conversion Factor*	
Compound	**(min)**	**(µmol/bottle)**	**R^2**	**(µmol/btl to µM)**	**(µmol/btl to mg/L)**
Methane	0.49	2.0574E-06	0.9950	0.01	0.00
Ethene	0.70	1.2051E-06	0.9979	1.31	0.04
Ethane	0.79	1.0749E-06	0.9873	0.58	0.02
VC	2.60	2.8180E-06	0.9894	6.71	0.42
cis-DCE	6.84	8.6345E-06	0.9956	15.65	1.52
TCE	10.0	4.6154E-06	0.9952	11.85	1.55
PCE	14.6	3.4975E-06	0.9897	8.81	1.46

* Assumes 50 mL liquid, 98.5 mL headspace, 23^{o}C

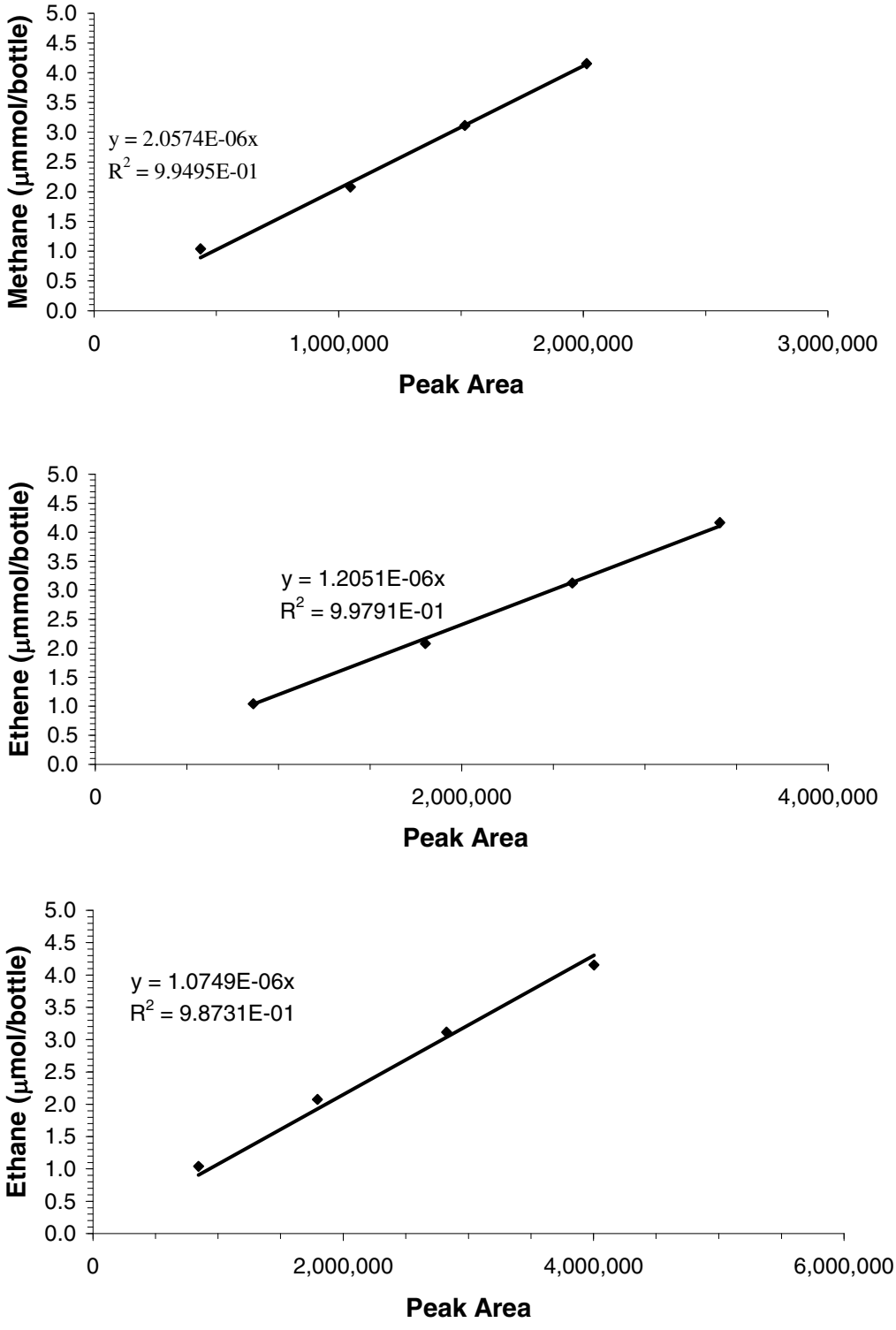

Figure B.4 GC response curves for methane (a), ethene (b) and ethane (c).

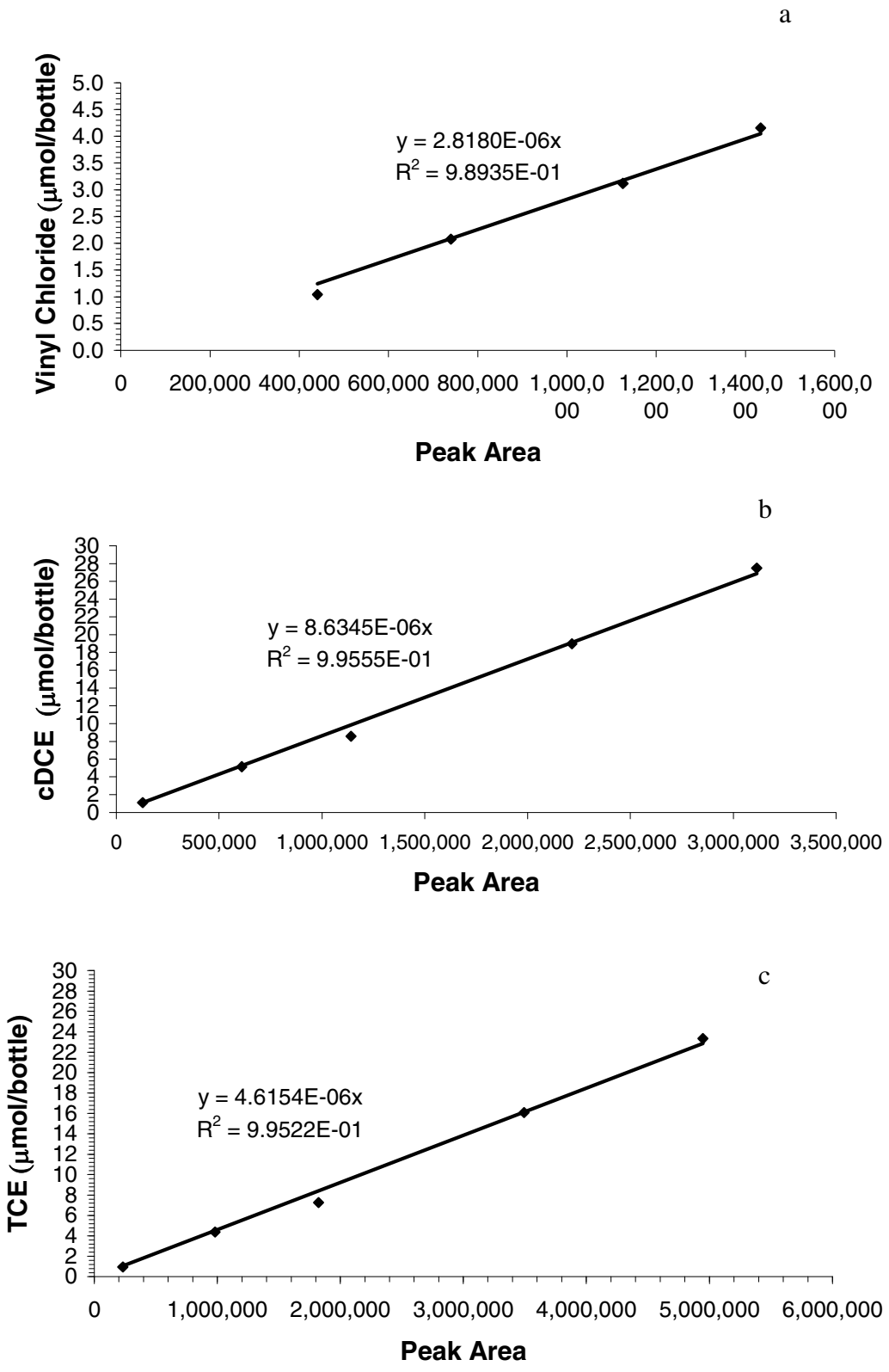

Figure B.4 GC response curves for VC (a), cDCE (b) and TCE (c).

