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EVALUATION OF DICHLOROMETHANE AS AN ELECTRON DONOR FOR REDUCTIVE DECHLORINATION OF TETRACHLOROETHENE TO ETHENE

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EVALUATION OF DICHLOROMETHANE AS AN ELECTRON DONOR FOR
REDUCTIVE DECHLORINATION OF TETRACHLOROETHENE TO ETHENE

A Thesis
Presented to
the Graduate School of
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by
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ABSTRACT

Tetrachloroethene (PCE) and trichloroethene (TCE) are the predominant contaminants at hazardous waste sites in the United States. Although less prevalent, dichloromethane (DCM) is also found at a number of sites. EPA classifies PCE and DCM as likely to be carcinogenic in humans by all routes of exposure, while TCE is classified as carcinogenic to humans by all routes. At some sites, releases of PCE, TCE and DCM comingle in the groundwater. Field evidence from one such site in California suggests that DCM is used as the electron donor for reductive dechlorination of TCE. Nevertheless, definitive evidence that DCM can serve as an electron donor for complete reduction of chlorinated ethenes to ethene is lacking. The primary objective of this thesis was to evaluate the use of DCM as an electron donor for reductive dechlorination of PCE to ethene. Two anaerobic enrichment cultures were used. One grows by organohalide respiration of PCE and TCE to ethene, with lactate as the electron donor. The other uses DCM as its sole source of carbon and energy and releases formate and acetate as fermentation products. The experimental design included treatments with a combination of the two cultures and addition of only DCM and PCE. A secondary objective was to perform a preliminary assessment of the microbe responsible for biodegrading DCM.

In the treatment inoculated with both cultures and provided with only PCE (2.4 mg/L) and DCM (9.7 mg/L), biodegradation of DCM and reductive dechlorination of PCE started at the same time. Repeated additions of DCM were consumed in 4-7 days, with only minor accumulation of chloromethane. Repeated additions of PCE were also consumed, with increases and then decreases of chlorinated ethene daughter products.

Ethene started to accumulate after approximately three months and by the end of the incubation period (80-130 days), ethene was the only daughter product detected. Formate, acetate, and propionate were detected as products from biodegradation of DCM. Other treatments confirmed that the chlorinated ethene culture can use formate and hydrogen as electron donors, but not acetate; which is consistent with the observed use of DCM as a sole electron donor. A treatment inoculated with the chlorinated ethene culture that received no electron donor failed to reduce PCE. Furthermore, the chloroethene culture was unable to biodegrade DCM and the DCM culture was unable to reduce PCE (with lactate provided as the electron donor).

The only isolate obtained in previous research that is able to grow anaerobically on DCM as a sole carbon and energy source via fermentation is *Dehalobacterium formicoaceticum* strain DMC. Preliminary attempts were made to evaluate if the DCM enrichment culture developed during this research also contains *Dehalobacterium* spp. PCR analysis of the enrichment culture tested positively for the presence of members of the phylum Firmicutes, which includes *Dehalobacterium*. Microscopic evaluation of the enrichment revealed an abundance of short rods, which were gram positive, which is also consistent with *Dehalobacterium*. Nevertheless, additional research is needed to determine a more specific identification of the microbe responsible.

The results of this study provide definitive evidence that it is possible for DCM to serve as an electron donor for reductive dechlorination of PCE to ethene. While it is inconceivable that DCM would ever be intentionally added to serve as an electron donor, the results are relevant to those sites where these contaminants are comingled.

DEDICATION

This thesis is dedicated to my mother, Ranjana Kanitkar and my father, Hemant Kanitkar.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
CM	Chloromethane
cDCE	*cis*-1, 2-Dichloroethene
DCM	Dichloromethane
DDI	Distilled, Deionized
EPA	Environmental Protection Agency
FID	Flame Ionization Detector
GC	Gas Chromatograph
HPLC	High Performance Liquid Chromatography
MCL	Maximum Contaminant Level
MCLG	Maximum Contaminant Level Goal
NCBI	National Center for Biotechnology Information
PCE	Perchloroethylene (tetrachloroethene)
PCR	Polymerase Chain Reaction
SRS	Savannah River Site
TCE	Trichloroethene
THF	Tetrahydrofolate
VC	Vinyl Chloride

CHAPTER ONE

1.0   INTRODUCTION

Tetrachloroethene (PCE) and trichloroethene (TCE) are the predominant contaminants at hazardous waste sites in the United States (33). Although less prevalent, dichloromethane (DCM) is also found at a number of sites. Occasionally, the two types of contaminants are found comingled. PCE, TCE and DCM are excellent organic solvents and were used widely in dry cleaning, paint stripping and degreasing after 1960 (2). As of January 2000, the Environmental Protection Agency (EPA) classified PCE and DCM as likely to be human carcinogens by all routes of exposure while TCE is classified as a carcinogenic to humans by all routes of exposure (2). Studies indicate that chronic exposure to DCM can increase the probability of getting liver and lung cancer and benign growths in humans. The maximum contaminant level goal (MCLG) and the maximum contaminant level (MCL) for all three contaminants have been set to 0 and 0.005 mg/L, respectively (8).

Chlorinated ethenes and DCM undergo anaerobic biodegradation via different pathways. Chlorinated ethenes primarily undergo sequential reductive dechlorination to *cis*-1,2-dichloroethene (cDCE), vinyl chloride (VC), and ethene. At each step a chlorine atom is replaced by hydrogen. When the chlorinated compound is used as a terminal electron acceptor linked to growth, the process is referred to as organohalide respiration. Halorespiration of PCE and TCE to cDCE is mediated by a variety of genera (12, 18, 29, 31, 32), however, cDCE is an environmentally unacceptable endpoint. The only genus that is able to metabolically reduce cDCE and VC to ethene is *Dehalococcoides*. *Dehalococcoides ethenogenes* strain 195 was the first microbe

discovered that is able to reductively dechlorinate PCE to ethene; however, transformation of VC to ethene is cometabolic (23-25) and consequently occurs at a slow rate. Other *Dehalococcoides* strains have since been identified that are able to halorespire VC to ethene at a high rate (15, 17, 27, 31). Some types of *Dehalococcoides* can also use PCE and TCE as terminal electron acceptors but not all can use VC by organohalide respiration. The unifying characteristic of *Dehalococcoides* is their ability to metabolically reduce cDCE to VC. All known strains of *Dehalococcoides* use only hydrogen as the electron donor and acetate as a carbon source. Because of their very specific nutritional requirements, *Dehalococcoides* typically grow best in the presence of fermentative microbes that convert organic substrates to acetate, hydrogen, and various growth factors.

On the other hand, DCM undergoes anaerobic biodegradation as a sole carbon and energy source and yields formate and acetate as products (21). Freedman and Gossett (10) developed an anaerobic enrichment culture that consumed DCM as the sole carbon and energy source. The culture produced stoichiometric amounts of methane (i.e., 0.5 mol CH_4/mol DCM) at low concentrations of DCM, while acetate and hydrogen accumulated at DCM concentrations that inhibited methanogenesis. Mägli et al. (22) subsequently isolated a novel anaerobic bacterium, *Dehalobacterium formicoaceticum* strain DMC, that uses DCM as its sole source of organic carbon and energy, forming acetate and formate as products. Strain DMC was isolated from a two-component culture, with the other member being a *Desulfovibrio* sp. (21). The *Desulfovibrio* sp. provided strain DMC with growth factors, which made isolation of strain DMC that much more challenging. Phylogenetic analysis of the 16S rDNA from *Dehalobacterium formicoaceticum* strain DMC revealed that it grouped closely

to *Clostridium bacillus* and showed 89% sequence similarity with *Desulfotomaculum orientis* and *Desulfitobacterium dehalogenans* (22). *Dehalobacterium formicoaceticum* strain DMC is a gram-positive rod that occurs in pairs (22).

Mägli et al. (20) proposed a pathway for DCM biodegradation by strain DMC (Figure 1.1). During the reaction of three moles of DCM with three moles of tetrahydrofolate, all of the chlorine atoms are removed as six moles of HCl and three moles of methylene tetrahydrofolate are formed. Two moles of the methylene tetrahydrofolate form two moles of formate; the other mole goes through a series of reactions, including a condensation with carbon dioxide, yielding one mole of acetate. Thus, the overall stoichiometry is 0.67 mol of formate plus 0.33 mole acetate per mole of DCM (plus 0.33 mol CO_2). ATP is formed during the final step of formate and acetate formation.

Since fermentative anaerobic biogradation of DCM yields acetate and formate, the possibility exists that this process could be beneficial to reductive dehalogenation. Acetate can serve as an electron donor for several types of microbes that reduce PCE and TCE to cDCE (19), and acetate is also the required carbon source for *Dehalococcoides* (17). Formate can be used directly as an electron donor (19), or via disproportionation to carbon dioxide and hydrogen, which is generally regarded as the universal electron donor for reductive dechlorination (9). Field evidence from an aquifer in California suggests that DCM is used as the electron donor for reductive dechlorination of TCE (personal communication, L. G. Lehmicke). Plumes of DCM and TCE are comingled at the site, and DCM is the only signficant source of potential electron donor. The plume of TCE has not migrated appreciably, and cDCE and VC have been detected. Nevertheless, definitive evidence

that DCM can serve as an electron donor for complete reduction of chlorinated ethenes to ethene is lacking.

The primary objective of this research was to evaluate the use of DCM as an electron donor for reductive dechlorination of PCE to ethene under controlled laboratory conditions. To do so, two anaerobic enrichment cultures were used. One grows by organohalide respiration of PCE and TCE to ethene, with lactate serving as the electron donor. The other uses DCM as its sole source of carbon and energy. A variety of treatments were evaluated, including a combination of the two cultures and addition of only DCM and PCE. A secondary objective of the research was to perform preliminary characterization of the DCM enrichment culture, including an assessment of the type of microbe most likely responsible for anaerobic biodegradation of DCM.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1   Chemicals and Media

VC (99.5%) was obtained from Fluka. Ethene (polymer grade, 99.9%) and methane (Chemical Parameter Grade, 99%) were obtained from Matheson. Chloromethane (CM; 99.5%) was obtained from Praxair. PCE (99.9%) was obtained from Sigma-Aldrich, TCE (99.5%) from Fisher, cDCE (99%) from TCI America, DCM (99.5%) from Fisher Scientific. PCE and DCM were added to experimental bottles as saturated solutions of mineral salts medium, containing approximately 0.90 and 235 mM, respectively. Sodium lactate syrup (containing 58.8-61.2% sodium lactate; specific gravity = 1.31) was obtained from EM Science. Sodium formate and sodium acetate (99.9%) were obtained from Mallinckrodt. High purity hydrogen (99.99%) was obtained from National. All other chemicals used were reagent grade, unless indicated otherwise.

The two enrichment cultures that were used as inoculum were maintained in an anaerobic mineral salts medium adapted from Edwards and Grbić-Galić (7), as follows: $MgSO_4$ (62.50 g/L) was replaced with an equimolar amount of $MgCl_2$ (49.43 g/L) and $Fe(NH_4SO_4)$ (2.84 g/L) was replaced with $FeCl_2 \cdot 4H_2O$ (1.98 g/L) and $Na_2S \cdot 9H_2O$ (0.24 g/L). The intent of these changes was to reduce the concentration of sulfate and thereby reduce consumption of electron donor by sulfate reducing bacteria.

2.2   Enrichment Cultures

Two obligate anaerobic enrichment cultures were used for this research. One grows by organohalide respiration of chlorinated ethenes, with lactate serving as the

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electron donor, and will be referred to as the MicroCED culture. The other uses DCM as its sole source of carbon and energy, and will be referred to as the DCM enrichment. A brief description of each culture is given below.

The MicroCED culture was started from microcosms consisting of soil and groundwater from the Twin Lakes area at the Savannah River Site(4). Wood (35) used the microcosms as an inoculum to develop a sediment-free enrichment culture in an anaerobic mineral medium similar to the one used in this research. Eaddy (5) subsequently characterized the enrichment culture with respect to the range of halogenated compounds that can be used as terminal electron acceptors (all of the chlorinated ethenes, 1,2-dichloroethane, 1,2-dibromoethane, and vinyl bromide), the types of electron donors that can be used (lactate and emulsified vegetable oil), pathogenicity, and the effects of exposure to oxygen, 1,1,1-trichloroethane, and pH levels below and above circumneutral. Peethambaram (28) quantified the yield of *Dehalococcoides* during growth of the MicroCED culture on PCE, 1,2-dichloroethane, and 1,2-dibromoethane. No significant growth of *Dehalobacter* or *Desulfitobacterium* occurred. A patent on the culture was obtained by the Savannah River National Laboratory.

The MicroCED culture was grown in 20 L canisters, as previously described (5). Maintenance consisted of additions of neat PCE and TCE (resulting in aqueous phase concentrations of ~15 and 40 mg/L, respectively), addition of lactate on a biweekly basis, addition of NaOH to maintain the pH between 6.6 and 7.1, and monitoring of the dechlorination process based on gas chromatography (GC) analysis of headspace samples (see below). The pH was measured in 0.5 mL samples using a Corning 345 pH meter and VWR SympHony probe. The meter was calibrated with

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4.0 and 7.0 buffer solutions. If the pH of a sample was below 6.6, 8 M NaOH was added to the enrichment culture. After equilibrating for 30 min, a new sample was removed and the pH was re-measured. The process was repeated until the culture pH was approximately 7. After approximately two weeks of incubation, the PCE and TCE were completely dechlorinated to ethene and more PCE and TCE was added. Approximately once every three months, 3-4 L of the culture was removed and replaced with fresh mineral medium.

The DCM enrichment culture was developed by Wang (34) using inoculum from a microcosm study conducted at Clemson University. DCM at a concentration of 13-15 mg/L was biodegraded in the microcosms without accumulation of CM. DCM biodegradation in the microcosms was enriched by gradually increasing the dose to 500 mg/L, which also inhibited methanogenesis. An aliquot from the microcosm was transferred to anaerobic mineral salts medium (1% v/v) to begin development of a sediment-free enrichment culture. Maintenance of the DCM culture was continued as part of the research for this thesis. The fourth transfer of the DCM culture was repeatedly spiked with DCM prior to its use as inoculum for this thesis, in order to increase the population of the DCM degrader The pH of the DCM enrichment culture was kept between 6.6-7.1 using the same procedure described above.

Repeated transfers of the DCM culture without addition of any other electron donor or organic carbon source provided evidence that DCM was used to support growth. A preliminary evaluation of the culture indicated that acetate, formate and hydrogen formed during DCM biodegradation, and less than stoichiometric amounts of methane formed (i.e., well below 0.5 mol CH_4/mol DCM). Microscopic evaluation

of the culture indicated the prevalence of short rods. A gram stain was predominantly gram positive. These properties are consistent with *Dehalobacterium formicoaceticum* strain DMC. Initial efforts to identify the microbe responsible for DCM biodegradation in the enrichment culture used in this research are described in Chapter 3.

2.3   Experimental Design

Table 2.1 summarizes the ten treatments that were prepared to determine if DCM can serve as the only electron donor for reductive dechlorination of PCE to ethene. Treatments varied with respect to the source of inoculum (one or both of the enrichment cultures), whether DCM, PCE or both were added, and if other potential electron donors were added (lactate, acetate, formate, or hydrogen). Triplicates of each treatment were prepared.

The objective of each treatment is summarized as follows.

Treatment #1 served to directly assess if DCM can serve as the sole electron donor for reductive dechlorination of PCE to ethene. As such, both enrichment cultures were added (5% v/v each), and both PCE and DCM were added; no other substrates were added.

Treatment #2 served as a positive control for reductive dechlorination of PCE to ethene. It was operated in the same manner as the MicroCED enrichment culture itself, receiving lactate as the electron donor; DCM was not added.

Treatment #3 served as a positive control for anaerobic biodegradation of DCM. It was operated in the same manner as the DCM enrichment culture, receiving DCM as the only source of carbon and energy; PCE was not added.

Treatment #4 served as a negative control for reductive dechlorination of PCE. It was inoculated with the MicroCED culture and PCE was added, but no electron donor was added.

Treatment #5 was used to test if the activity of the DCM enrichment culture would interfere with the MicroCED culture when it was provided with both PCE and DCM. This treatment was the same as #1 except that lactate was added as an electron donor in addition to DCM. Of particular interest was the possibility that DCM might inhibit PCE dechlorination and, conversely, that PCE (or one of its daughter products) might inhibit biodegradation of DCM.

Treatment #6 was used to evaluate if the DCM enrichment culture possessed any ability to reductively dechlorinate PCE, with lactate added as the electron donor.

Treatment #7 was used to evaluate if the PCE enrichment culture possessed any ability to anaerobically biodegrade DCM, with no other electron donor added.

Treatments #8, #9 and #10 were used to evaluate the ability of the MicroCED culture to use acetate, formate or hydrogen as the electron donor. These three compounds are likely products from anaerobic biodegradation of DCM.

The initial doses of PCE and DCM were 2.4 and 9.7 mg/L, respectively, which are equivalent to 2.0 and 12.5 µmol/bottle. These amounts of PCE and DCM were below the upper limit of each enrichment culture and it was anticipated that the concentrations would not be high enough for PCE to inhibit the DCM enrichment culture, and vice versa. When either compound was consumed, more was added.

The amounts of lactate, acetate and formate added were based on achieiving a 100 fold excess of the electron equivalents needed to reduce PCE to ethene. Each 2 µmol dose of PCE was, therefore, accompanied by a targeted amount of 1.6 meq: 42

µL of a 3.2 M solution of lactate (assuming 12 meq/mmol); 50 µL of a 4.0 M solution of acetate (assuming 8 meq/mmol); and 100 µL of a 8.0 M solution of formate (assuming 2 meq/mmol). Adding an equivalent amount of hydrogen (20 mL) was considered to be excessive, since hydrogen is directly available as an electron donor; such a high dose of hydrogen may have led to an increase in the amount diverted for processes other than reductive dechlorination (e.g., acetogenesis). Consequently, each dose of PCE to treatment #10 was accompanied by 2 mL of hydrogen, or 0.016 meq of electron donor.

