SCALE-UP AND CHARACTERIZATION OF AN ENRICHMENT CULTURE FOR BIOAUGMENTATION OF THE P-AREA CHLORINATED ETHENE PLUME AT THE SAVANNAH RIVER SITE

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ABSTRACT

Bioaugmentation has become an increasingly popular remediation strategy for groundwater sites contaminated with chlorinated solvents. When biostimulation is not an option due to the lack of necessary microorganisms required for dechlorination of the contaminants, bioaugmentation is an attractive option for remediation. The P-Area groundwater plumes at the Savannah River Site (SRS) are just such a case. The P-Area site is contaminated with tetrachloroethene (PCE), trichloroethene (TCE), and *cis*-dichloroethene (cDCE), and no dechlorination past cDCE is occurring. A similar site, the C-Area site, is near one of the P-Area site's source zones but displays complete reduction of TCE to ethene. An enrichment culture was developed from the C-Area wetland for possible use as an indigenous bioaugmentation culture for the P-Area site. The culture underwent characterization in terms of potential terminal electron acceptors, pathogenicity, susceptibility to 1,1,1-trichloroethane (1,1,1-TCA), potential use of emulsified vegetable oil as an electron donor, and response to oxygen exposure and a range of pH levels.

The ability of the SRS culture, which was enriched on PCE and TCE, to use other halogenated alkenes and alkanes as terminal electron acceptors was investigated. The SRS culture is capable of utilizing PCE, TCE, cDCE, *trans*-dichloroethene, 1,1-dichloroethene, vinyl chloride (VC), 1,2-dichloroethane, 1,2-dibromoethane, and vinyl bromide as electron acceptors. Additionally, the culture's ability to dechlorinate several chlorinated benzenes was investigated. The SRS culture can dechlorinate hexachlorobenzene, pentachlorobenzene, 1,2,4,5-tetrachlorobenzene, and 1,2,4-

trichlorobenzene; however, further testing is required to determine if these electron acceptors are used metabolically as with the halogenated alkenes and alkanes. The culture cannot dechlorinate the dichlorobenzene isomers or chlorobenzene.

The SRS culture was tested for potential pathogenicity, which would hinder its regulatory approval as a bioaugmentation culture. Initially, the culture's ability to grow on a rich substrate (trypticase soy broth) at the temperature of the human body was tested. The culture grew aerobically at 37ºC, and further analysis using commercial coliform and *E. coli* testing kits revealed that the SRS culture does contain coliforms. However, *E. coli* is not present in the culture. Further molecular testing is being conducted at the Savannah River National Laboratory (SRNL) to determine if other pathogenic species might be present in the culture.

As 1,1,1-TCA has been shown to be inhibitory to many dechlorinating cultures, the SRS culture's susceptibility to this known inhibitor was evaluated. The culture's ability to completely dechlorinate TCE to ethene was inhibited by 300 µM 1,1,1-TCA; a mixture of VC and ethene were produced as end products. Lower concentrations of 1,1,1-TCA (0.7 and 3.6 µM) did not inhibit TCE conversion to ethene. Additionally, the SRS culture was not capable of dechlorinating 1,1,1-TCA.

The SRS culture was enriched on lactate as an electron donor, however, the use of emulsified oil substrate (EOS®) as an electron donor was investigated as it is a longer lasting electron donor. A microcosm evaluation suggested that EOS® is a better electron donor than lactate. Reductive dechlorination of PCE and TCE occurred faster and with

less accumulation of daughter products in treatments amended with EOS® than in those amended with lactate.

The SRS culture was tested for its vulnerability to oxygen exposure, as it is an anaerobic culture, and exposure to oxygen could be detrimental to the success of the culture in the field. Quiescent exposure to air (21% oxygen in headspace) for 24 hours slowed the dechlorination of PCE and TCE. However, the culture was able to overcome the aerobic conditions and completely dechlorinate PCE and TCE to ethene. The low redox conditions provided by the media in which the culture is maintained allowed for anaerobic conditions in the bottles to be reestablished. Given low redox conditions in contaminated groundwater, the SRS culture should be able to sustain brief oxygen exposure and retain its reductive dechlorinating ability.