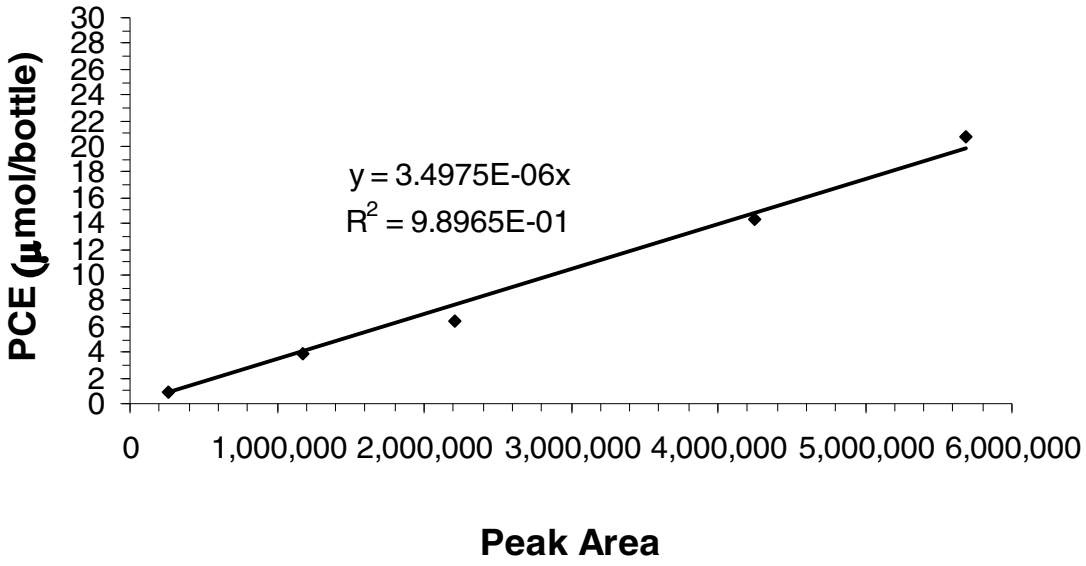

Figure B.5 GC response curves for PCE.

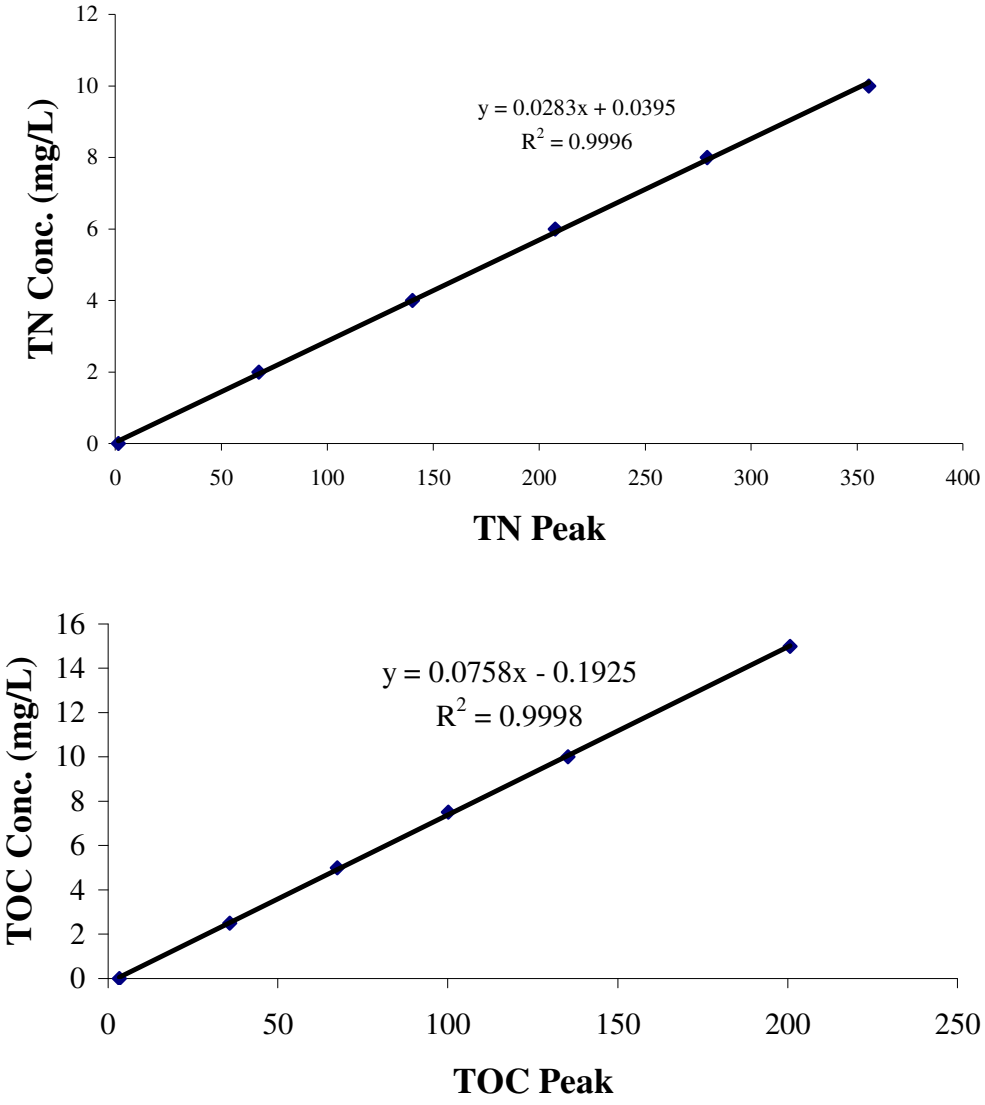

Figure B.6 Response curves for TOC and total nitrogen (TN).

Table B.3 HPLC response factors.