All treatments were prepared in 160 mL serum bottles containing 100 mL of liquid. After transferring approximately 150 mL of the MicroCED and DCM enrichment cultures into an anaerobic chamber (containing approximately 98% N_2 and 2% H_2), the inocula and mineral medium were added to the serum bottles. Aseptic techniques were used. The bottles were then capped with grey butyl rubber septa, removed from the anaerobic chamber, and sparged with 30% CO_2/70% N_2 to adjust the pH and remove the hydrogen. The pH of each bottle was measured and adjusted to neutral, as needed, in the same manner described above for the enrichment cultures. After adding the appropriate amendments (PCE, DCM, lactate, acetate, formate or hydrogen), the bottles were incubated quiescently, shielded from light, at room temperature (~22-24°C), and with the liquid in contact with the septum to minimize the loss of volatile compounds.

2.4   Analysis of Volatile Organic Compounds and Hydrogen

PCE, TCE, cDCE, VC, ethene, DCM, CM, and methane were monitored by headspace analysis using a Hewlett Packard Series II 5890 GC, as previously described (10, 11). 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 (FID) in conjunction with a column packed with 1% SP-1000 on 60/80 Carbopack-B (Supelco, Inc.). The carrier gas was nitrogen (13). Aqueous phase concentrations were calculated based on the total mass present in the bottle, the volumes of the aqueous phase (100 mL) and gas phase (60 mL), and Henry's law constants, as previously described (13). Detection limits for the chlorinated compounds were less than their MCL. Representative response curves are shown in Appendix A.1.

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 was nitrogen with the column and reference flow rates were 16 and 35 mL/min, respectively. The column temperature was isothermal (105°C) and the injector and detector temperatures were 200°C. Representative response curve is shown in Appendix A.2.

2.5   Organic Acids and Sulfate

Organic acids were measured by high performance liquid chromatography (HPLC) using a 3000 Ultimate Dionex HPLC system and an Aminex$^{®}$ HPX-87H ion exclusion column (300-mm×7.8-mm; BioRad). Eluent (5 mM H_2SO_4) was pumped (0.6 mL/min) through the column into a UV/Vis detector set at 210 nm. The injection volume was 100 μL. After GC monitoring of a bottle was terminated, 1 mL of the aqueous phase was removed and filtered (0.2 μm) into 250 μL inserts within HPLC autosampler vials. Excess filtered sample was stored at 4ºC. Representative response curves for organic acids are shown in Appendix A.3.

Sulfate was measured using a Dionex DX-2100 Ion Chromatograph (IC) (Sunnyvale, CA). A degassed sodium carbonate/bicarbonate eluent (4.5 mM/0.8 mM, respectively) was used with an IonPac® AS9 guard column (AG11, 4 mm x 50 mm), followed by an IonPac® AS9-HC anion-exchange column (4 mm x 250 mm), at a flow rate of 1.0 mL/min. Samples (0.5 mL) from the microcosms were filtered (0.45 μm PTFE, NALGENE®) and 250 μL was injected onto the IonPac column. A representative response curve for sulfate is shown in Appendix A.4.

2.6 PCR

DNA was extracted from 5 mL samples removed, using the FastDNA® SPIN Kit (MP Biomedicals) according to the protocol provided by the manufacturer. After extraction, the 16S rRNA gene was selectively amplified by PCR, using oligodeoxynucleotide primers designed to anneal to conserved regions of the eubacterial 16S rRNA gene. The forward primer corresponded to positions 8 to 27 of *Escherichia coli* 16s rRNA (5'-AGAGTTTGATCCTGGCTCAG-3'), and the reverse primer corresponded to the complement of positions 1510 to 1492 (5'-GGTTACCTTGTTACGACTT-3'). DNA (3 μL, containing 10 ng) was mixed with $MgCl_2$ (6 μL, 2.5 mM), dNTPs (0.5 μL, 0.20 mM), forward and reverse primers (1.2 μL, 1 μM), PCR buffer (5 μL), and Taq polymerase (0.2 μL, 2.5u). The volume of the mixture was brought to 50 μL with DNA/RNA free water. The reaction was performed in a 0.2 mL PCR tube with a flat cap in an Eppendorf master cycler gradient. The mixture was subjected to 40 cycles, with each cycle consisting of denaturation for 1.5 min at 92ºC, primer annealing at 37°C for 1 min, and chain extension at 72°C for 2 min (first cycle) or more; 5 s was added to the extension time per cycle (6). The reaction products were checked using gel electrophoresis.

Since a member of the *Dehalobacterium* genus is currently the only known microbe capable of growing anaerobically on DCM as the sole source of carbon and energy, it was of interest to determine if the same type of microbe is responsible for DCM biodegradation in the enrichment culture used in this study. The first step taken was to determine if Firmicutes are present, since the *Dehalobacterium* genus is part of this phylum. The primer set used was 944F (5'-GGAGYATGTGGTTTAATTCGAA GCA-3') and 1070R (5'-AGCTGACGACAACCATGCAC-'3) (14). DNA extracted from *Clostridium beijerinckii* 8052 was used as a positive control and the negative control was DNA/RNA free water.

As the results will show, the DCM enrichment culture was positive for Firmicutes. The next step was to evaluate the culture using a primer set specific for *Dehalobacterium*. The following primer set was used: DF1038F (5'-GGCGAAGGA GTGATCTGGAG-3') and DF1262R (5'- CACCTTCCGATACGGCTACC -'3). The NCBI primer BLAST tool was used to identify the primer. An attempt was made to obtain a sample of *Dehalobacterium formicoaceticum* strain DMC (DSM 10151) from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), to serve as a positive control. Unfortunately, DSMZ was unable to grow *Dehalobacterium formicoaceticum* strain DMC and, therefore, was unable to provide a sample (personal communication, Dr. R. Pukall, Curator Gram-positive Bacteria). Consequently, the PCR was run without a positive control.

CHAPTER THREE

3.0 RESULTS

3.1 Reductive Dechlorination of PCE with DCM as the Sole Electron Donor

The prospect of DCM serving as the sole electron donor for reductive dechlorination of PCE was confirmed with treatment #1, as shown in Figures 3.1-3.3 for the triplicate bottles. Biodegradation of DCM and reductive dechlorination started at approximately the same time. Repeat additions of DCM were consumed in 4-7 days, with only a minor amount of CM accumulation (i.e., <0.005 mol CM/mol DCM consumed). Reductive dechlorination of PCE followed the expected patern, i.e., an accumulattion and then decline in TCE, followed by cDCE and then VC. Ethene started to accumulate between days 80 and 130, after VC reached a plateau.

The treatment #1 bottles were only terminated after it was clear that complete dechlorination of PCE to ethene with DCM as the electron donor was confirmed. This took the least amount of time with bottle #1 since the expected pattern of PCE and DCM additions was maintained throughout. With bottles #2 and #3, however, an unintended addition of PCE was made after day 150. The PCE addition to bottle #2 was below the previously targeted amount of 2.0 μmol/bottle. It is noteworthy that the subsequent decrease in PCE resulted in only a minor increase in VC; most of the PCE was reduced to ethene with only a minor accumulation of daughter products. With bottle #3, the last addition of PCE on day 174 was approximately 10 times higher than the targeted amount, resulting in an aqueous phase concentration of 24 mg/L. As in bottle #2, most of the PCE was reduced to ethene with only a minor accumulation of daughter products. This indicated that the DCM enrichment culture can tolerate a relatively high concentration of PCE (~15% of its solubility in water),

and that once the two enrichment cultures are in sync, most of the PCE is dechlorinated to ethene with only a minor transient increase in VC.

3.2 Comparison of PCE and DCM Consumption

Complete results for treatments #2-10 are presented in Appendix B.1. Figure 3.4 summarizes the performance of all of the treatments with respect to the cumulative amount of PCE consumed, ethene formed, and DCM consumed. Treatments #2, 5, 9 and 10 consumed similar amounts of PCE (42-48 µmol/bottle). Active consumption of PCE was expected for treatment #2, since it served as a postive control for the MicroCED culture (i.e., lactate was the only electron donor added). The performance of treatment #5, which received both lactate and DCM, confirmed that DCM (at the concentration added) was not inhibitory to PCE consumption; instead, consumption of DCM appears to have initially enhanced PCE removal (compared to the treatments with no DCM added). Active consumption of PCE in treatments #9 and 10 confirmed that the MicroCED culture uses formate and hydrogen as electron donors, respectively, yielding similar results to use of lactate (treatment #5). Cumulative consumption of PCE in treatment #1 exceeded the others due to the inadvertant addition of PCE after day 150 (Figures 3.2 and 3.3).

The lack of significant PCE consumption by treatment #6 confirmed that the DCM enrichment culture alone was unable to perform reductive dechlorination. A minor level of PCE consumption occurred with treatment #7, indicating that acetate was not an effective electron donor for the MicroCED culture.

Treatments #2, 5, 9 and 10 yielded similar amounts of ethene (32-34 µmol/bottle); the total formed in treatment #1 was higher due to the inadvertently higher amount of PCE added. There was noticeable variability among treatments in

the onset of ethene accumulation. The shortest lag time occurred in treatment #5, followed by treatment #2 (the positive control for the MicroCED culture). Next were treatments #9 and 10, which received formate and hydrogen as electron donors, respectively. The slowest onset of ethene accumulation was in treatment #1, which received only DCM as an electron donor.

DCM was actively biodegraded in treatments #1, 3, and 5. Treatment #3 served as a positive control; it was inoculated with the DCM enrichment culture and DCM was the only compound added. Once it was established that DCM enrichment culture was performing as expected, DCM additions were stopped, even though treatments #1 and 5 were continued. Although DCM consumption started at similar rates in each of these treatments, the rate of conumption after ~day 40 was higher in the treatments #1 and 5, which were simultaneously consuming PCE. The lack of DCM consumption by treatment #7 confirmed that the MicroCED culture does not contain the microbe needed for anaerobic biodegradation of DCM.

Table 3.1 summarizes results for the overall incubation period. The recovery of ethene in relation to the amount of PCE consumed ranged from 66-77% for treatments #1, 2, 5, 9 and 10. Although lower than anticipated, ethene was the only product remaining at the end of the incubation period and TCE, cDCE and VC were below their MCL levels. Diffusional losses through the septa may have contributed, along with potential errors in the GC response factors used to quantify the volatile compounds. Regardless, it is evident that ethene was the only signficant product from PCE in all of the treatments in which signficant amounts of PCE were consumed.

The rate at which *Dehalococcoides* (presumptively) grew in treatments #1, 2, 5, 9 and 10 may be related to the extent of daughter product accumulation. The

highest amount of cDCE and VC that accumulated is shown in Table 3.1; TCE was relatively minor throughout. The lowest amount of cDCE accumulated in treatment #5, while the highest amounts accumulated in treatments #1 and 2. The lowest level of VC that accumulated was in treatment #2, while the highest amount was in treatment #1. These results suggest that although DCM can be used as an electron donor for reductive dechlorination of PCE, the rate of growth of *Dehalococcoides* is slower than with conventional electron donors; the slower rate of ethene accumulation by treatment #1 is consistent with these observations (Figure 3.4b).

Table 3.1 also presents the amounts of electron donor consumed, expressed in terms of electron equivalents. All of the treatments consumed electron donor in considerable excess of the stoichiometric amount needed for complete dechlorination of PCE to ethene, which ranged from 0.34-0.38 meq/bottle. The electron equivalents of DCM consumed in treatment #1 was notably lower than the amount of lactate consumed by treatment #2 and the amount of formate used by treatment #9, but was similar to the amount of hydrogen consumed by treatment #10. The lower amount of DCM consumed may be related to the slower onset of ethene accumulation in treatment #1 compared to treatments #2, 5, and 9.

3.3 Methane, Organic Acids, Hydrogen, and Sulfate

Methane output was highest in treatments #1 (DCM added), #5 (DCM + lacatate added), and #10 (H_2 added) (Figure 3.5). The rate of accumulation roughly paralleled that for ethene, which was fastest for treatment #5, followed by #10 and #1 (Figure 3.4). Methanogenesis accounted for ~20% of the electron donor added (i.e., eeq of CH_4 formed/total eeq of e^- donor added) for treatments #1 and 10, 3-5% for treatments #3 and 5, and less than 2% in the remaining treatments.

The concentration of organic acids present in each treatment at the end of the incubation period is shown in Figure 3.6. Acetate was highest in the treatments that received lactate as the electron donor (#2 and 5) and in the treatments that received formate (#9) and hydrogen (#10). Acetate was added as the electron donor in treatment #8, which explains the residual level at the end of the incubation period. Formate accumulated above 0.14 mM only in the treatments that actively consumed DCM (#1, 3 and 5; Figure 3.4c). Propionate accumulated above 0.54 mM in the treatments that actively consumed PCE and/or DCM (#1, 2, 3, 5, 9 and 10).

Hydrogen was measured on the last day of incubation for all treatments and was below 0.002 μmol/bottle (Appendix B.2, Table B-1), suggesting it was consumed as it formed (or was added, in the case of treatment #10) and, therefore, did not accumulate. Sulfate was measured in the treatments that actively consumed PCE and/or DCM (#1, 2, 3, 5, 9 and 10); final concentrations ranged from 0.01-0.06 mM; this compares to 0.28 mM that was present in the mineral salts medium at the start of the experiment, indicating that sulfate reduction did occur. Sulfidogenesis consumed ~17% of the electron donor added (i.e., eeq of SO_4^{2-} presumptively reduced to H_2S/total eeq of e^- donor added) for treatment #3 (DCM only), ~6% for treatments #1 (DCM + PCE) and #10 (H_2 + PCE), and less than 2% in the remaining treatments; complete results for sulfate are shown in Appendix B.2, Table B-2.

3.4 Molecular Characterization of DCM Enrichment Culture

As previously stated, the diversity of microbial community within the DCM culture was unknown. The first step in characterizing the culture was to establish if bacteria in the Firmicute phylum are present, since *Dehalobacterium* are a part of this phylum and *Dehalobacterium* is the only genus known thus far that grows

anaerobically on DCM as a sole carbon and energy source. DNA extracted from treatment #3 (inoculated with the DCM culture and provided with only DCM) was PCR amplified using universal Eubacteria primers (8F 1492R). The same DNA was then evaluated using Firmicute specific primers. As shown in Figure 3.7, the presence of a 126 bp amplification product confirmed the presence of Firmicutes. The positive control for this reaction was DNA extracted from *Clostridium beijerinckii* 8052 and the negative control was DNA/RNA free water.

A preliminary clone library was constructed with DNA from the DCM enrichment culture. The protocols and the results are included in Appendix B.3. Out of the 15 clones that were sequenced, five were Firmicutes, lending further support for the hypothesis that the microbe responsibe for DCM biodegradation in the DCM enrichment culture is a member of this phylum.

4.0 DISCUSSION

The results of this study provide definitive evidence that it is possible for DCM to serve as an electron donor for reductive dechlorination of PCE to ethene. This was accomplished by combining two distinct enrichment cultures, one that grows on DCM as its sole carbon and energy source and releases acetate and formate as products, and one that uses chlorinated ethenes as terminal electron acceptors via organohalide respiration. While it is inconceivable that DCM would ever be intentionally added to serve as an electron donor, the results are relevant to those sites in which the two types of contaminants are comingled.

In the DCM enrichment culture developed for this study, formate, acetate and propionate were the main organic acids that accumulated during fermentation of DCM. Although only a minor amount of hydrogen was detected at the end of the incubation period in the treatments with DCM added (#1, 3 and 5), prior evaluation of the culture indicated a higher level of hydrogen accumulation during periods of active DCM consumption (data not shown). Given what is known about the DCM biodegradation pathway for *Dehalobacterium formicoaceticum* strain DMC, it appears unlikely that propionate was a direct product from DCM in the enrichment culture used for this study. Instead, propionate was probably produced by other microbes; accumulation of propionate may indicate an excess of hydrogen (30).

Of the organic acids that accumulated, acetate is the least likely to have served as an electron donor for reductive dechlorination of PCE to ethene, which was confirmed by the results for treatment #8, which was inoculated with the MicroCED culture and received acetate as the electron donor. Only a minor amount of PCE was

consumed, with cDCE being the main product. Various studies have shown that several chlororespiring microbes can use acetate as an electron donor for reduction of PCE and TCE to cDCE (3, 16). The lack of significant dechlorination activity in treatment #8 suggests such microbes are largely absent from the MicroCED culture. Propionate can serve as an electron donor via its fermentation to acetate and hydrogen. Utilization of formate as an electron donor for reductive dechlorination has not been widely studied. *Dehalococcoides* species that reductively dehalogenate cDCE and VC cannot use formate directly as an electron donor (24). However, formate disproportionation to hydrogen and carbon dioxide by mixed cultures can support complete reductive dechlorination of PCE (26). Besides serving as an electron donor, formate offers an advantage in terms of buffering capacity. Release of HCl via dechlorination combined with accumulation of organic acids from fermentation of an organic electron donor serves to depress alkalinity and potentially lead to a decrease in pH outside the range regarded as favorable for *Dehalococcoides* (i.e., ~6.5-7.5). Since enzymatic disproportionation of formate also produces bicarbonate, it acts as a buffer and thereby moderates changes in pH.

Although DCM was successful as an electron donor, the rate of VC reduction to ethene was slower in comparison to other treatments (Figure 3.4b). The treatment with formate added (#9) also lagged in comparison to treatments with lactate added (#1 and 5), suggesting there was some acclimation of the MicroCED culture to use of formate as an electron donor. Furthermore, the amount of formate added to treatment #9 (14.40 mmol/bottle) was considerably higher than the formate yield from the DCM consumed in treatment #1 (0.44 mmol/bottle).

The higher rate of DCM consumption in treatments #1 and 5 (which also consumed PCE) in comparison to treatment #3 (DCM only added) was unexpected. Fermentation of DCM is a thermodynamically favorable reaction, even at 1 atm of hydrogen (20, 22). Thus, consumption of the products from DCM fermentation is not required to sustain the process. Nevertheless, consumption of formate in treatments #1 and 5 (as evidenced by a lack of stoichiometric accumulation) improved the rate of DCM consumption. This is an advantageous outcome for in situ conditions in which PCE and DCM are comingled.

Compared to other halogenated organic compounds, the environmental fate of DCM is usually very favorable. This is reflected in the fact that at most hazardous waste sites where DCM has been released, it does not move very far from the source zone, even though DCM is very soluble (~20,000 mg/L, versus 150 mg/L for PCE) and does not adsorb strongly (K_{OC} = 8.80 mL/g, versus 364 mL/g for PCE). Presumably this is due to its biodegradability as a sole carbon and energy source. DCM is used as a substrate under a variety of redox conditions, including aerobic, denitrifying, and fermentative, and the biodegradation products (CO_2, Cl^- and organic acids) are non-toxic. This compares to other halogenated organic compounds including PCE and TCE, whose daughter products may persist and pose an even greater health risk than the parent compounds (as is the case with VC). The results of this study point to another advantageous feature of DCM in terms of its environmental fate, i.e., it can facilitate reductive dehalogenation of other compounds via the generation of organic acids that are readily used for organohalide respiration.

5.0 CONCLUSIONS AND RECOMMENDATIONS

Based on the experiments performed for this thesis research, the following conclusions were reached:

1. Results from treatment #1, (inoculated with the MicroCED and DCM enrichment cultures, provided with only PCE and DCM) show conclusively that DCM can serve as an electron donor for reductive dechlorination of PCE to ethene. The unintended spike to a much higher concentration of 24 mg/L, shows that microbes in the DCM were able to resist the higher concentration of PCE. These results are applicable to sites that have PCE, TCE and DCM plumes comingled with each other.

2. Formate, acetate and propionate were the major organic acids formed in each of the treatments. Formate was observed in treatments that actively degraded DCM which suggests that it was the likely to be a product of DCM degradation. It could be speculated that the formate disproportionated and supplied the hydrogen necessary for the reductive dechlorination to occur. This argument is supported by the performance of other treatments, which suggest the MicroCED culture (PCE → ethene) can use formate and H_2 as electron donors but not acetate.

3. The rate of DCM degradation was faster in Treatments #1 and #5 which showed active reductive dechlorination of PCE as well as DCM consumption, in contrast to treatment #3 which consumed DCM as its sole carbon and energy source. This too suggests that the consumption of the metabolic products of DCM degradation as electron donors for reductive dechlorination may have caused the rate of DCM degradation to be greater than observed in the DCM enrichment culture alone.

4. The successful amplification of the DNA extracted from the DCM degrading culture using Firmicute specific PCR primer suggests that a Firmicute is present in the DCM enrichment culture. Other indicators such as the presence of formate at the end of incubation period and the abundance of gram positive rods suggest that the microbe responsible for DCM degradation could be *D. formicoaceticum* or related to it. However, further investigation is needed to identify the microbe responsible for DCM degradation.

The following recommendations are offered:

1. Additional information is needed for the maximum concentration of PCE and DCM that results in inhibition of biodegradation. It is conceivable that higher concentrations of PCE and DCM than the ones used in this research could be encountered in situ, e.g., near a source zone containing DNAPLs of both compounds.

2. Further research is needed to identify the microbe responsible for DCM biodegradation, and if the biodegradation pathway it uses is the same or different from the one used by strain DMC.

TABLES

Table 2.1 Experimental design.

Treatment No.	Inoculum (mL)		DCM Addition[a]			PCE Addition[a]			Electron Donor Addition[a]			
	MicroCED Culture	DCM Culture	(μmol)	(μL saturated MSM)	(mg/L)	(μmol)	(mL saturated MSM)	(mg/L)	Lactate (μL)	Acetate (μL)	Formate (μL)	H$_2$ (mL)
1	5	5	12	50	9.7	2.0	2.2	2.4	0	0	0	0
2	5	0	0	0	0	2.0	2.2	2.4	50	0	0	0
3	0	5	12	50	9.7	0	0	0	0	0	0	0
4	5	0	0	0	0	2.0	2.2	2.4	0	0	0	0
5	5	5	12	50	9.7	2.0	2.2	2.4	42	0	0	0
6	0	5	0	0	0	2.0	2.2	2.4	42	0	0	0
7	5	0	12	50	9.7	0	0	0	0	0	0	0
8	5	0	0	0	0	2.0	2.2	2.4	0	42	0	0
9	5	0	0	0	0	2.0	2.2	2.4	0	0	100	0
10	5	0	0	0	0	2.0	2.2	2.4	0	0	0	2

[a]Initial amounts added; repeat additions of equal amounts were added.

Table 3.1 Summary of final data.

Treatment #	PCE consumed (µmol/ bottle)	Ethene produced (µmol/ bottle)	% Rec[a]	Max Formed (µmol/bottle)		Electron Donor Added (meq/bottle)				
				cDCE	VC	DCM	Lactate	Acetate	Formate	H$_2$
1	61.4±11.4[b]	46.0±15.5	74±11	20.3±6.7	15.8± 1.7	2.68±0.44	-	-	-	-
2	41.7±0.7	31.8±0.7	76±2	18.3±0.9	4.3± 0.1	-	5.40±1.88	-	-	-
3	-[c]	-	-	-	-	0.84±0.04	-	-	-	-
4	4.7±1.3	<0.2	-	0.4±0.4	0.3±0.6	-	-	-	-	-
5	47.6±1.6	31.5±1.6	66±5	5.9±3.8	8.2±0.7	1.44±0.05	5.76±0.20	-	-	-
6	2.2±0.1	<0.2	-	<0.05	-	-	3.40±0.04	-	-	-
7	-	-	-	-	-	0.02±0.003	-	-	-	-
8	5.3±0.6	<0.2	-	1.9±1.8	0.4±0.6	-	-	28.80±0	-	-
9	44.3±0.3	33.6±1.1	75±2	13.3±1.9	13.2± 2.6	-	-	-	28.80± 0	-
10	43.4±0.4	33.4±6.1	77±4	12.2±3.6	10.5±0.9	-	-	-	-	2.88±0

[a] % Rec = (moles of ethene formed)/(moles of PCE consumed)*100.

[b] ± = Standard deviation for triplicate bottles.

[c] A dash indicates PCE and/or electron donor was not added, and therefore there was no ethene, VC, or cDCE formed .