Std conc. (mM)	Peak Area		
	lactate	**acetate**	**propionate**
Retention time (min	12.8-12.9	15.3-15.5	17.9-18.0
0.07614	23110	10278	11558
0.38069	117704	64434	74827
0.7614	236832	150633	178750
3.8069	1207667	754340	950466
Response factor	3.155E-06	6.637E-06	5.281E-06

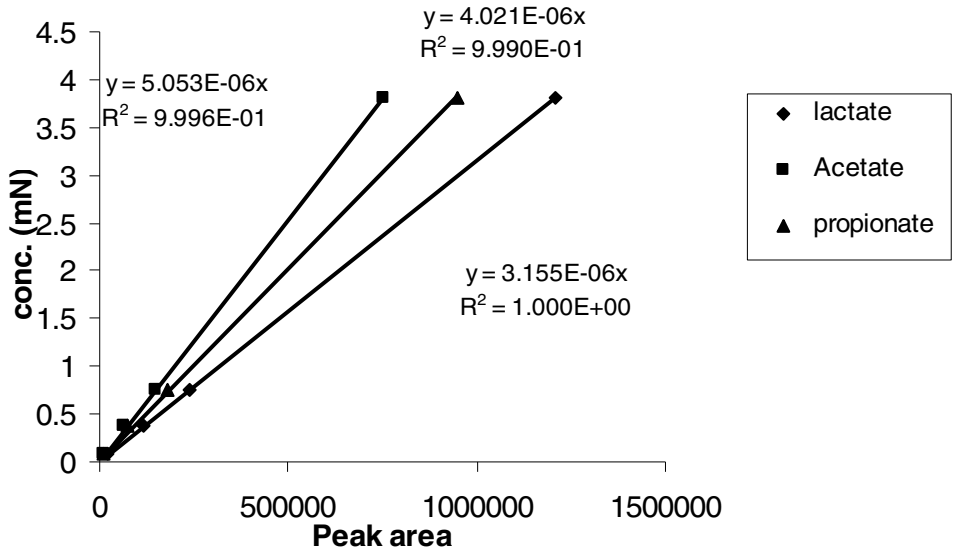

Figure B.7 HPLC response curves for lactate, acetate and propionate

Appendix C

Microcosm Results

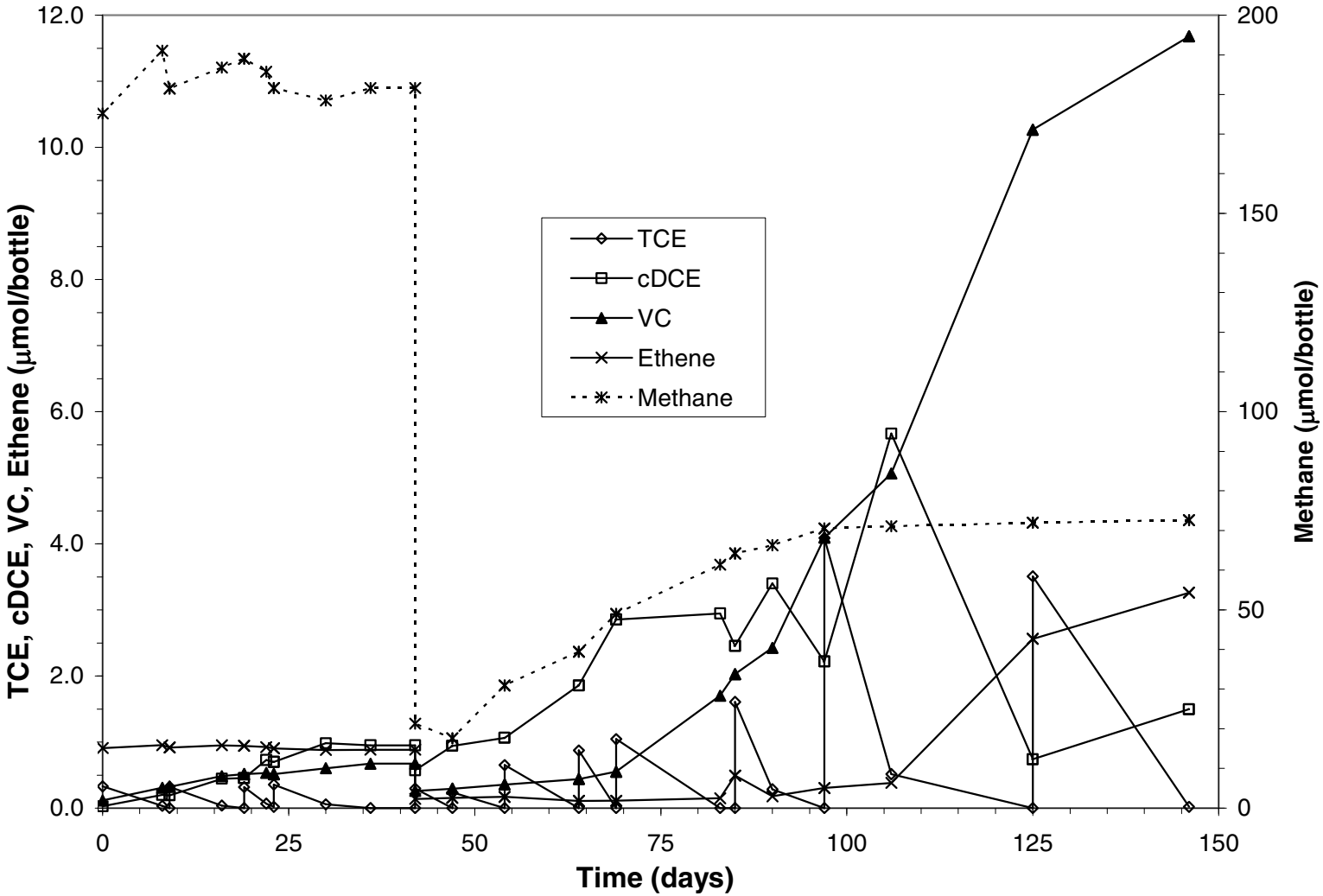

Figure C.1 Results for one of the CRP-44 microcosms (CRP-44-2).