FIGURES
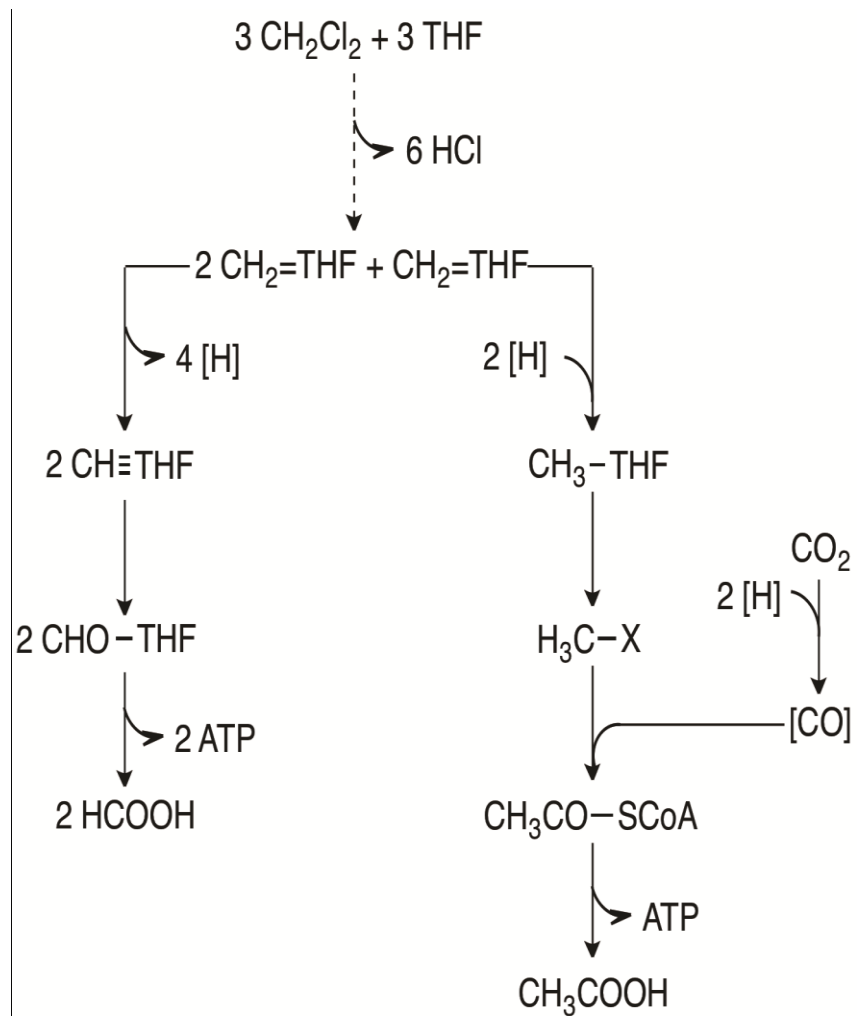
Figure 1.1 Pathway proposed by Mägli et al. (20) for anaerobic biodegradation of DCM by strain DMC; THF = tetrahydrofolate.
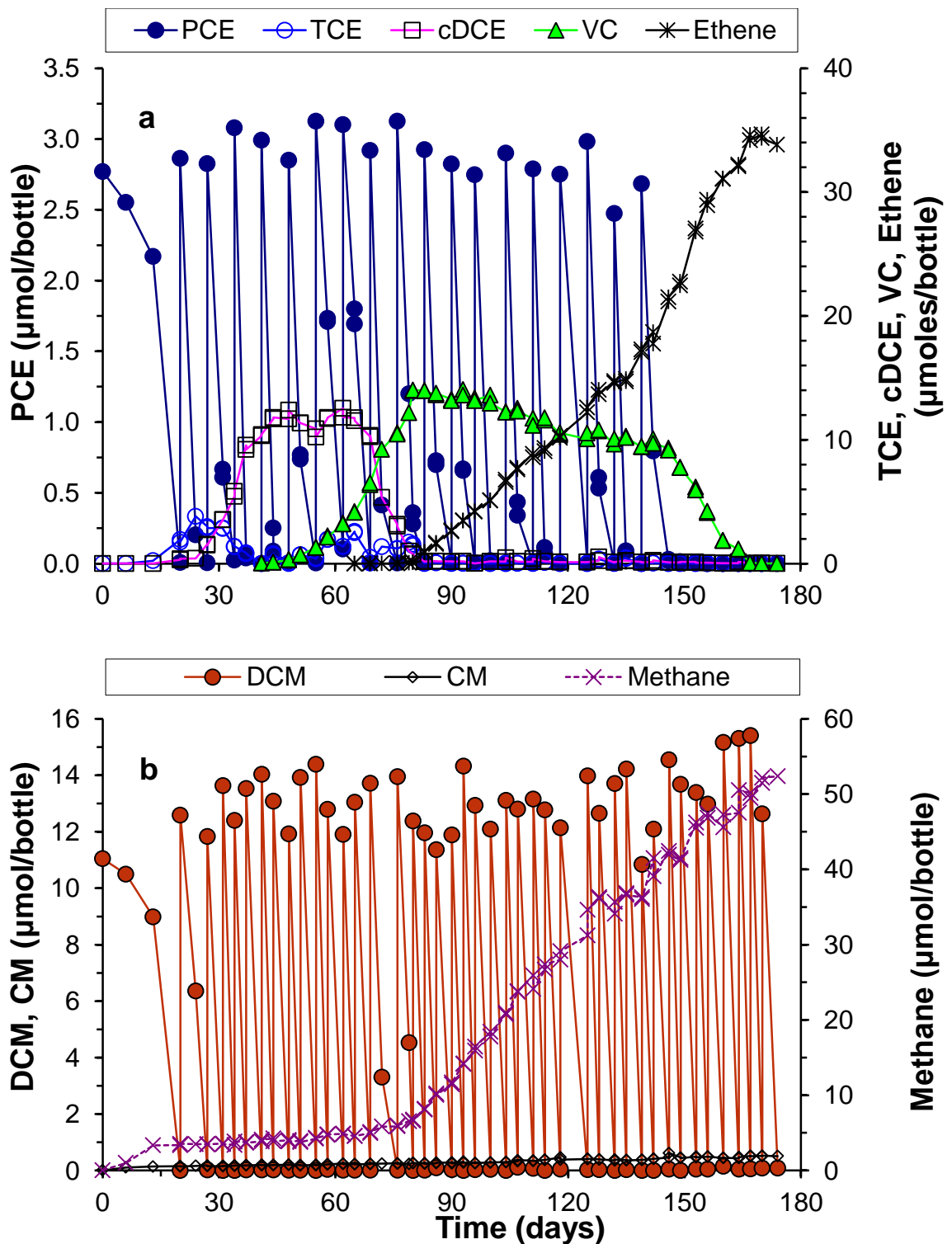
Figure 3.1 Results for treatment #1, bottle #1 (inoculated with MicroCED + DCM cultures and fed with PCE + DCM) showing **a)** the chlorinated ethenes and ethene; and **b)** DCM, CM and methane.
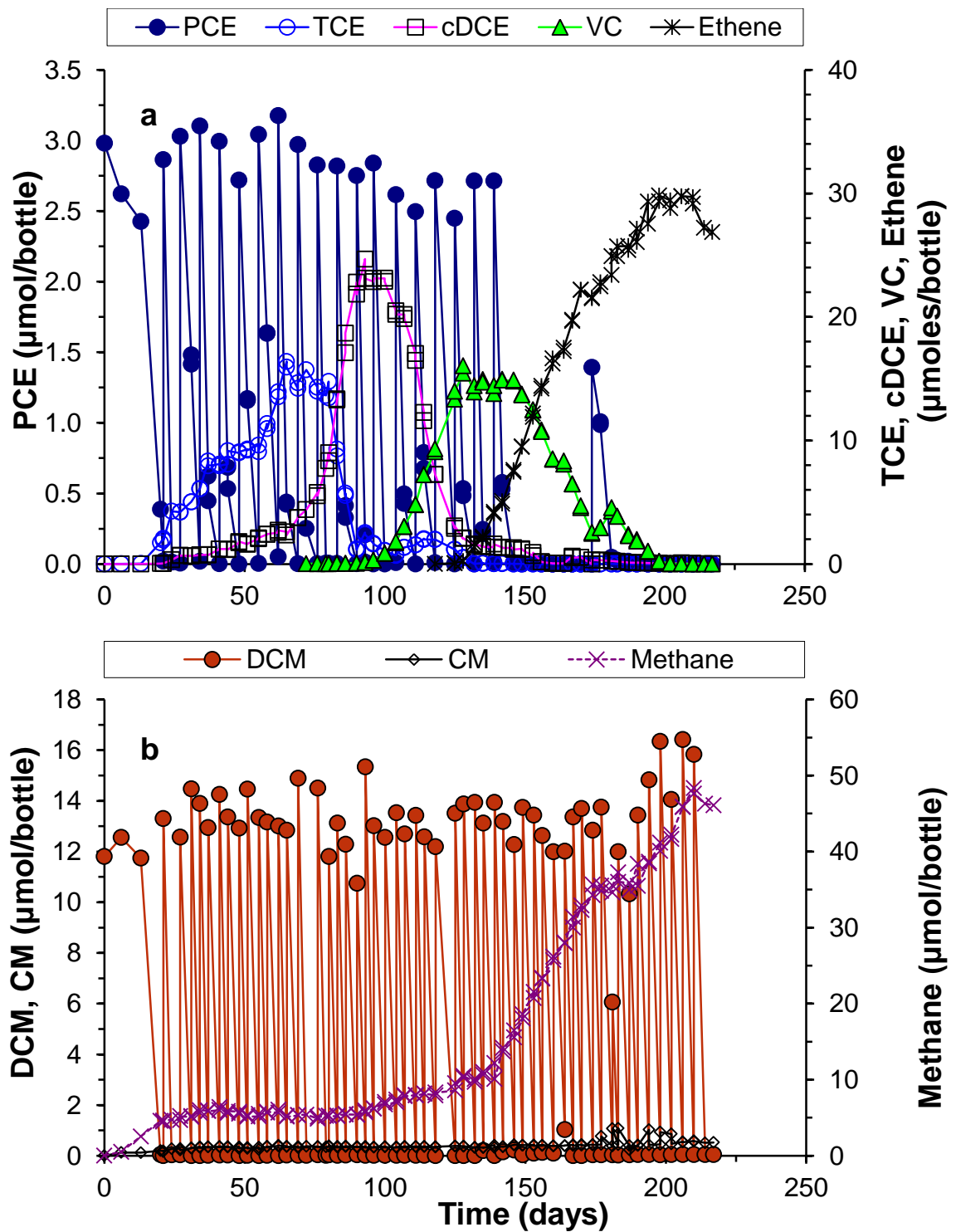
Figure 3.2 Results for treatment #1, bottle #2 (inoculated with MicroCED + DCM cultures and fed with PCE + DCM) showing **a)** the chlorinated ethenes and ethene; and **b)** DCM, CM and methane.
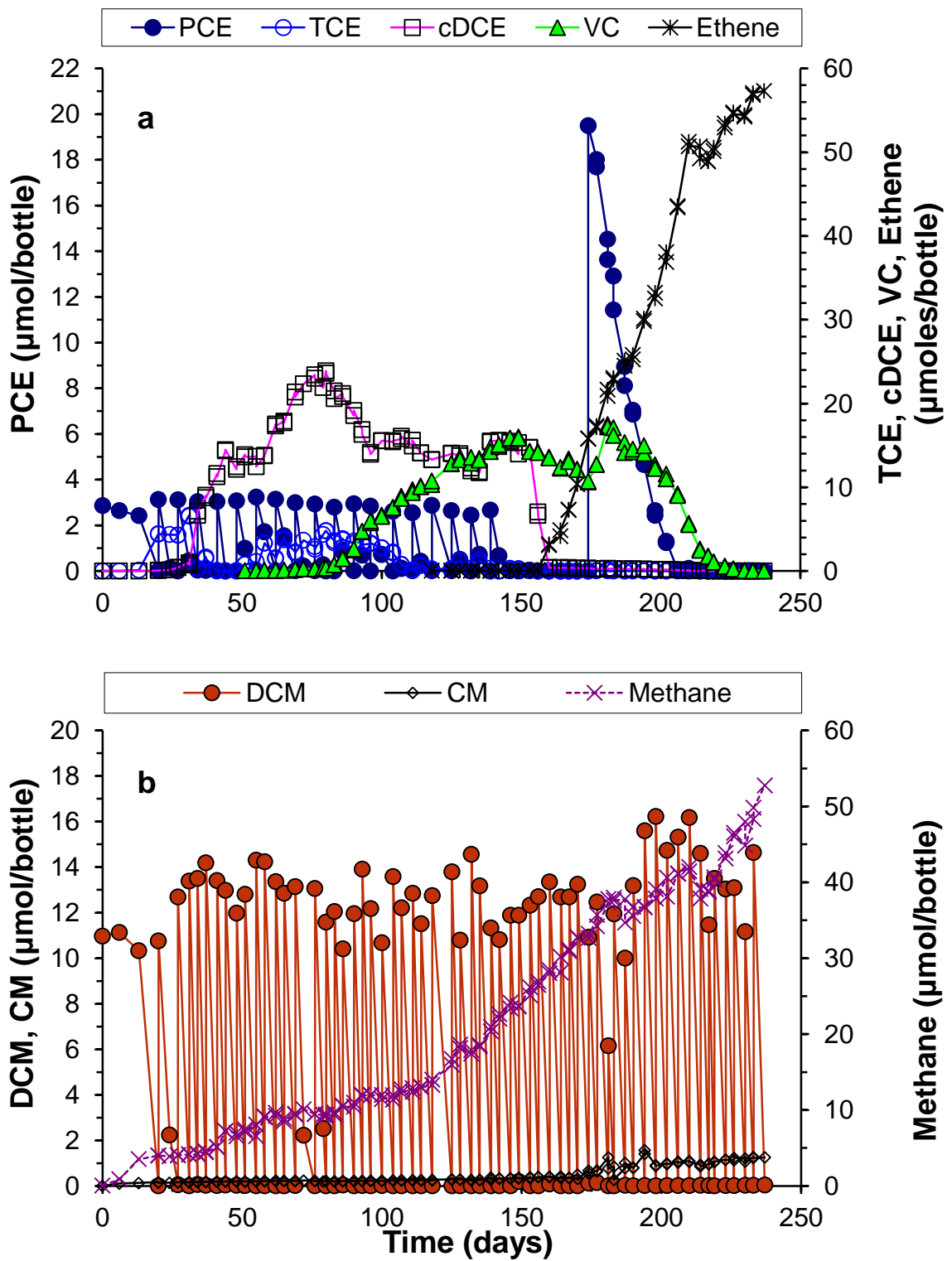
Figure 3.3 Results for treatment #1, bottle #3 (inoculated with MicroCED + DCM cultures and fed with PCE + DCM) showing **a)** the chlorinated ethenes and ethene; and **b)** DCM, CM and methane.
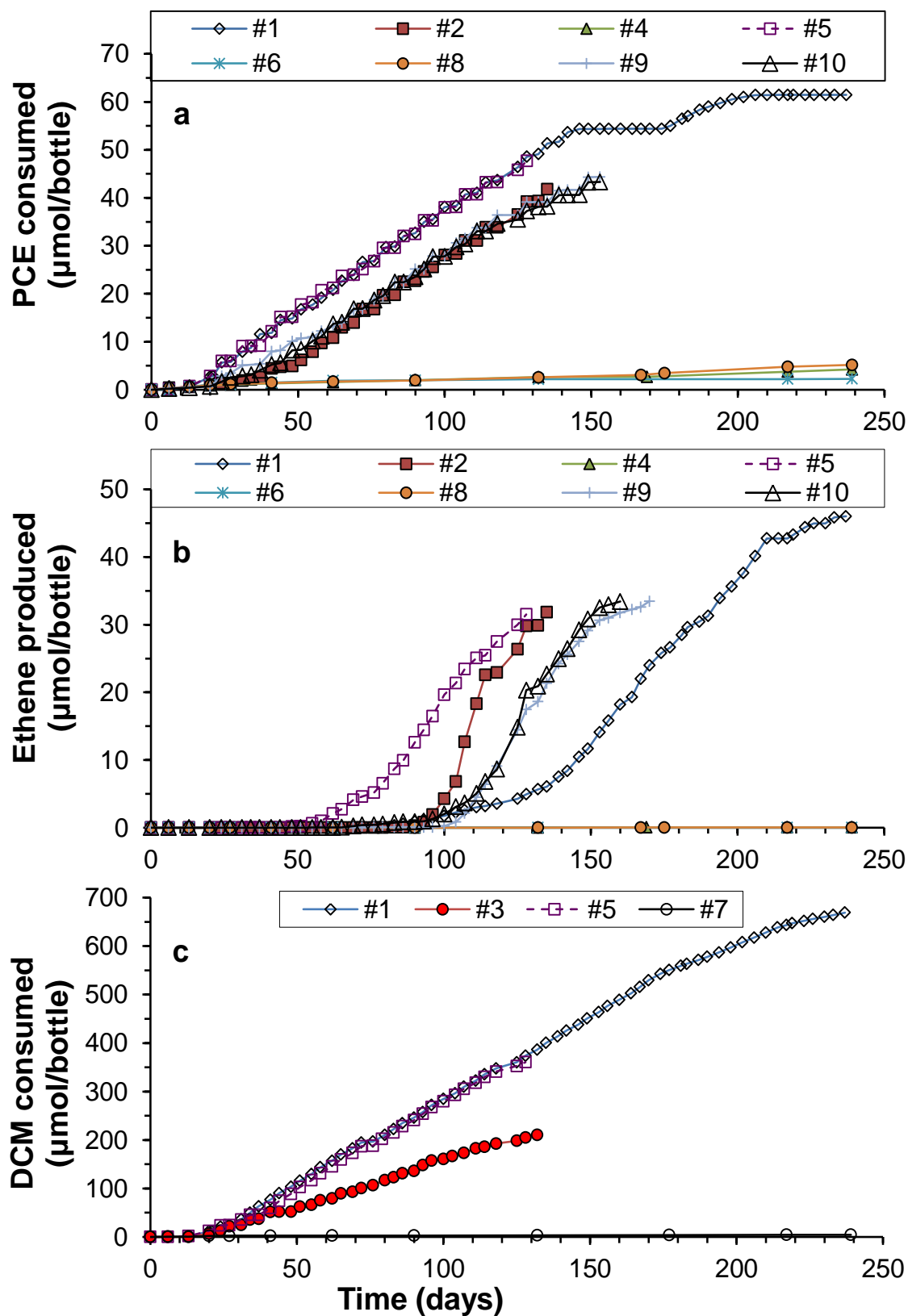
Figure 3.4 Summary of results for all treatments, showing the cumulative amount of **a)** PCE consumed; **b)** ethene formed; and **c)** DCM consumed.
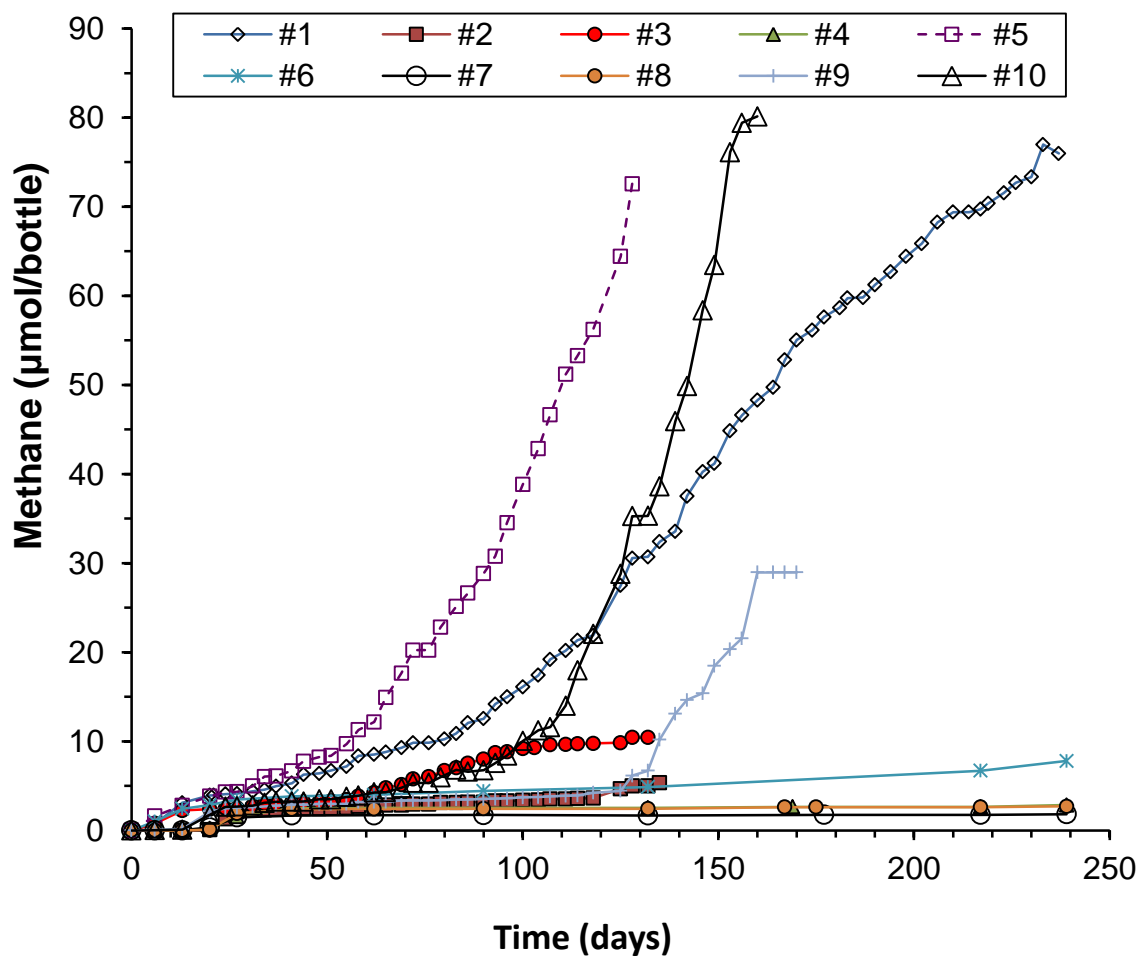
Figure 3.5 Summary of results for all treatments, showing the cumulative amount of methane formed.
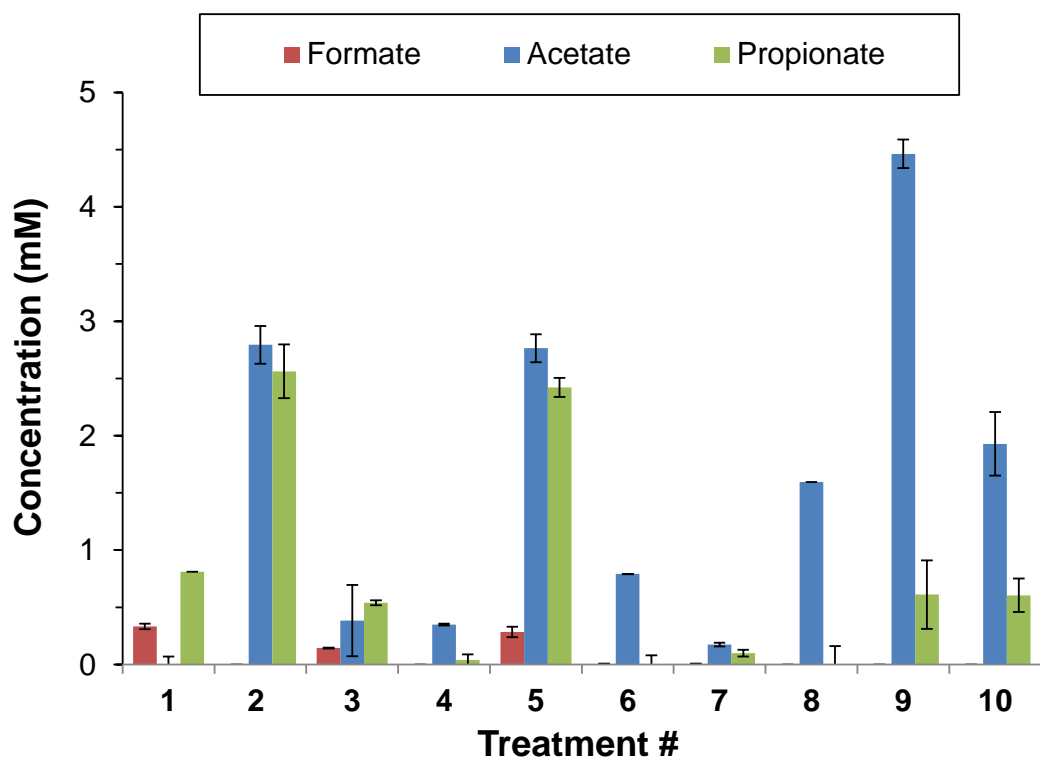
Figure 3.6 Summary of results for all treatments, showing the average level of organic acids present on the final day of sampling. Error bars represent the standard deviation for triplicate bottles.
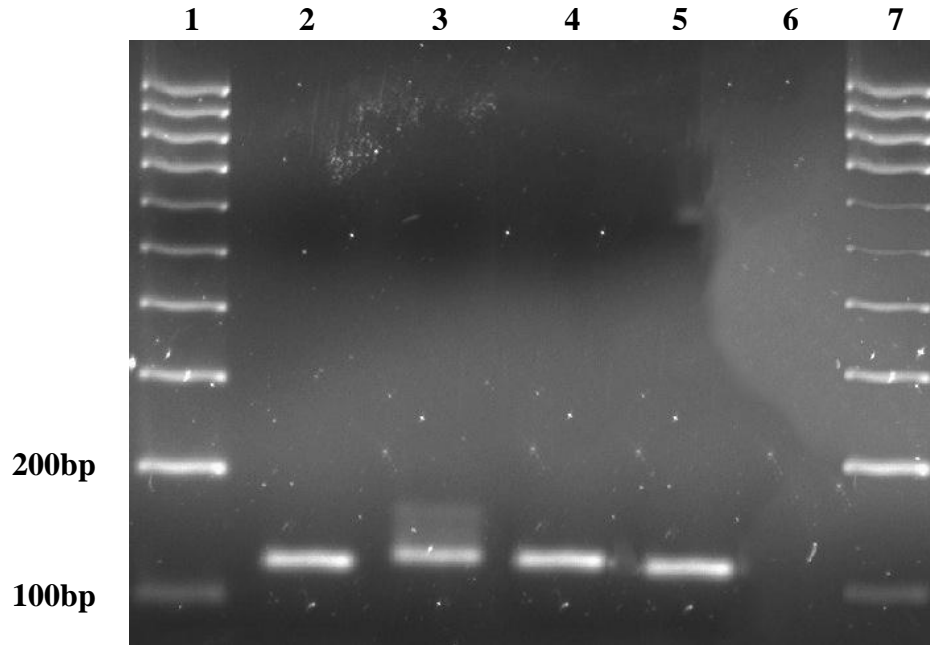
Lane	Description
1	λ ladder marker
2	Template DNA from Treatment 3, bottle 1.
3	Template DNA from Treatment 3, bottle 2.
4	Template DNA from Treatment 3, bottle 3.
5	Positive control, DNA from *Clostridium beijerinckii* 8052
6	Negative control, DNA/RNA free water
7	λ ladder marker

Figure 3.7 Agarose gel electrophoresis for PCR conducted with Firmicute specific primers.

APPENDICES

APPENDIX A.1:

Response Factors and Standard Curves for GC - FID

Table A-1 GC FID response factors used for 160 mL serum bottles.