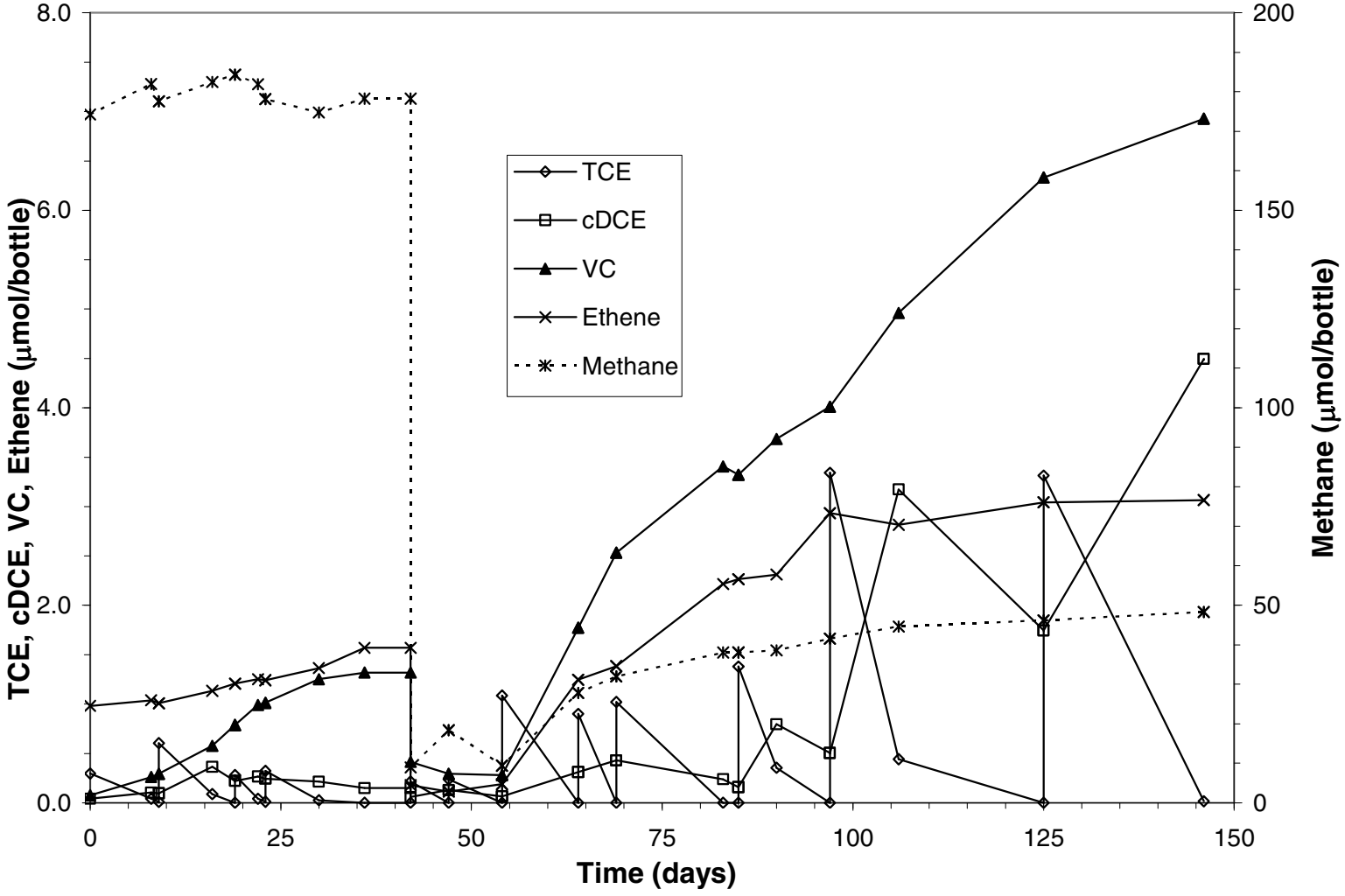

Figure C.2 Results for one of the CRP-44 microcosms (CRP-44-3).

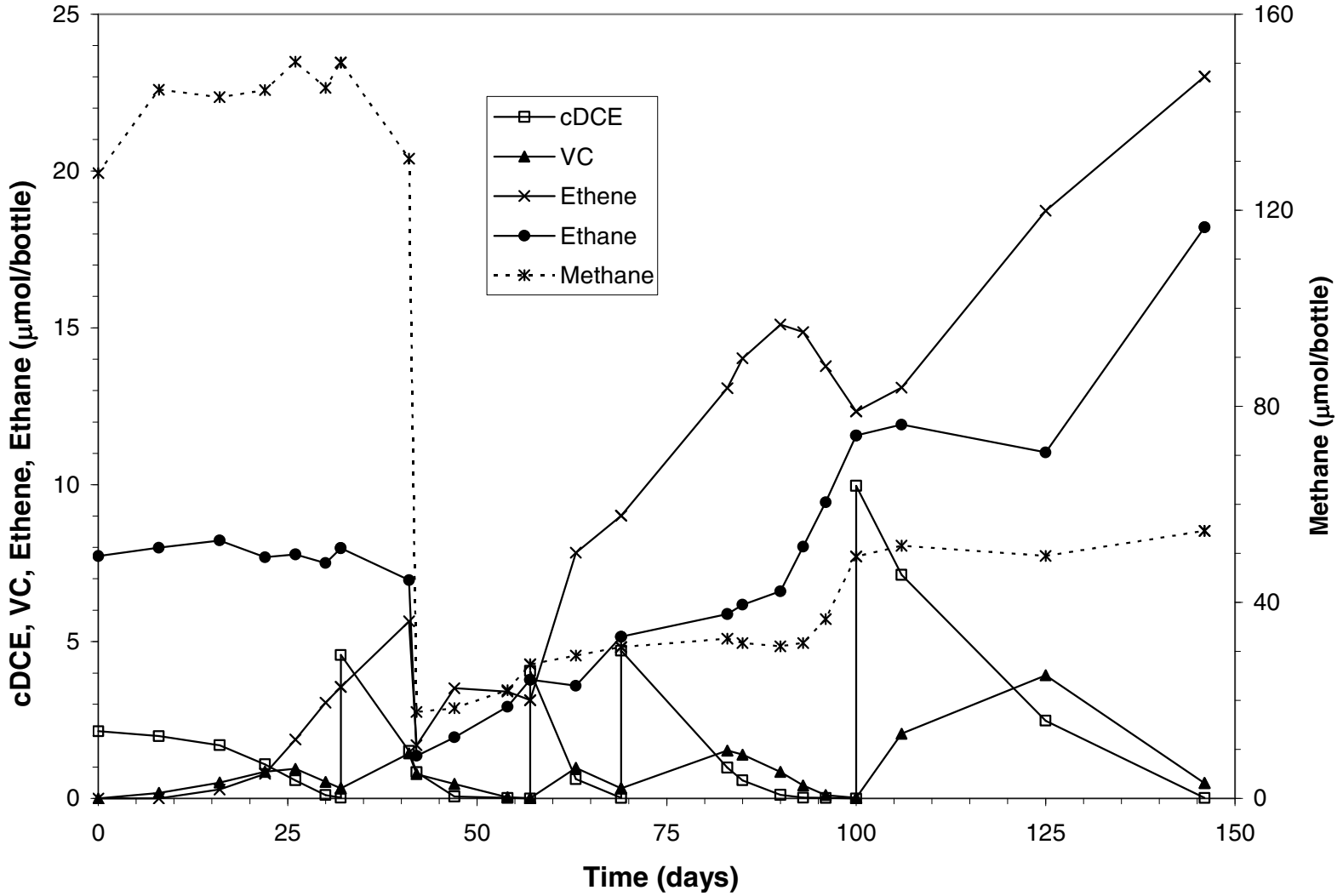

Figure C.3 Results for one of the CRP-48 microcosms (CRP-48-2)