Compound	GC Retention Time (min)	Response Factor (µmol/bottle/PAU)	R^2
Methane	0.5	1.6030E-06	9.9961E-01
Ethene	0.7	1.0616E-06	9.9943E-01
CM	2.1	1.2395E-05	9.9932E-01
VC	2.7	1.5109E-06	9.9647E-01
DCM	4.5	5.1415E-05	9.9410E-01
cDCE	7.4	1.6554E-05	9.9525E-01
TCE	10.8	6.8231E-06	9.9078E-01
PCE	14.5	4.3471E-06	9.9591E-01
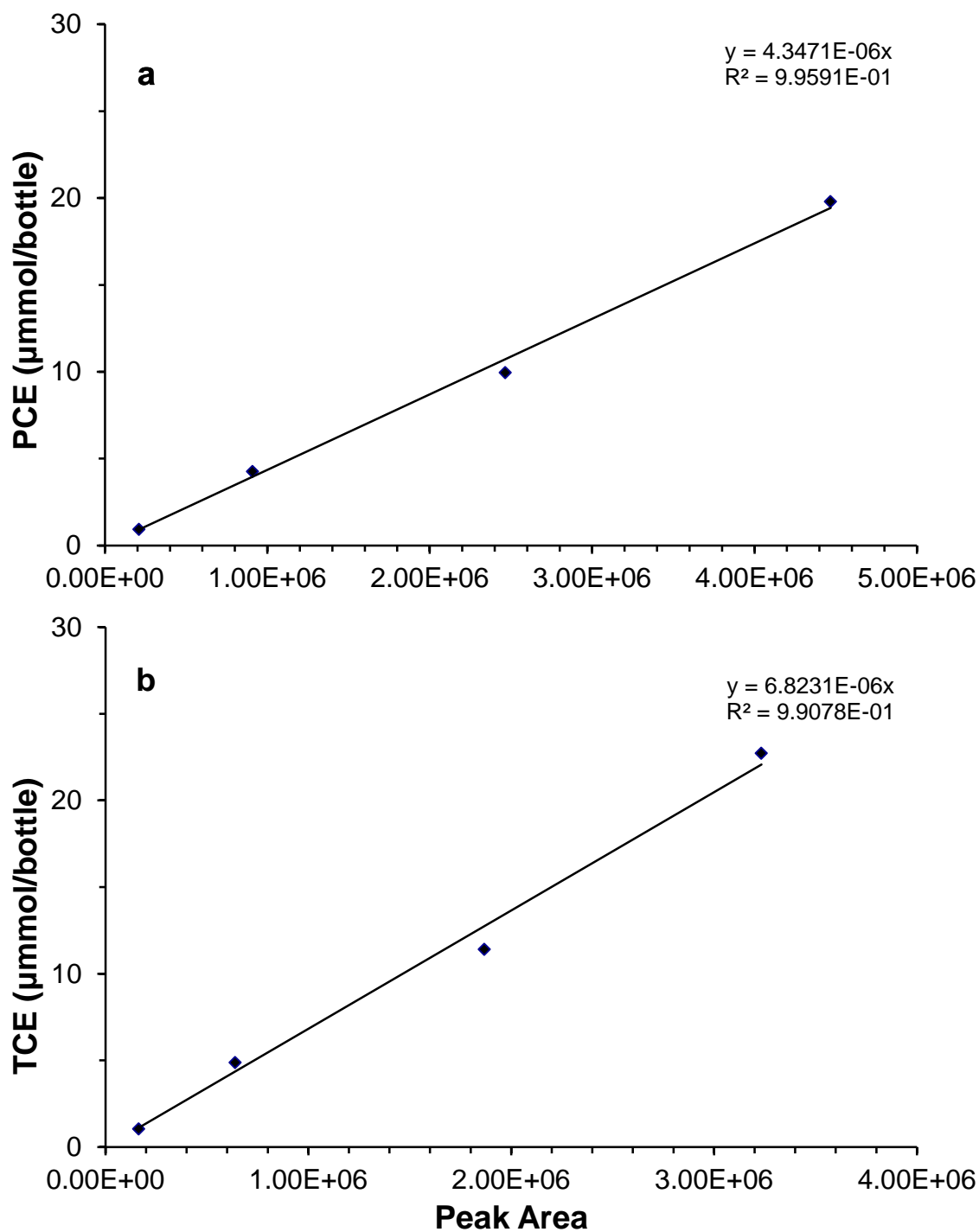
Figure A.1 GC FID response curves for a) PCE, b) TCE 160 mL serum bottle with 60 mL headspace.
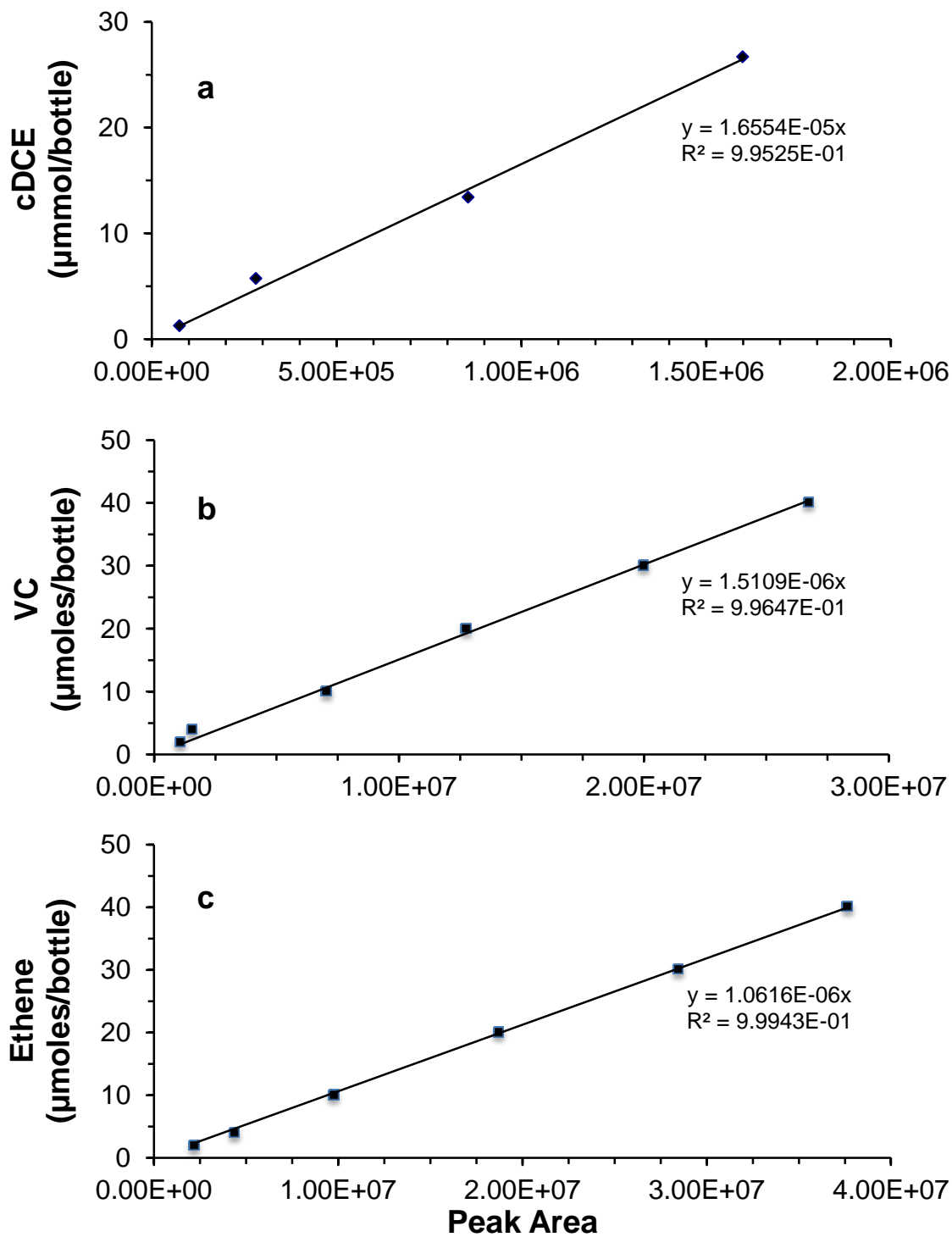
Figure A.2 GC FID response curves for a) cDCE, b) VC c) Ethene 160 mL serum bottle with 60 mL headspace.
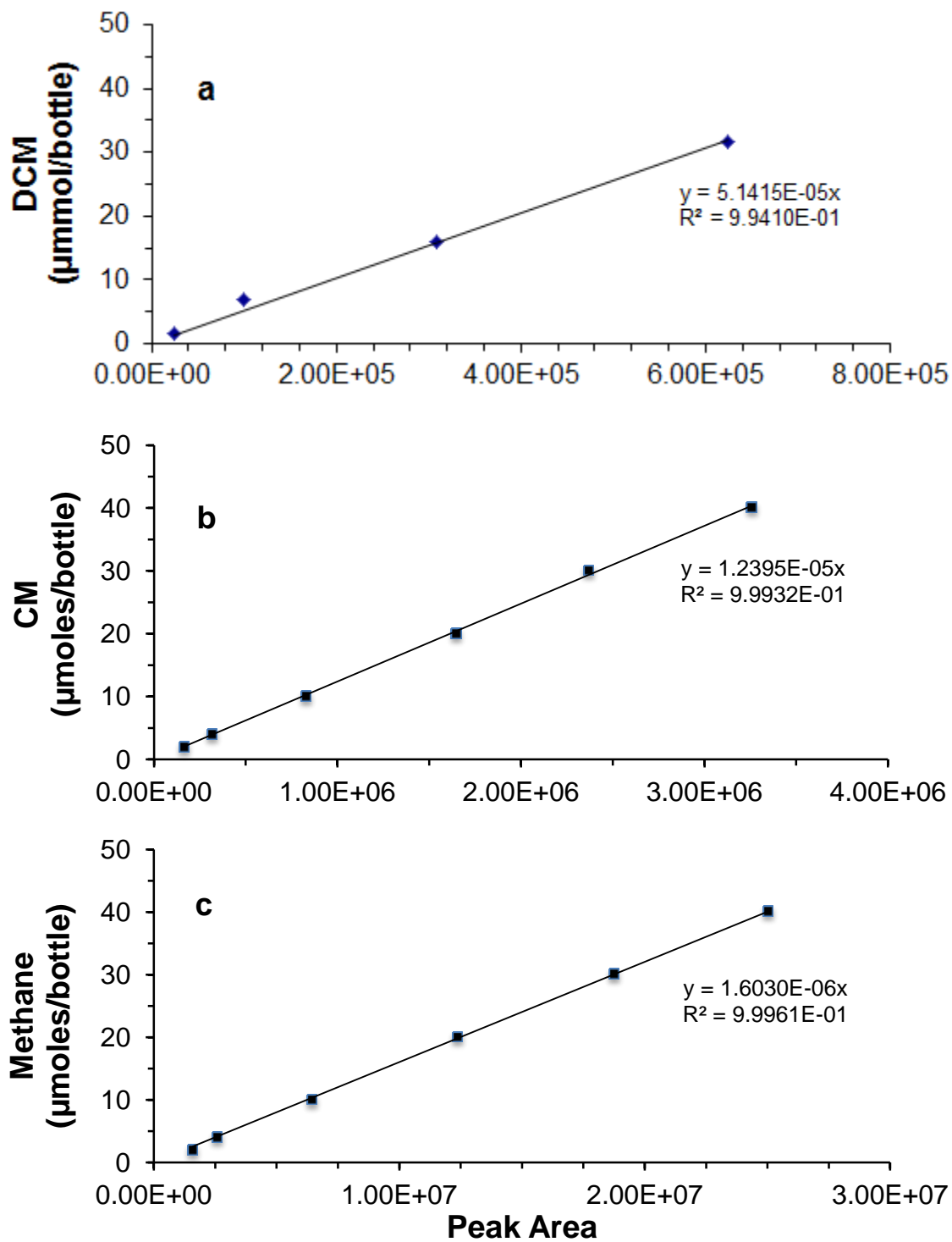
Figure A.3 GC FID response curves for a) DCM, b) CM c) Methane 160 mL serum bottle with 60 mL headspace.

Response Factor and Standard Curve for GC - TCD

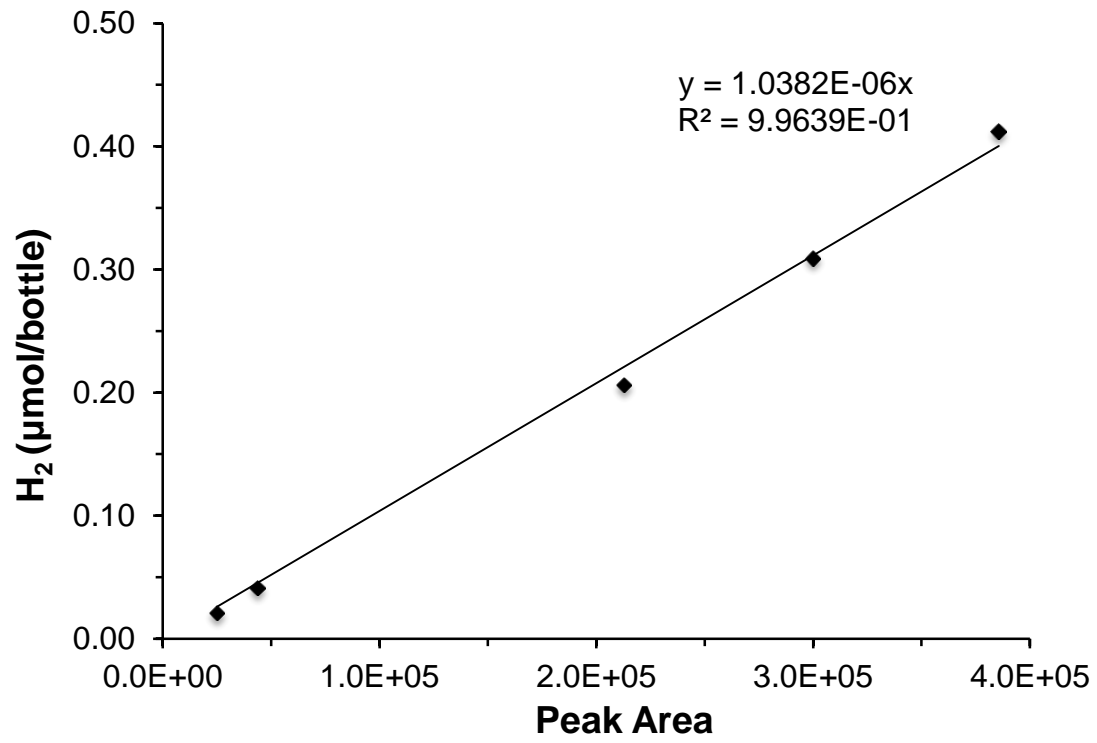**Figure A.2.1** GC TCD response curve for hydrogen.

Response Factors and Representative Response Curves for Organic Acids

Table A-2 HPLC response factors for organic acids.