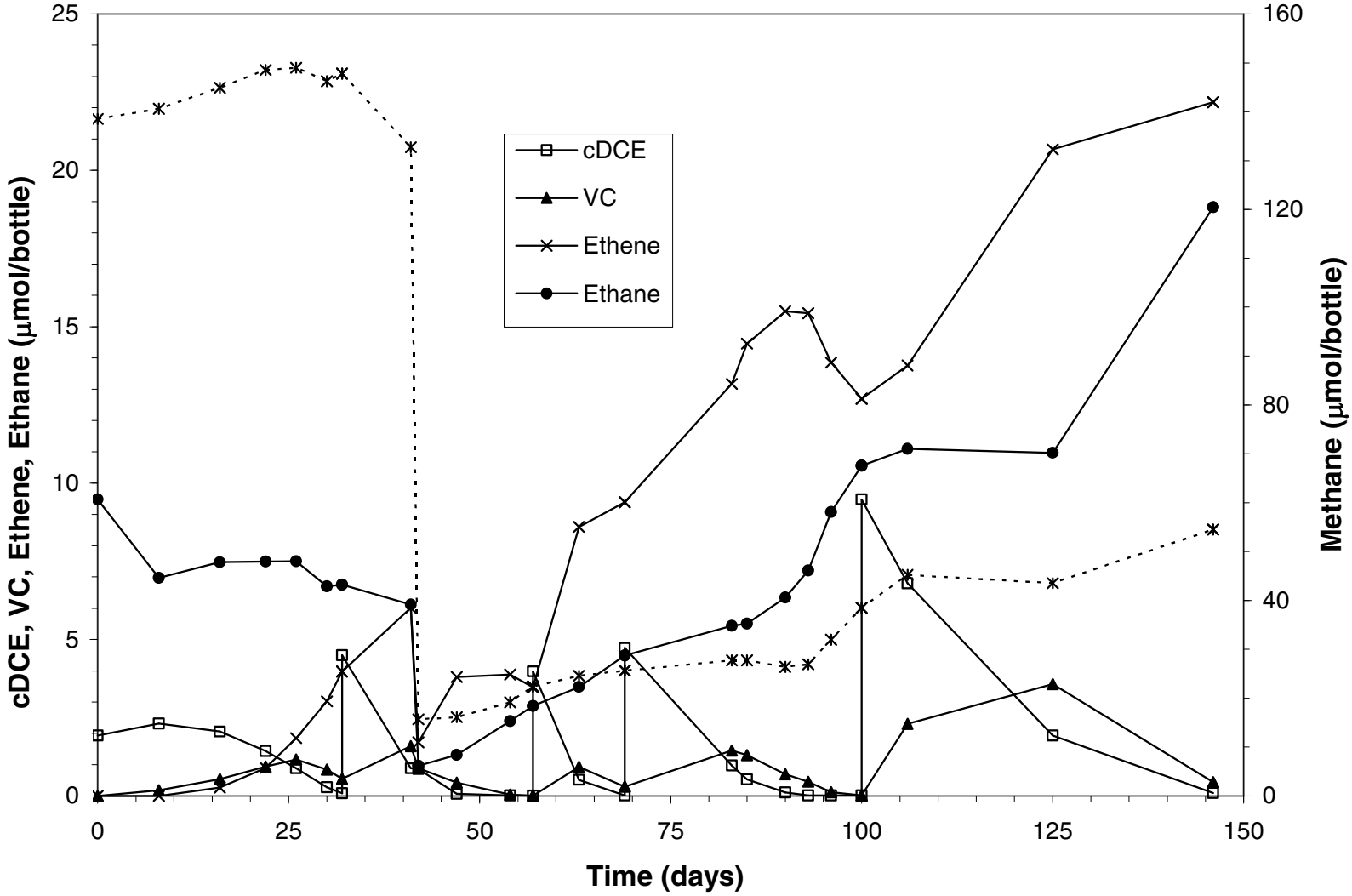

Figure C.4 Results for one of the CRP-48 microcosms (CRP-48-3).

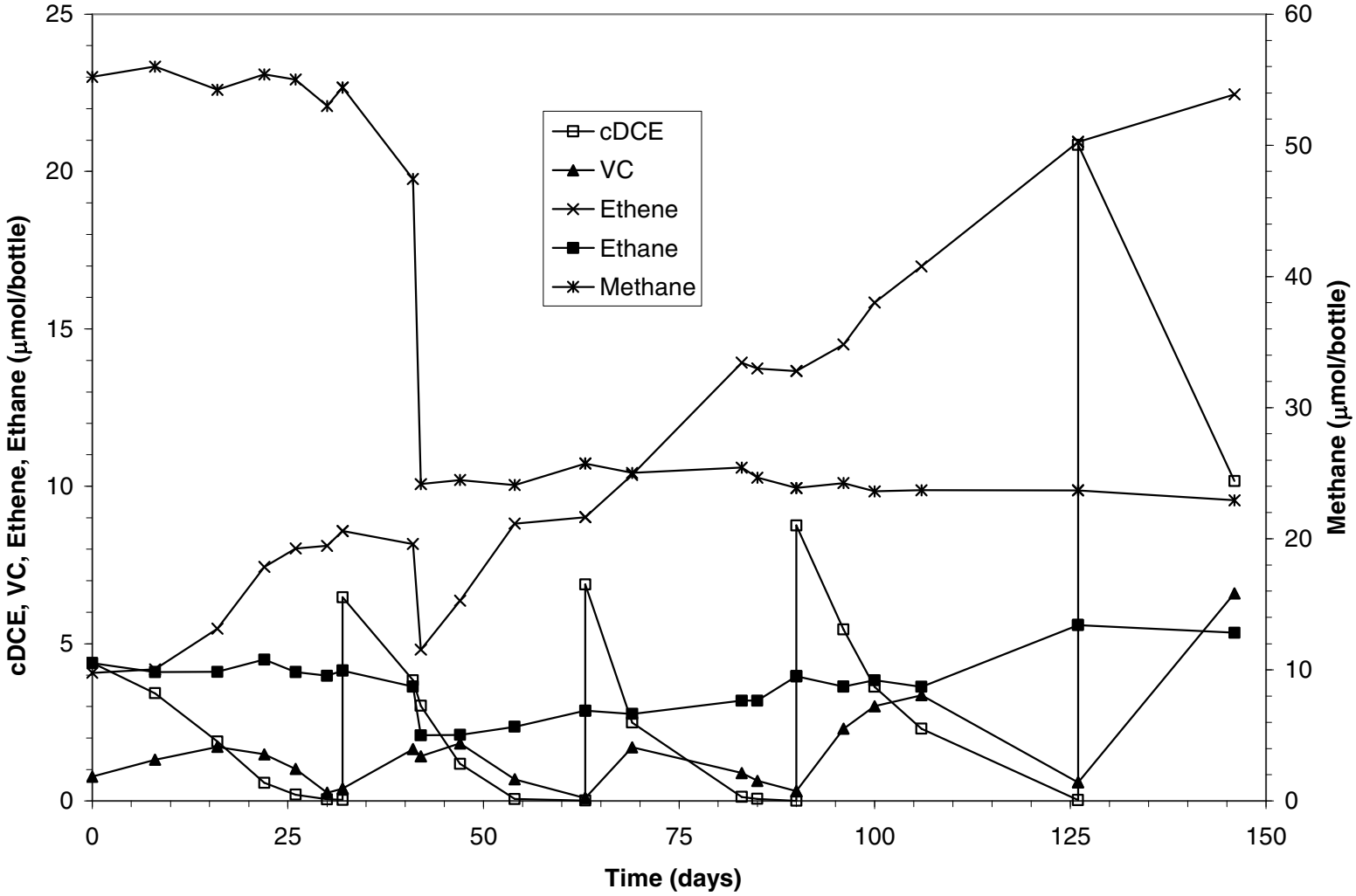

Figure C.5 Results for one of the CRP-50 microcosms (CRP-50-2).