Compound	Retention Time (min)	Response Factor (mM/PAU)	R^2
Lactate	12.84	0.0695	0.9968
Formate	14.22	0.1364	0.9990
Acetate	15.48	0.1960	0.9998
Propionate	18.23	0.1437	0.9995
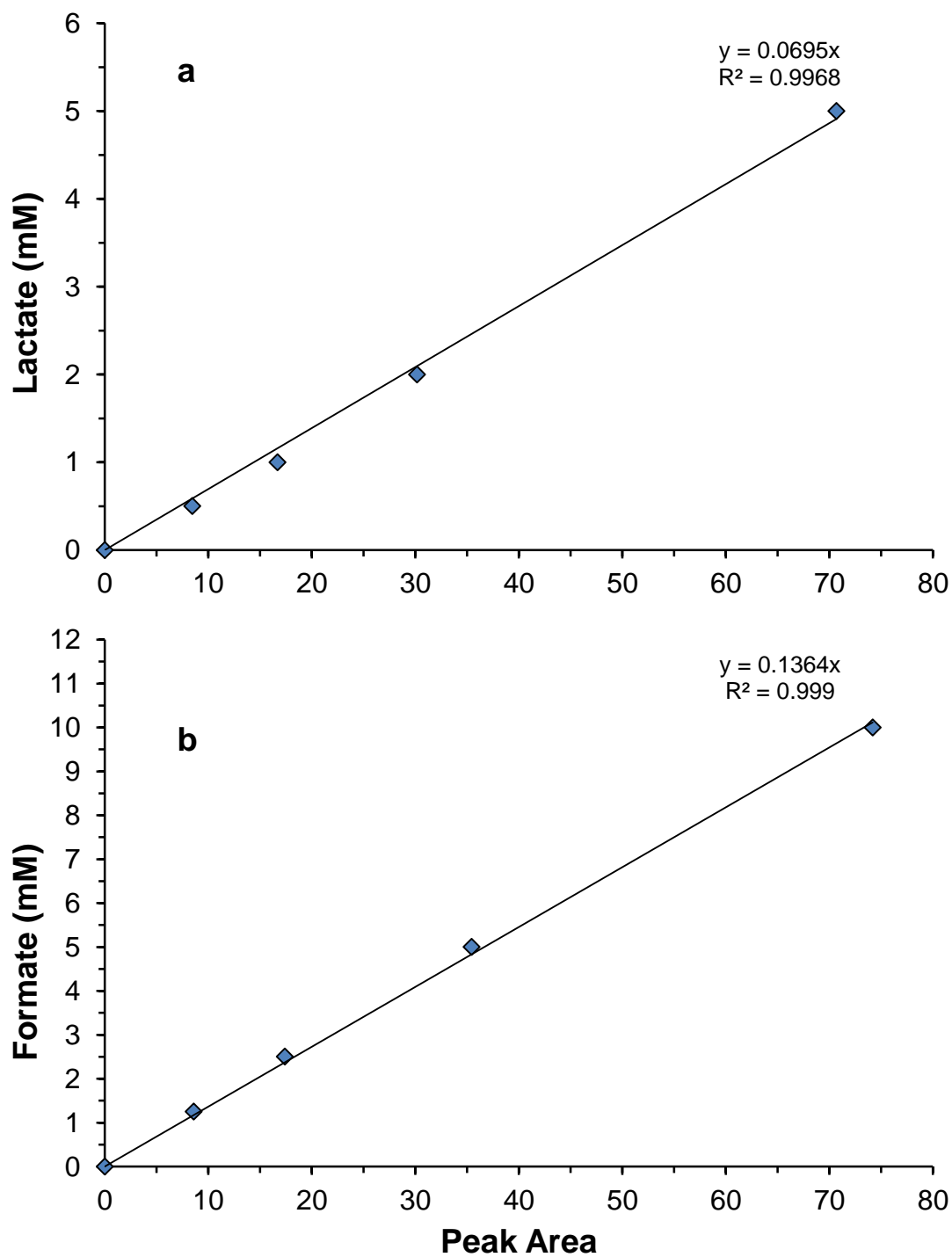
Figure A.3.1 HPLC response curves for a) lactate, b) formate.
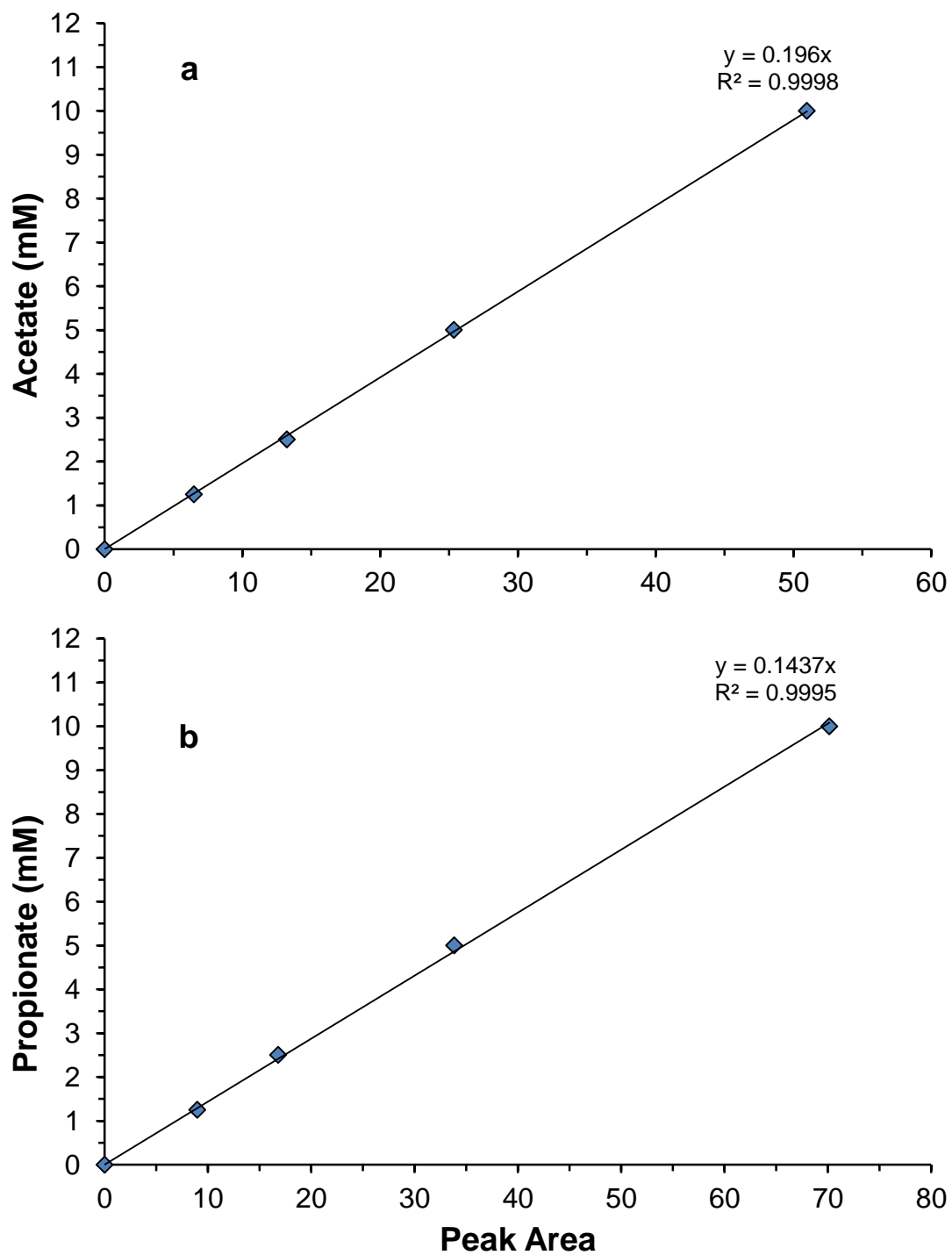
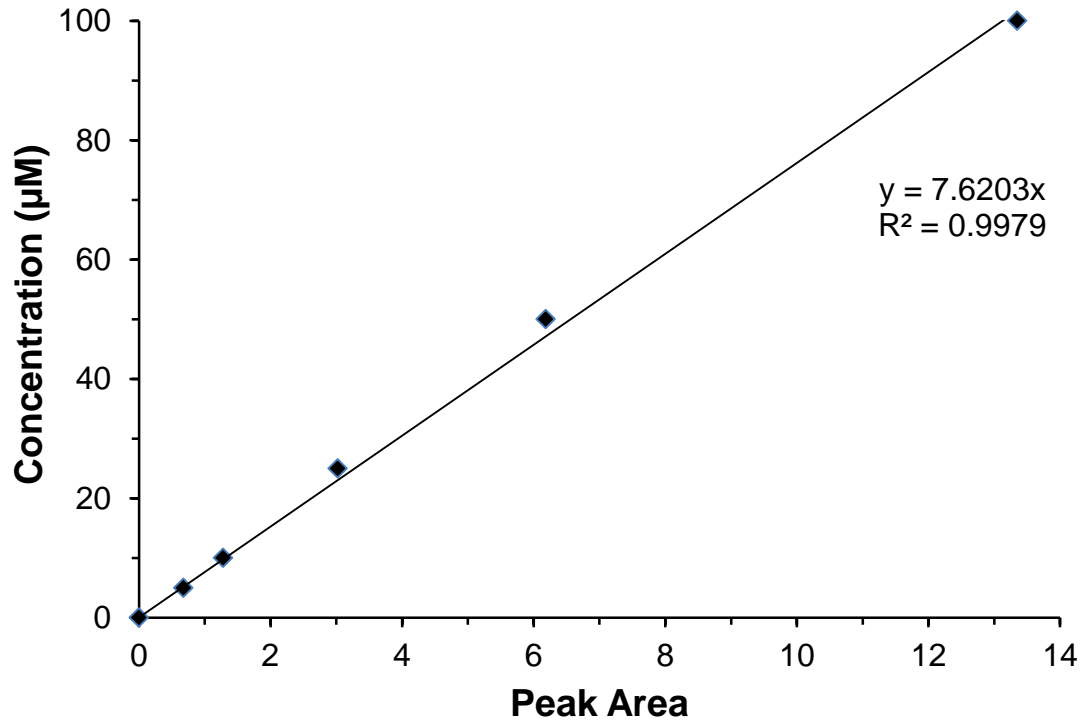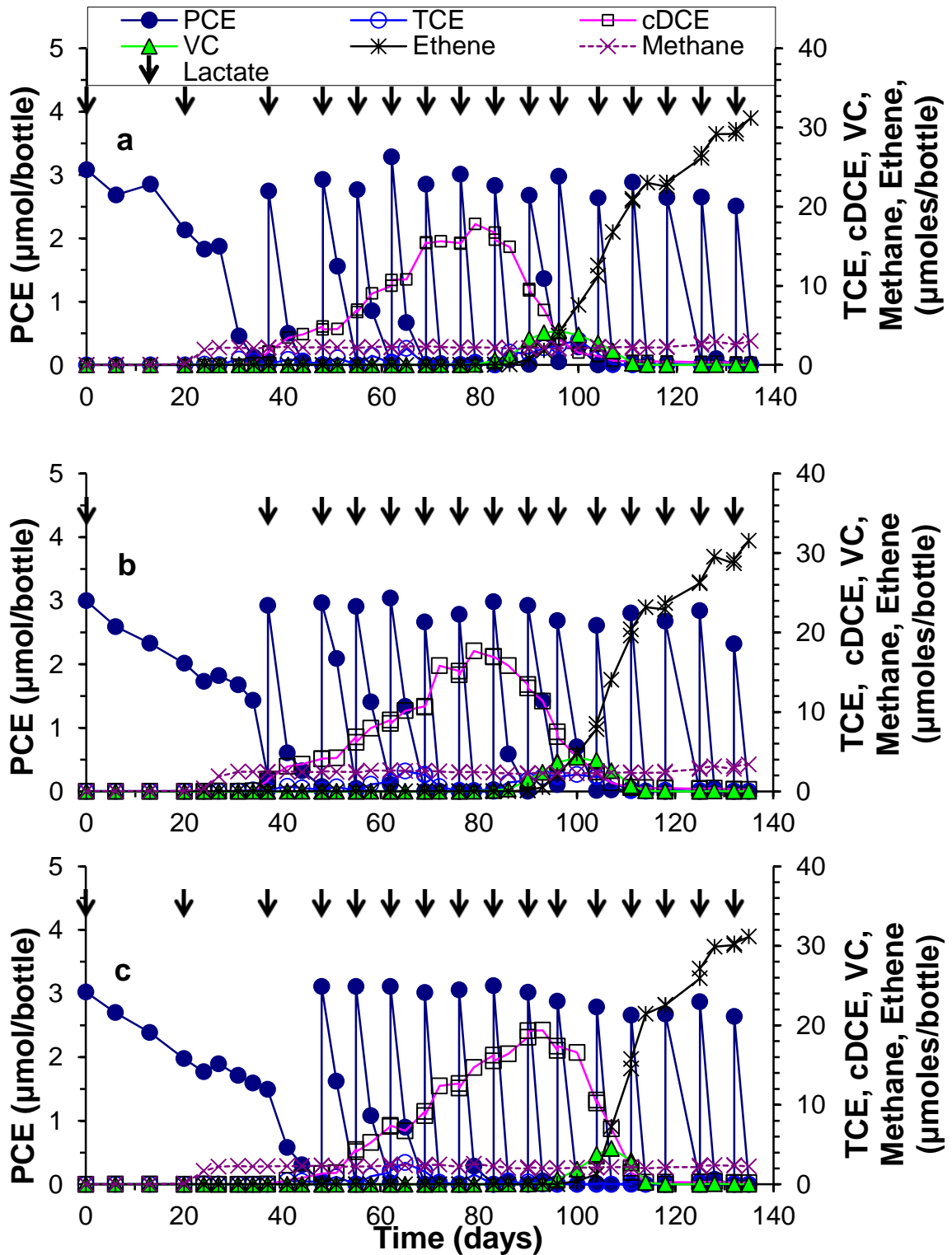**Figure A.3.2** HPLC response curves for a) acetate, b) propionate.
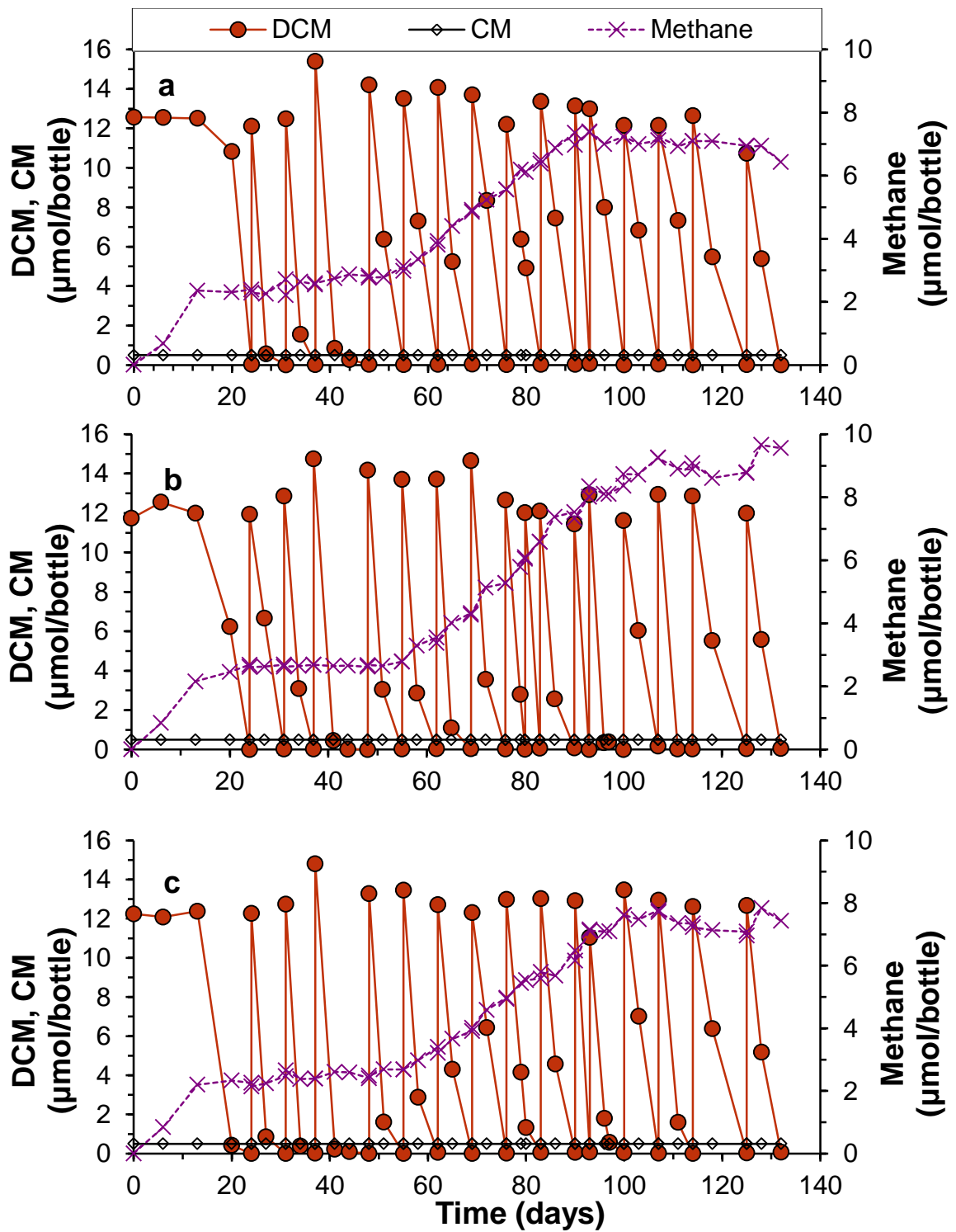
Response Factor and Representative Response Curve for Sulfate