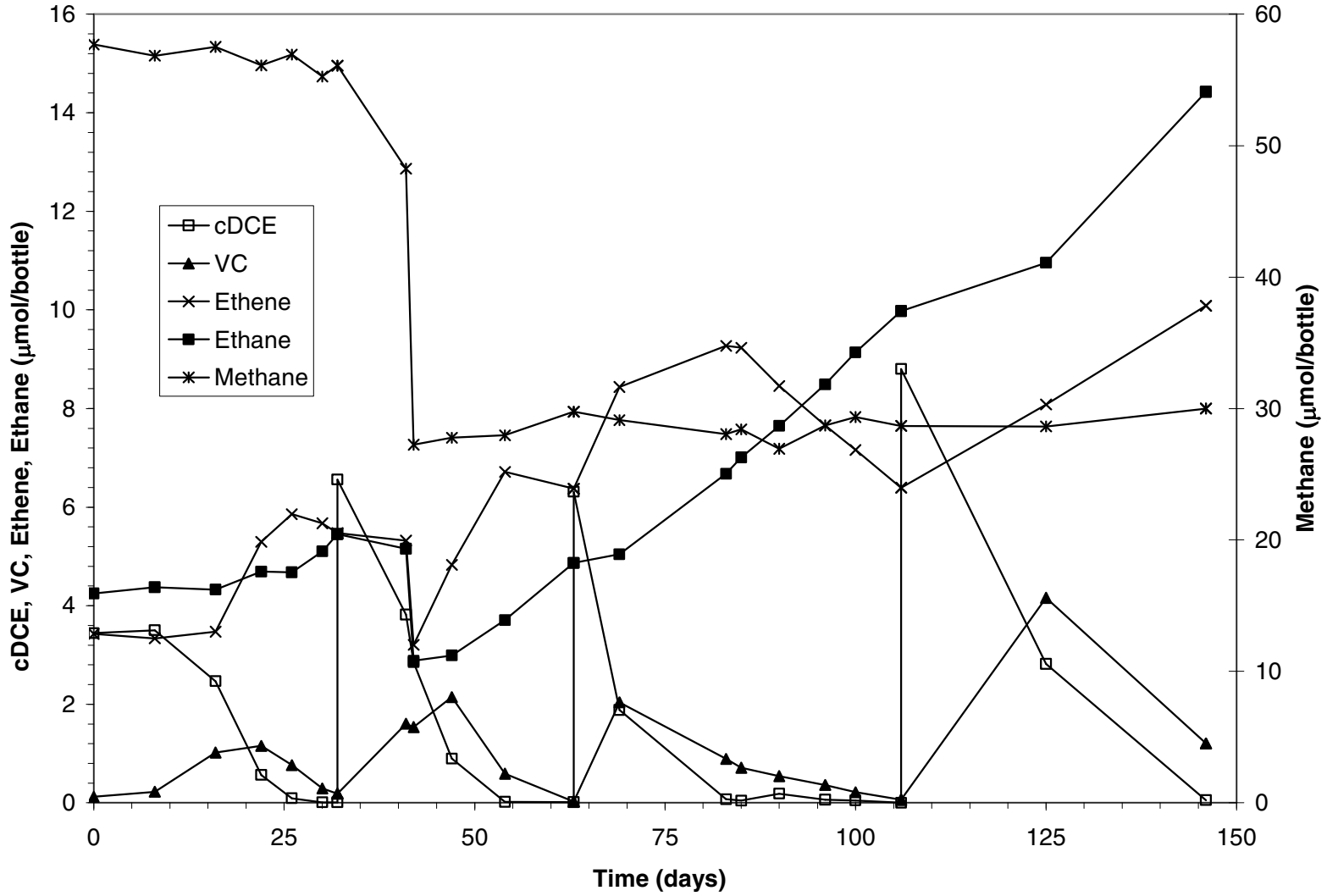

Figure C.6 Results for one of the CRP-50 microcosms (CRP-50-3).

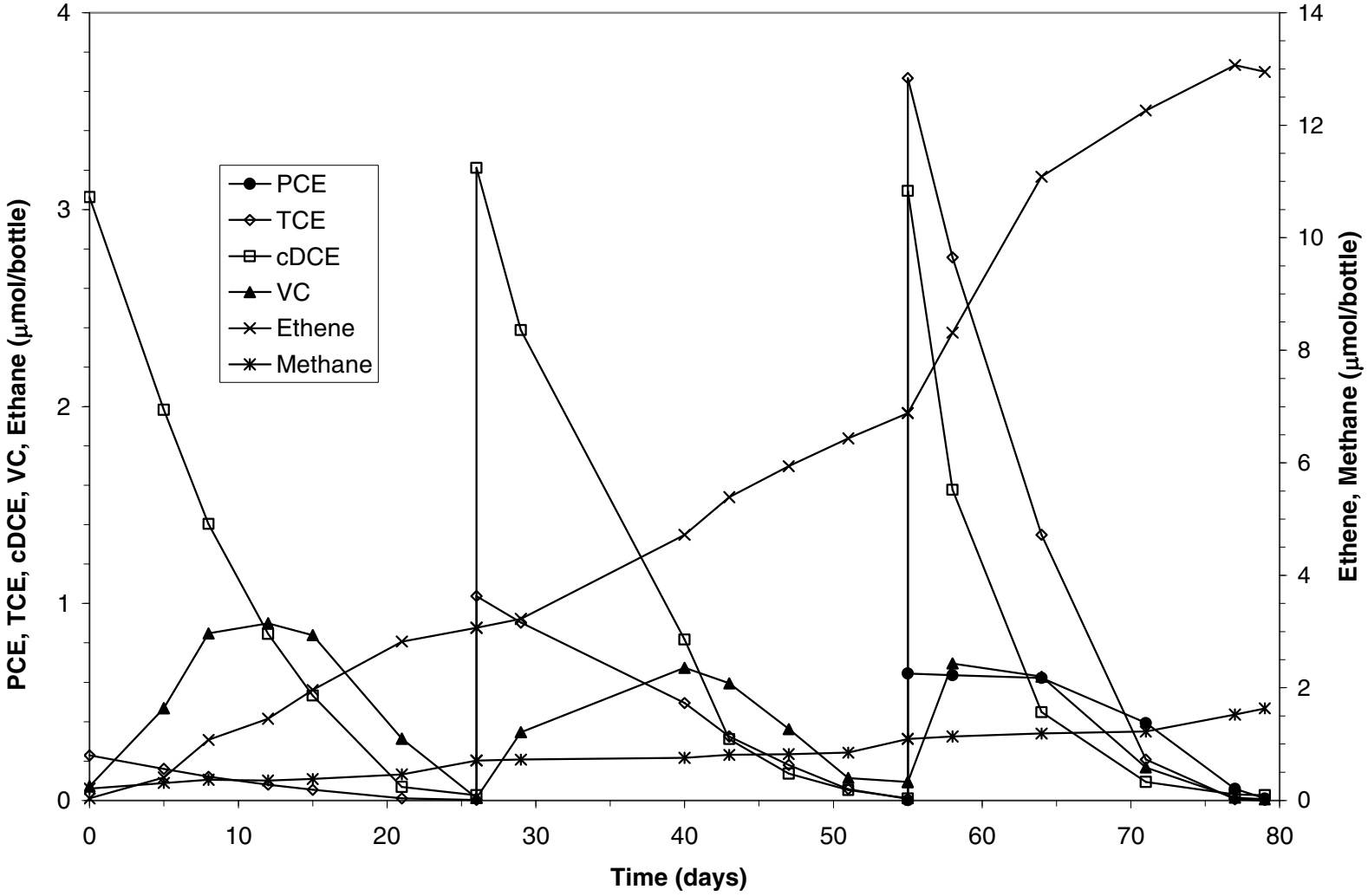

Figure C.7 Results for one of the Phase I enrichments (E-1B).

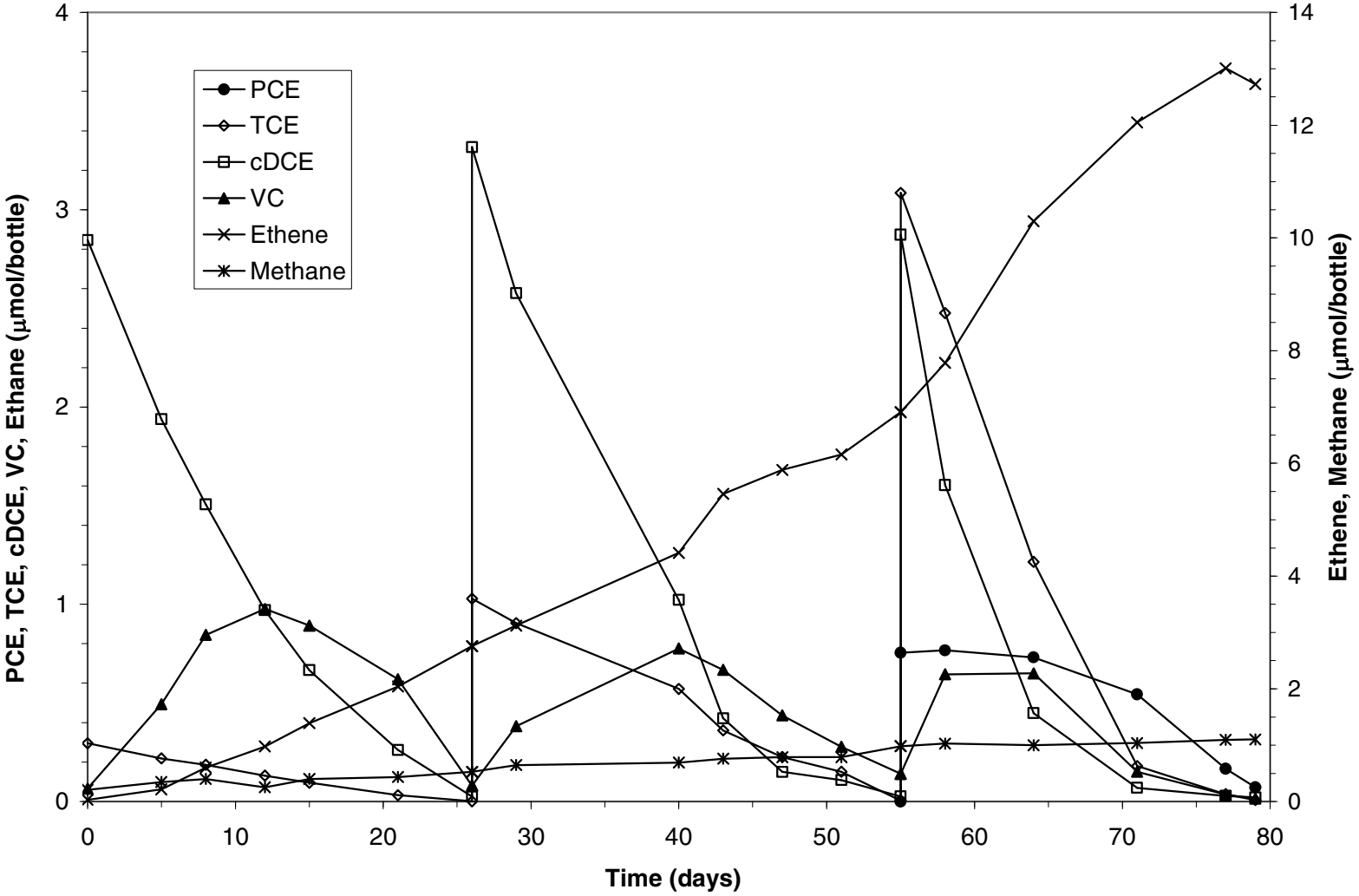

Figure C.8 Results for one of the Phase I enrichments (E-1C).

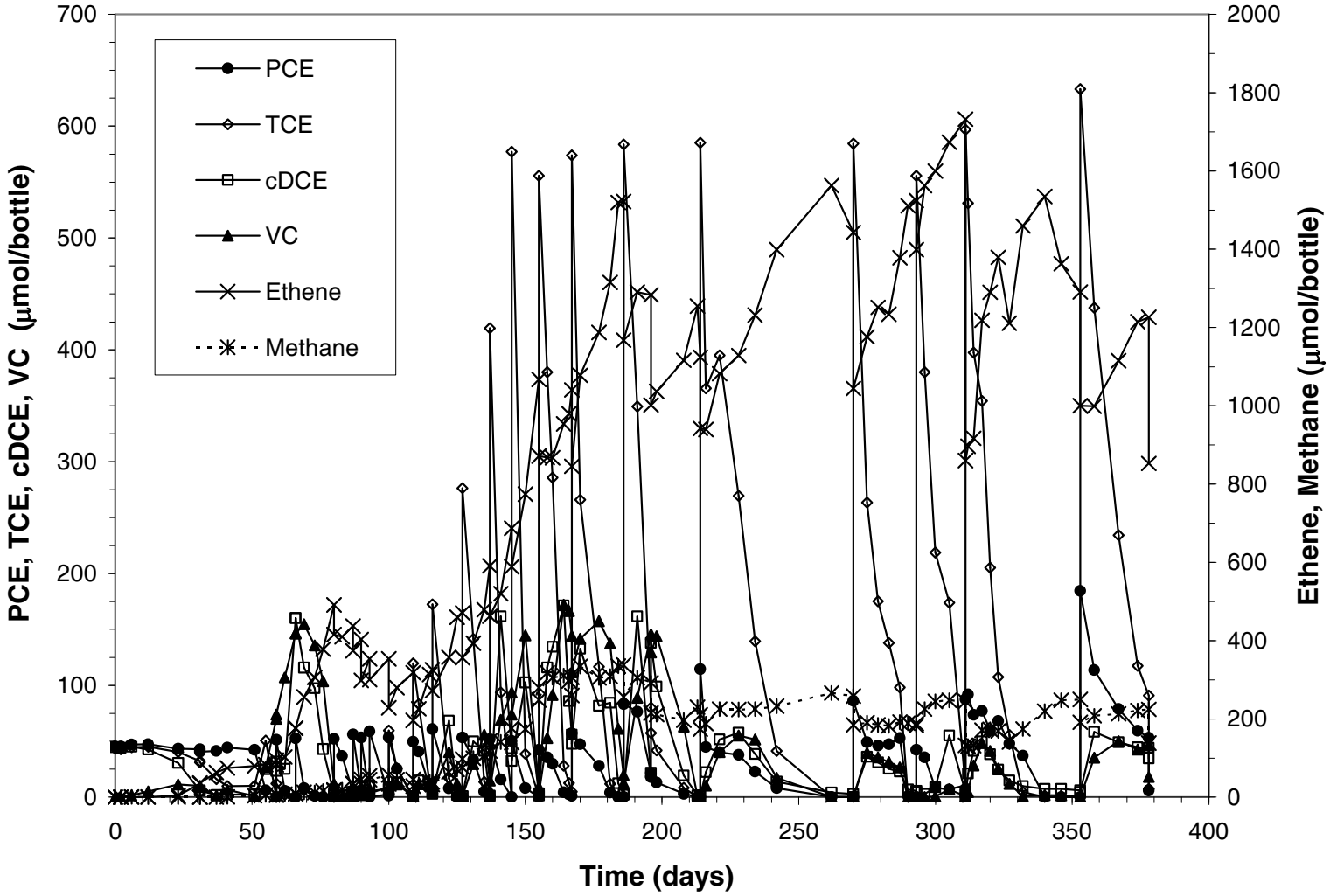

Figure C.9 Results for one of the Phase II enrichments (E-2B).