Figure A.4.1 IC response curve for sulfate. (The response factor for the IC was 7.6203 µM/Peak area).
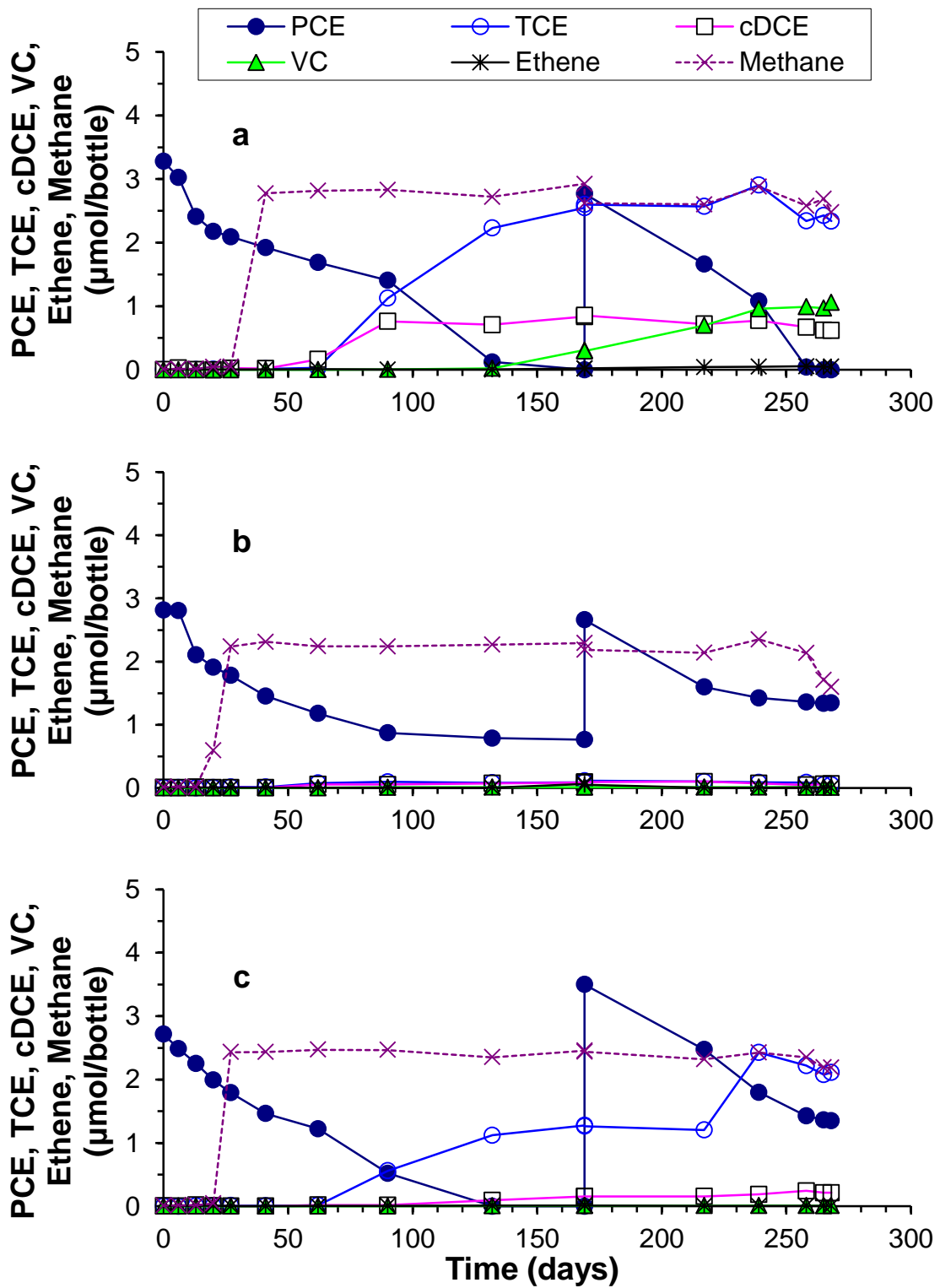
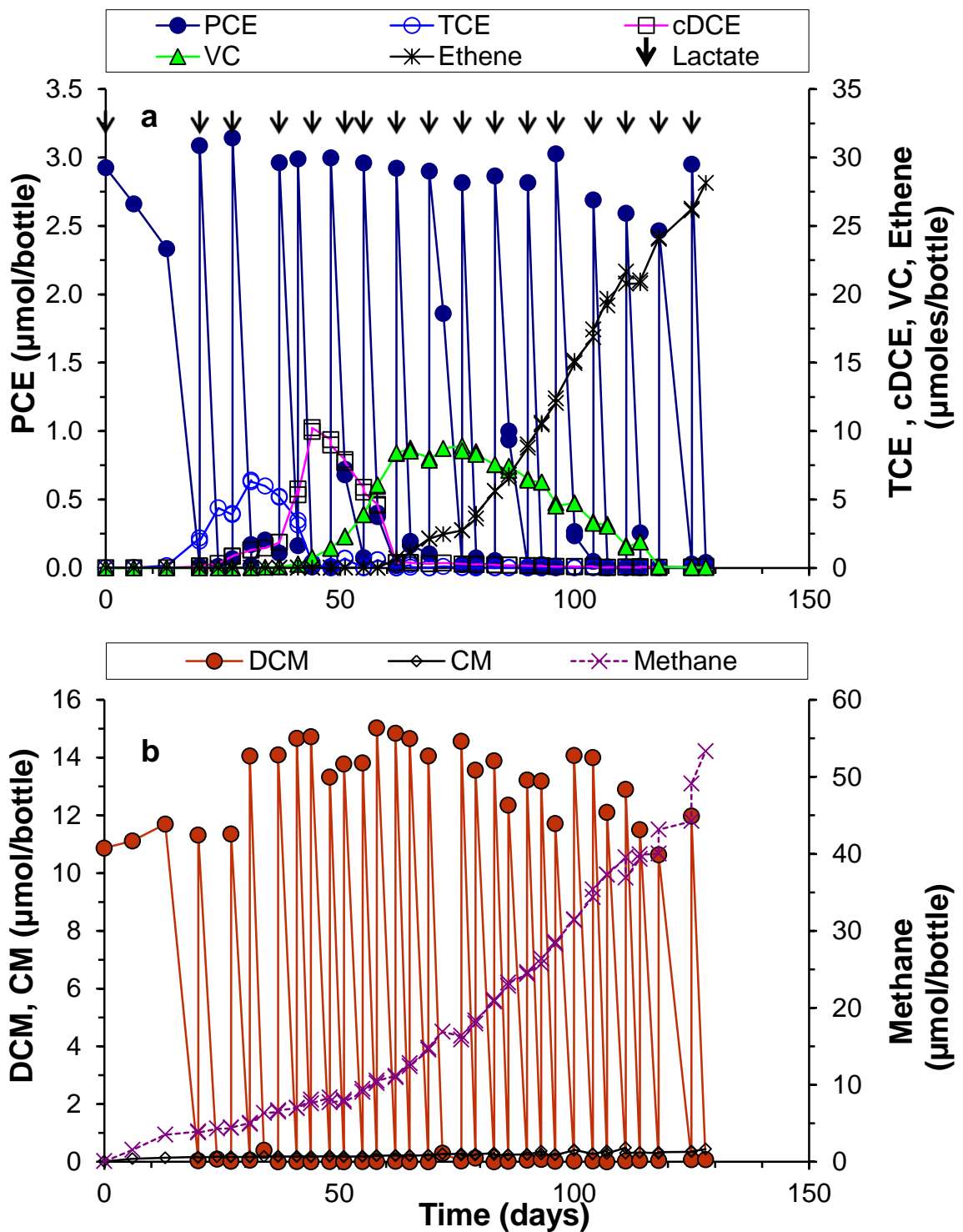GC Results for Treatments #2-10

Figure B.1.1 Results for treatment #2, (inoculated with SRS culture and fed with PCE and lactate) a) bottle#1, b) bottle#2, c) bottle #3.
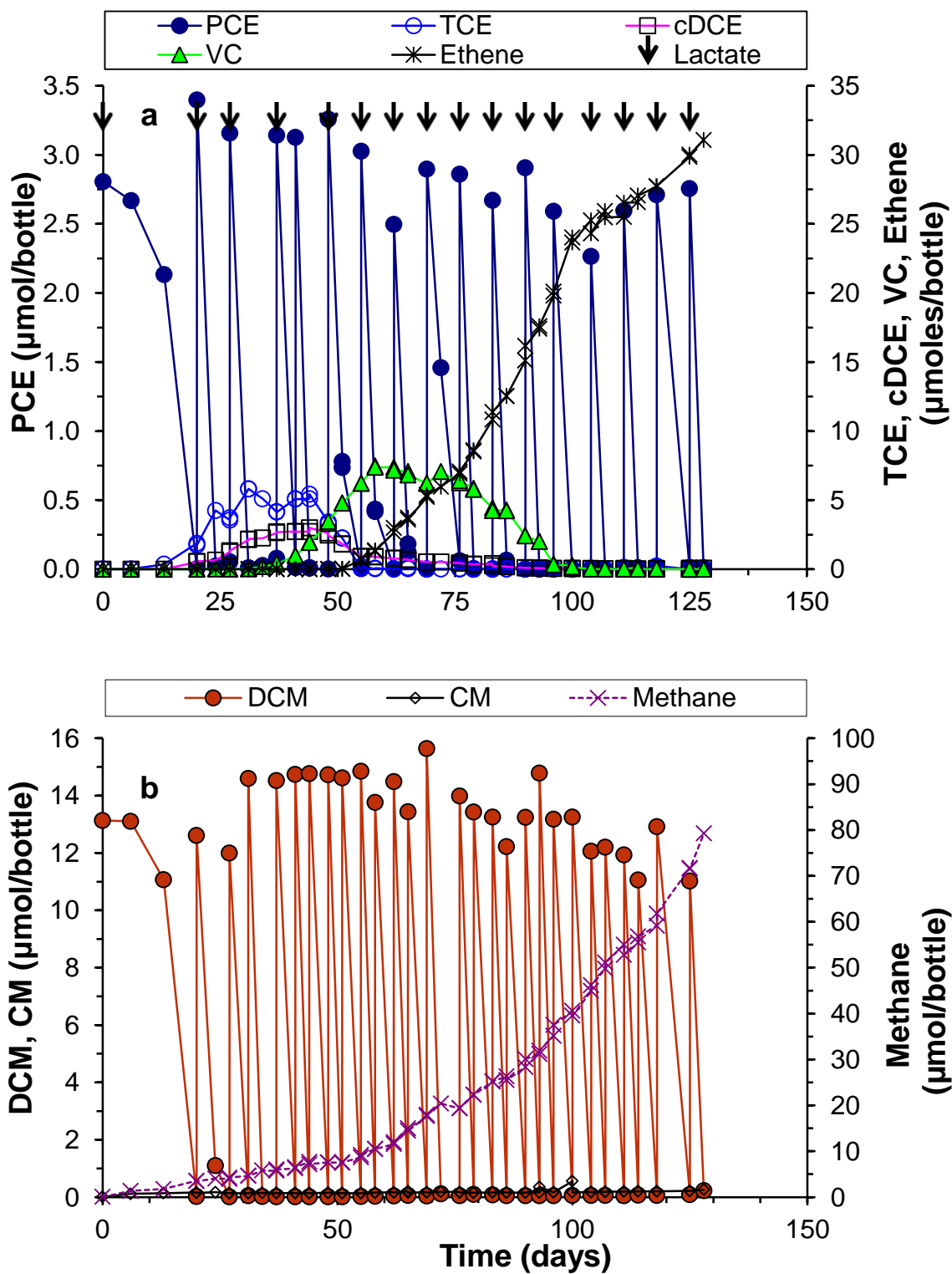
Figure B.1.2 Results for treatment #3, (inoculated with DCM culture and fed with DCM as sole carbon and energy source) a) bottle#1, b) bottle#2, c) bottle #3.

Figure B.1.3 Results for treatment #4, (inoculated with SRS culture and fed with PCE with no electron donor) a) bottle#1, b) bottle#2, c) bottle #3.

Figure B.1.4 Results for treatment #5, bottle #1 (inoculated with SRS, DCM cultures and fed with PCE, DCM and lactate); a) complete reductive dechlorination of PCE to ethene b) fermentation of DCM.

Figure B.1.5   Results for treatment #5, bottle #2 (inoculated with SRS, DCM cultures and fed with PCE, DCM and lactate); a) complete reductive dechlorination of PCE to ethene b) fermentation of DCM.

Figure B.1.6   Results for treatment #5, bottle #3 (inoculated with SRS, DCM cultures and fed with PCE, DCM and lactate); a) complete reductive dechlorination of PCE to ethene b) fermentation of DCM.
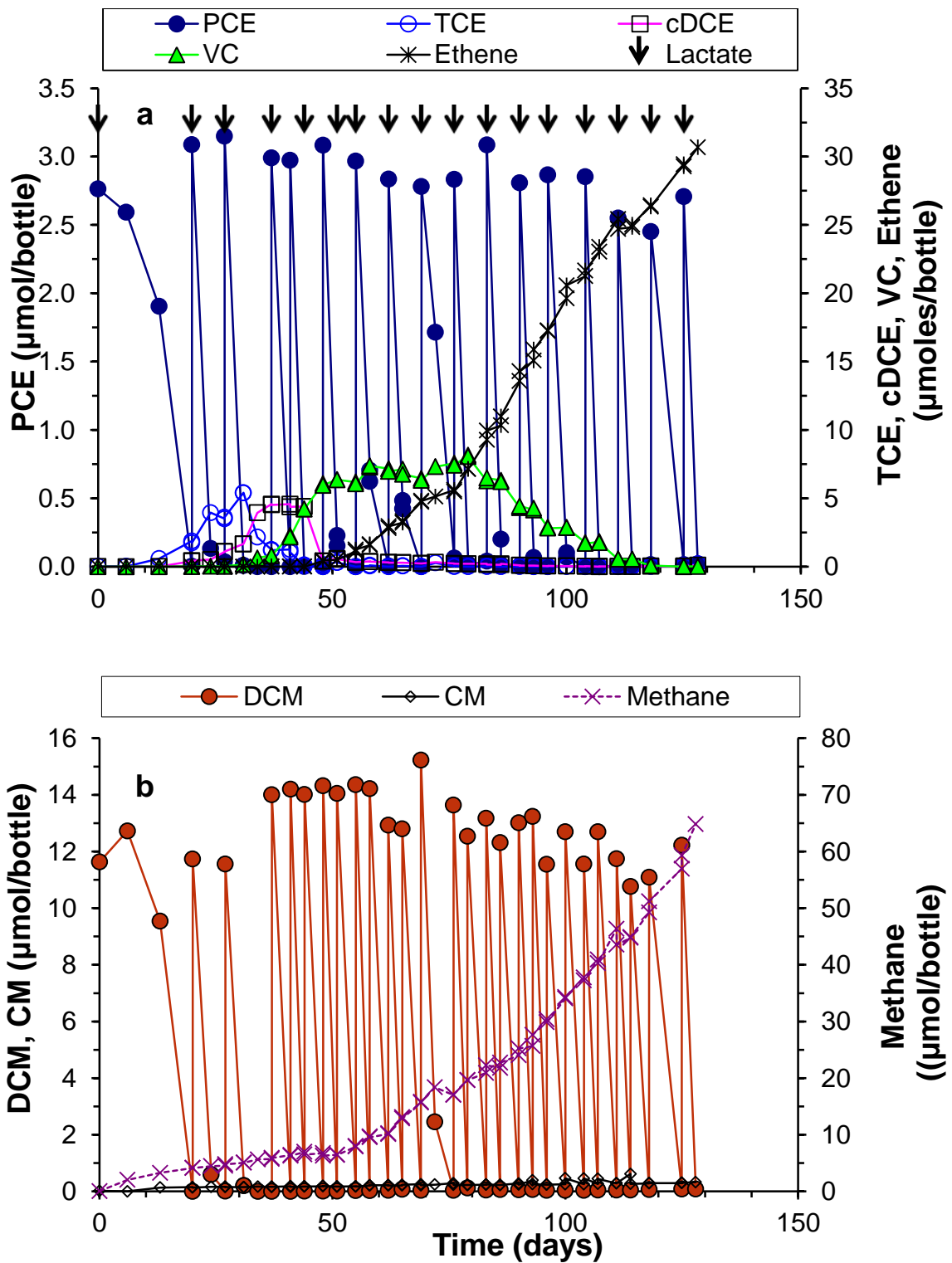
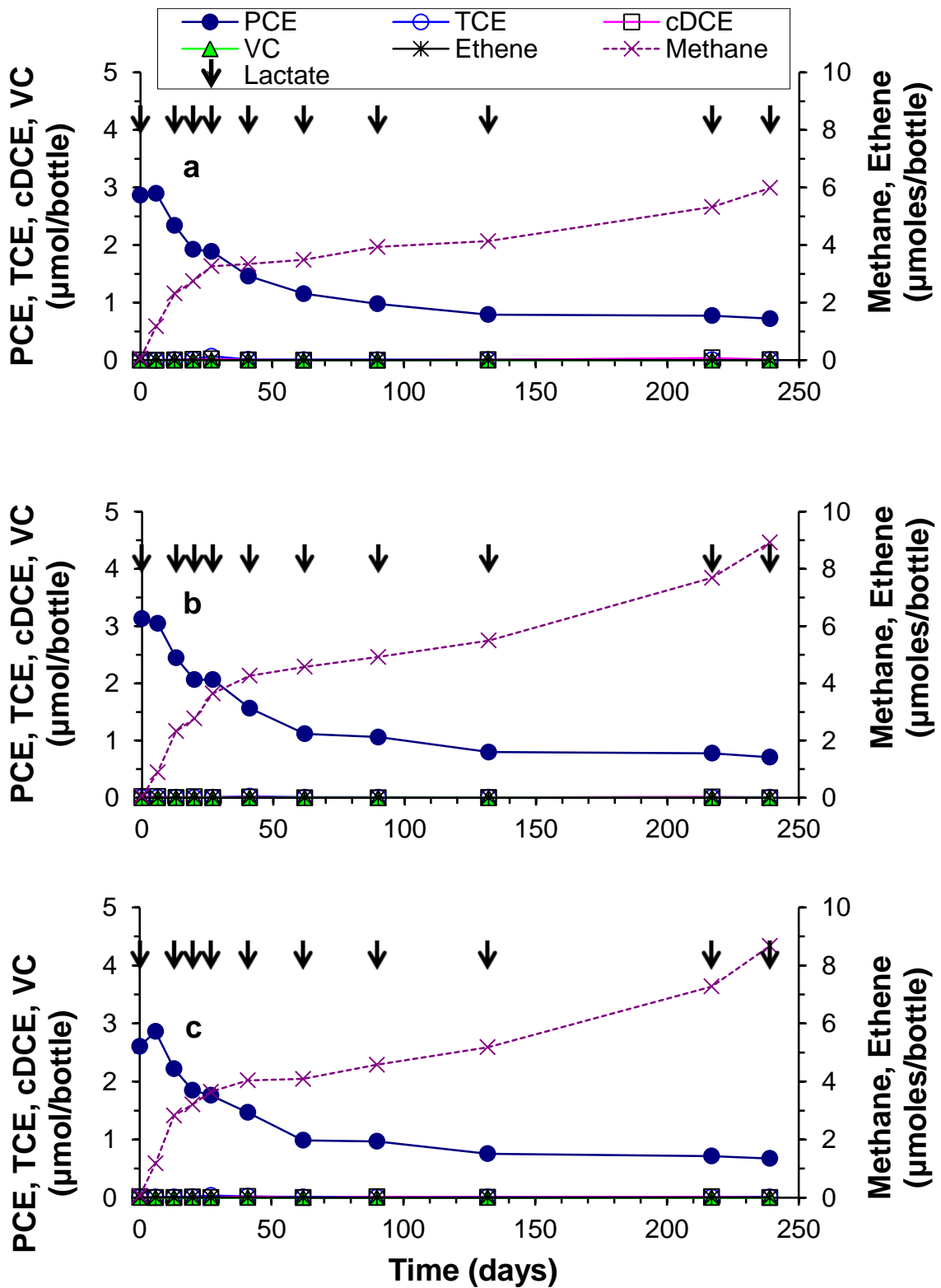**Figure B.1.7** Results for treatment #6, (inoculated with DCM culture and fed with PCE and lactate) a) bottle#1, b) bottle#2, c) bottle #3
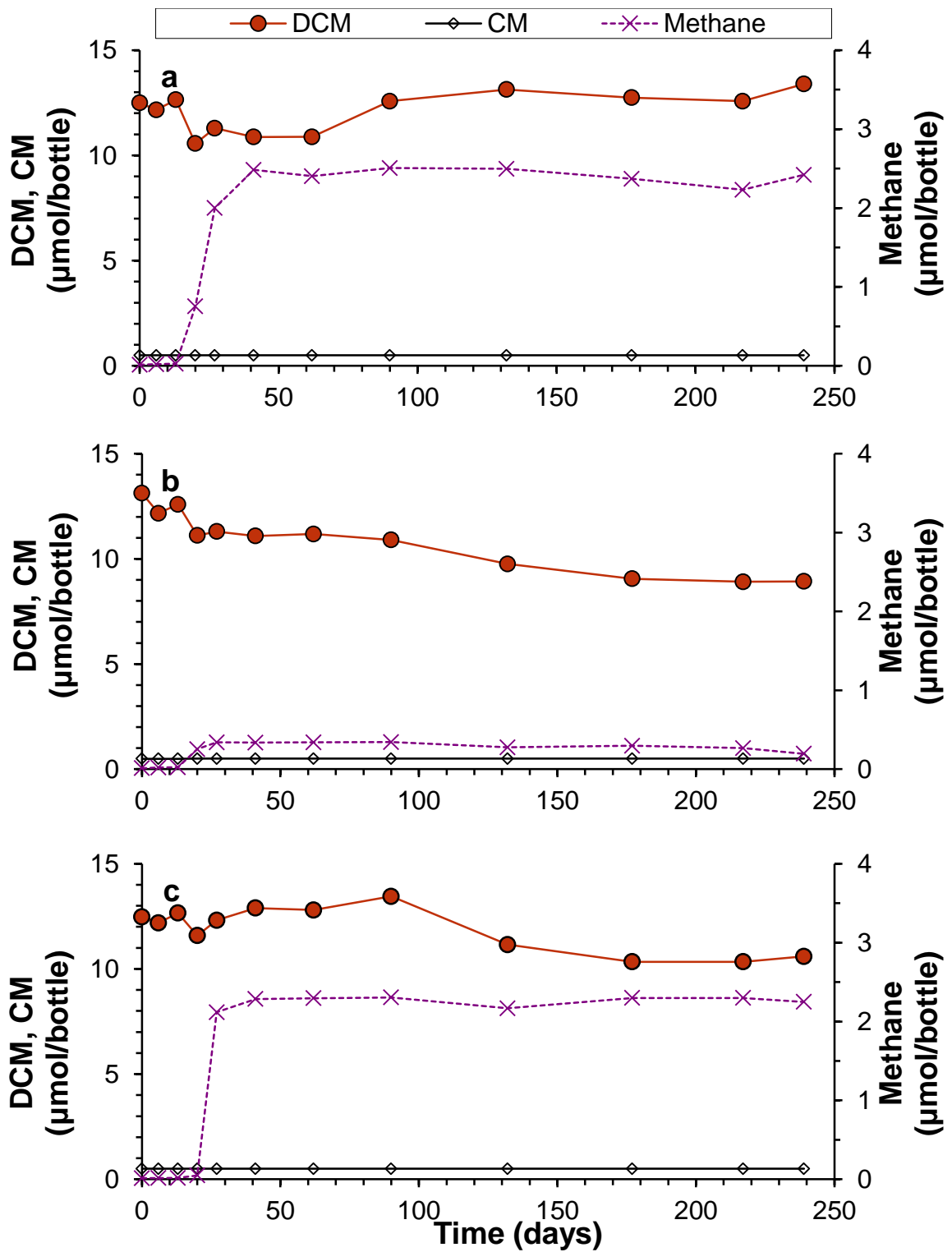
Figure B.1.8 Results for treatment #7, inoculated with SRS culture and fed with DCM and lactate a) bottle#1, b) bottle#2, c) bottle #3