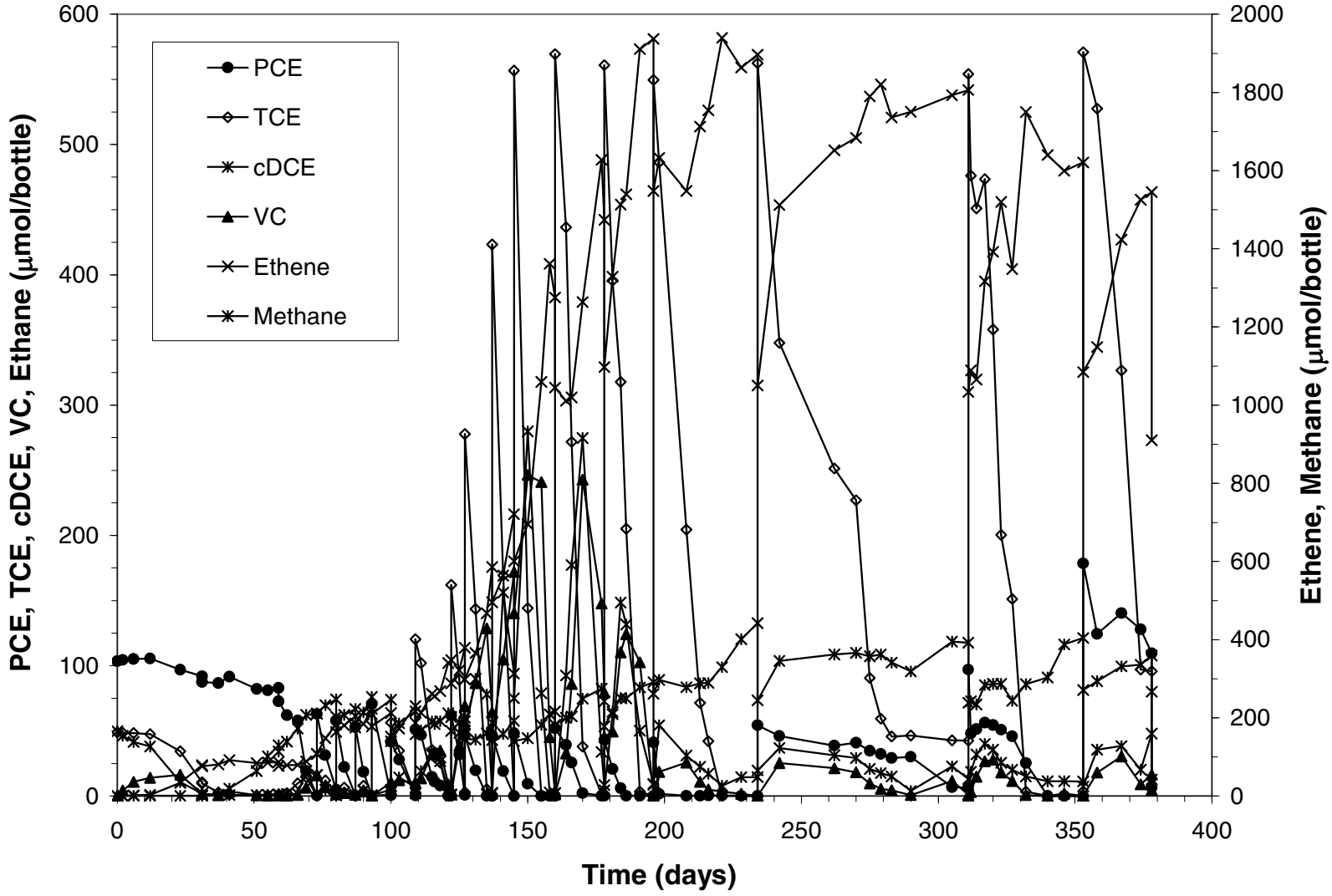

Figure C.10 Results for one of the Phase II enrichments (E-2C).

Appendix D

Complete Electron Donor Balance and Stoichiometry of CE

Table D.1 Complete Electron Donor Balance and Stoichiometry of CE

E-2A

Initial								Final								
Day	Cycle	PCE (umol/bottle)	TCE (umol/bottle)	cDCE (umol/bottle)	VC (umol/bottle)	Ethene (umol/bottle)	sum (umol/bottle)	Day	PCE (umol/bottle)	TCE (umol/bottle)	cDCE (umol/bottle)	VC (umol/bottle)	ethene (umol/bottle)	sum (umol/bottle)	% Recovery	% Ethene
145	1	50	577	32	74	589	1,322	155	0.2	0.9	3.5	8.9	1,067	1,081	81.7%	98.7%
155	2	42	556	1.2	2.8	872	1,474	167	1.2	4.9	56	144	1,040	1,246	84.6%	83.5%
167	3	57	574	48	112	845	1,636	186	0.2	0.7	2.6	20	1,522	1,545	94.4%	98.5%
186	4	83	584	2.1	13	1,167	1,849	214	0.0	0.0	1.4	0.1	1,124	1,126	60.9%	99.9%
214	5	115	585	0.9	0.2	942	1,643	270	0.0	0.0	2.9	0.0	1,443	1,446	88.0%	99.8%
270	6	86	584.5	0.0	0.0	1,044	1,714	293	0.0	0.0	5.7	0.1	1,524	1,530	89.3%	99.6%
293	7	42	556	4.0	0.0	1,399	2,001	311	0.8	9.2	4.8	10	1,732	1,758	87.8%	98.6%
311	8	88	597	0.0	0.9	860	1,545	353	0.0	0.0	5.9	0.0	1,291	1,297	83.9%	99.5%

E-2B

Initial								Final								
Day	Cycle	PCE (umol/bottle)	TCE (umol/bottle)	cDCE (umol/bottle)	VC (umol/bottle)	ethene (umol/bottle)	sum (umol/bottle)	Day	PCE (umol/bottle)	TCE (umol/bottle)	cDCE (umol/bottle)	VC (umol/bottle)	ethene (umol/bottle)	sum (umol/bottle)	% Recovery	% Ethene
145	1	54	571	176	168	458	1,428	167	0.0	0.0	8.1	19	1,187	1,214	85.0%	97.8%
167	2	56	561	6.2	11	883	1,517	186	0.0	0.9	50	67	1,403	1,521	100.3%	92.2%
186	3	86	535	22	41	985	1,669	208	0.1	1.2	13	13	1,176	1,203	72.1%	97.7%
208	4	77	586	11	11	973	1,659	234	0.0	0.1	24	0.2	1,503	1,528	92.1%	98.4%
234	5	75	570	17	0.0	1,085	1,747	293	0.0	0.1	24	0.2	1,503	1,528	87.5%	98.4%
293	6	75	570	17	0.0	1,422	2,084	311	0.0	4.0	4.6	0.9	1,638	1,647	79.1%	99.4%
311	7	92	581	0.0	0.1	912	1,585	353	0.0	0.0	14	0.0	1,262	1,276	80.5%	98.9%

E-2C

Initial								Final								
Day	Cycle	PCE (umol/bottle)	TCE (umol/bottle)	cDCE (umol/bottle)	VC (umol/bottle)	ethene (umol/bottle)	sum (umol/bottle)	Day	PCE (umol/bottle)	TCE (umol/bottle)	cDCE (umol/bottle)	VC (umol/bottle)	ethene (umol/bottle)	sum (umol/bottle)	% Recovery	% Ethene
145	1	48	557	75	140	600	1,421	160	0.1	0.1	2.1	2.4	1,275	1,280	90.1%	99.6%
160	2	52	569	2.3	1.2	1,045	1,670	178	0.1	1.6	8.4	79	1,474	1,563	93.6%	94.3%
178	3	43	561	4.3	45	1,097	1,750	196	0.1	0.0	9.4	0.7	1,937	1,947	111.2%	99.5%
196	4	41	550	8.7	0.2	1,548	2,147	234	0.0	0.0	14	0.0	1,896	1,911	89.0%	99.2%
234	5	54	562	20	0.0	1,050	1,686	311	6.5	43	13	1.2	1,806	1,870	110.9%	96.6%
311	6	97	554	0.9	0.1	1,034	1,686	353	0.0	0.0	11	0.0	1,621	1,632	96.8%	99.3%

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