Figure B.1.9 Results for treatment #8 (inoculated with SRS culture and fed with PCE and acetate); a) bottle#1, b) bottle#2, c) bottle #3.

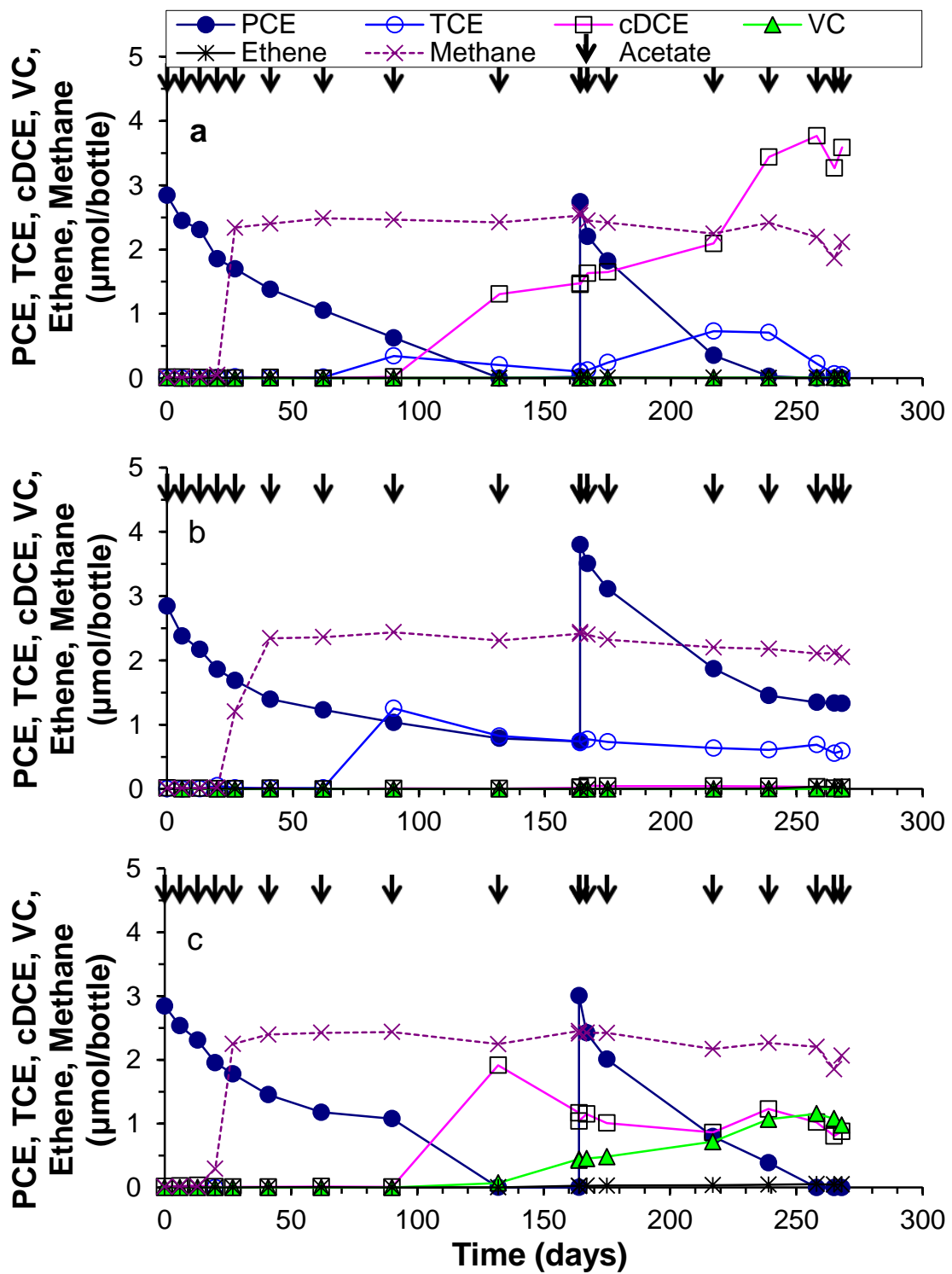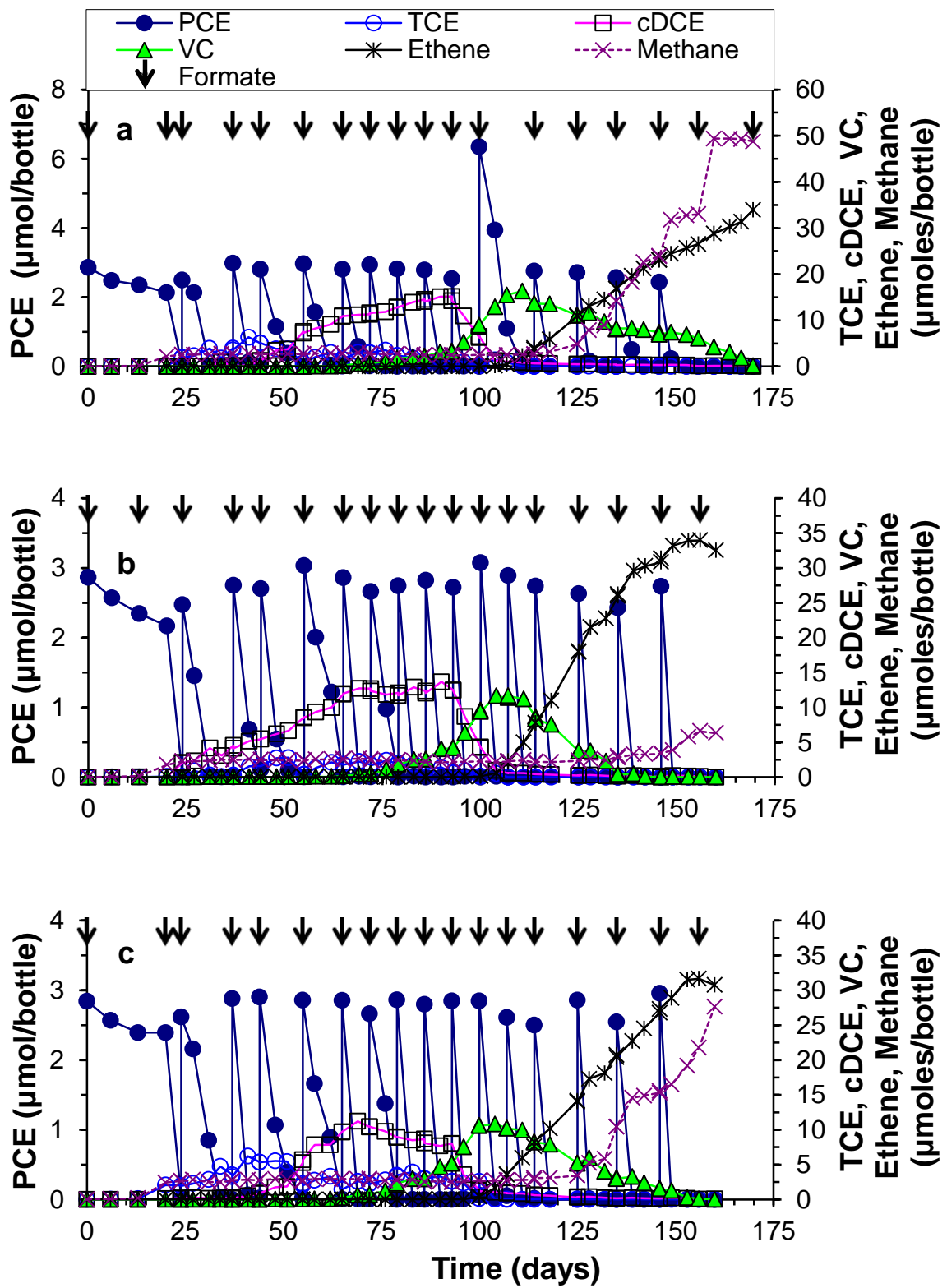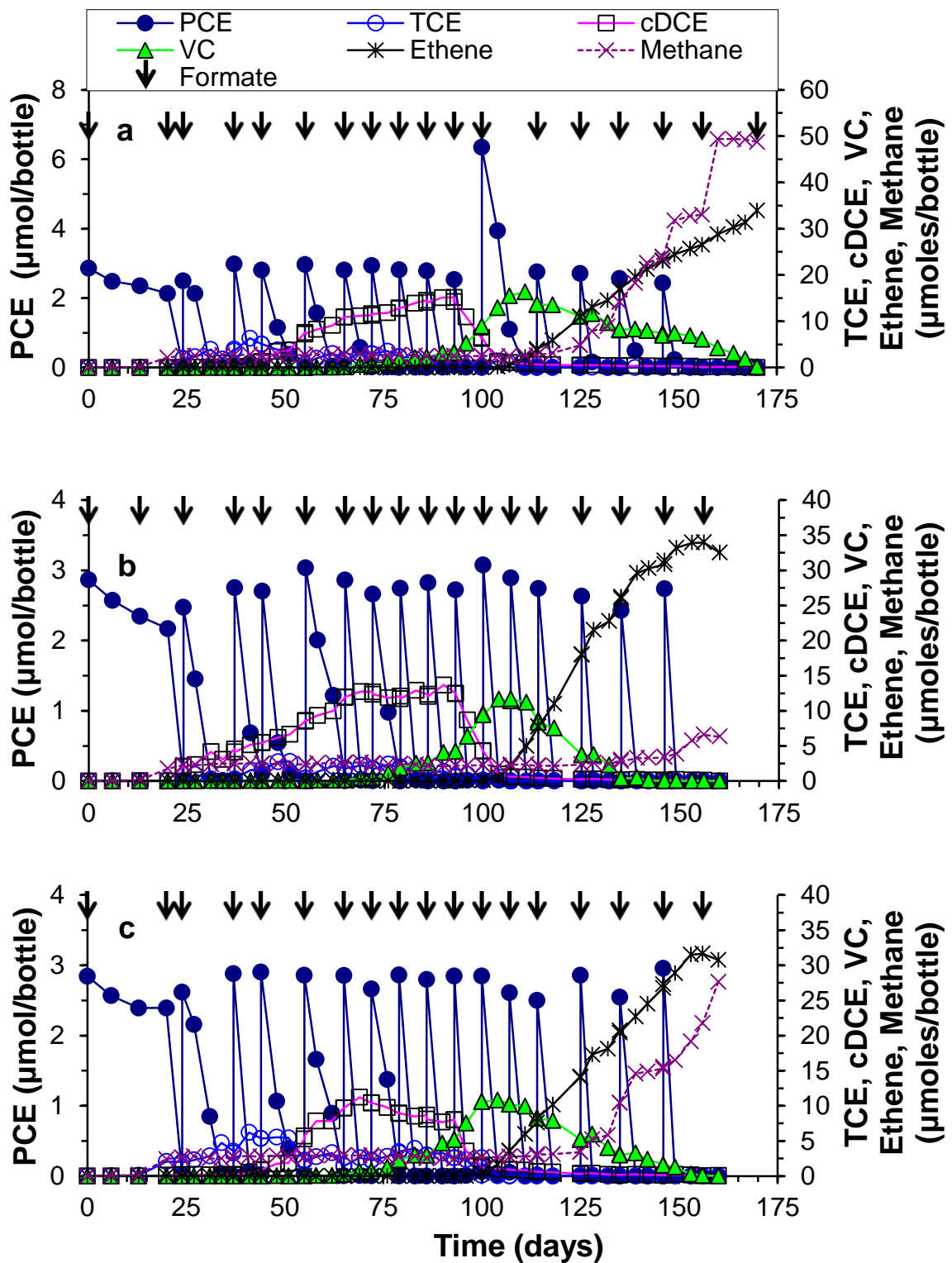**Figure B.1.10** Results for treatment #9 (inoculated with SRS culture and fed with PCE and formate); a) bottle#1, b) bottle# 2, c) bottle #3.

Figure B.1.11 Results for treatment #10, inoculated with SRS culture and fed with PCE and hydrogen a) bottle#1, b) bottle#2, c) bottle #3.

APPENDIX B.2:

Hydrogen and Sulfate Results

Table B-1: Hydrogen results for all treatments.

Treatment #	Average H_2 formed (μmol/bottle)	Standard deviation (μmol/bottle)
1	0.0008	0.0004
2	0.0004	0.0003
3	0.0003	0.0003
4	0.0002	0.0001
5	0.0008	0.0002
6	0.0010	0.0001
7	0.0005	0.0004
8	0.0013	0.0001
9	0.0002	0.0000
10	0.0008	0.0003

Table B-2: Sulfate results for treatments that showed reductive dechlorination.

Treatment #	Average sulfate remaining (μmol/bottle)	Standard deviation (μmol/bottle)
1	5.74	1.29
2	0.58	0.02
3	1.124	0.068
5	0.49	0.05
9	1.84	0.36
10	1.86	0.178

APPENDIX B.3:

Protocol and Results for the DCM Enrichment Culture Clone Library

Amplification products were cloned using the TOPO TA cloning kit (Invitrogen) and transformed into *E. coli* TOP10 cells. Transformants were selected using kanamycin (50 mg/ml) and screened by a complementation of the b-galactosidase gene. Positive clones were confirmed by EcoRI digest of mini-prepped plasmid DNA. Confirmed clones were sent to the Clemson University Genomics Institute for sequence analysis. Sequence data was trimmed and edited using Sequencher 4.1 and the resulting sequences were used to search the non-redundant GenBank Database using BLAST (1). Genus assignments were based upon the first 10 organisms listed (E-values = 0) or the Taxonomy browser available through GenBank (1).

A total of 15 positive clones were sequenced. The sequencing of the positive clones did not reveal a dominant phenotype with 5/15 of the clones returning firmicutes, 3/15 returning Chloroflexi and 7/15 returning miscellaneous sequences.

Table B-3 Results returned by the NCBI BLAST for the 15 sequenced clones

Clone	ID	Phylogenetic Group
T40	Uncultured	Bacteriodetes
T24	Uncultured	Chloroflexi
T31	Uncultured	Chloroflexi
T44	Uncultured Eubacterium	Chloroflexi
T46	*Desulfovibrio*	delta-Proteobacterium
T30	*Sufurospirillum*	epsilon-Proteobacterium
T57	*Sufurospirillum*	epsilon-Proteobacterium
T58	*Sufurospirillum*	epsilon-Proteobacterium
T62	*Sedimentibacter*	Firmicute
T25	*Synergistes*	Firmicute
T27	Uncultured	Firmicute
T28	*Synergistes*	Firmicute
T55	Uncultured	Firmicute
T26	*Pseudomonas*	gamma-Proteobacterium
T38	*Enterobacteriaceae*	gamma-Proteobacterium

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