Related to oxygen exposure, the SRS culture's susceptibility to extreme pH levels was investigated. The SRS culture is maintained in buffered minimal media within the pH range of 6.5-7.5. The culture was exposed to a range of pH levels (5.5, 6.0, 6.5, 7.0, and 8.5) as well as a treatment in which pH was allowed to decrease from neutral, as a result of HCl release. At pH 6.0, the dechlorinating activity of the SRS culture was slowed, and cDCE and VC accumulation was higher than at pH 7.0. At pH 5.5, reductive dechlorination stopped at cDCE, with no production of VC or ethene. When the pH was allowed to decrease from neutral, the culture exhibited a decrease in ethene production and accumulation of VC as the pH dropped below 6.0. The culture was most strongly inhibited at pH 8.5; some PCE was dechlorinated, but no TCE was consumed. Little to no cDCE or VC was produced. Given these results, it will be necessary for groundwater

pH to be adequately buffered at a pH of 6.5 or higher for successful bioaugmentation with the SRS culture.

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LIST OF ABBREVIATIONS

1,1,1-TCA	1,1,1-trichloroethane
1,1-DCA	1,1-dichlorethane
1,1-DCE	1,1-dichloroethene
1,2-DCA	1,2-dichloroethane
CA	chloroethane
CB	chlorobenzene
cDCE	*cis*-dichloroethene
DCB	dichlorobenzene
DDI	distilled, deionized
EDB	1,2-dibromoethane
EOS®	Emulsified Oil Substrate
GC	gas chromatograph
HCB	hexachlorobenzene
PBRP	P-Area Burning/Rubble Pit
PCE	tetrachloroethene
PeCB	pentachlorobenzene
RFs	response factors
SRNL	Savannah River National Laboratory
SRS	Savannah River Site
TCB	trichlorobenzene
TCE	trichloroethene

tDCE	*trans*-dichloroethene
TEA	terminal electron acceptor
TeCB	tetrachlorobenzene
TSB	trypticase soy broth
VB	vinyl bromide
VC	vinyl chloride
WCs	water controls

CHAPTER ONE

INTRODUCTION

Chlorinated ethenes are among the most common groundwater contaminants found at industrial sites throughout the United States. The Department of Energy's Savannah River Site (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 plumes, and currently, work is being done to characterize these plumes. Field data have shown this site to be contaminated with tetrachloroethene (PCE), trichloroethene (TCE), and *cis*-dichloroethene (cDCE). Maps of the PCE and TCE plumes are provided in Appendix A. P-Area groundwater discharges to Steel Creek where TCE levels above the current maximum contaminant level set by the United States Environmental Protection Agency have been detected (42, 47). There are two separate source zones for the P-Area plumes: 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 (42). After reaching capacity the pit and debris were covered with soil and no other actions were taken to remediate this site. As a direct result of these past disposal practices, chlorinated ethenes contaminated both the soil and groundwater beneath the PBRP (47).

The second area contributing to the P-Area plumes is the P-Area Reactor operable unit. In this area, both TCE and PCE have been detected at levels as high as 21,100 ppb and 365 ppb, respectively. These compounds have been detected in groundwater at a depth of approximately 15.2 m. While the exact source of the contamination is unknown, it is believed that a dense non-aqueous phase liquid source is located near the reactor facility (46).

A similar site at SRS, the C-Area Burning/Rubble Pit, is close to the P-area Burning/Rubble Pit and displays complete reduction of TCE to ethene. This plume extends approximately 1220 meters to the west and outcrops in the seepline along Twin Lakes and Fourmile Branch (42). Another branch of the plume extends to the south and outcrops at Castor Creek (48). In collaboration with SRNL, the Department of Environmental Engineering and Earth Sciences at Clemson University conducted a microcosm study using samples from the Twin Lakes seepline in the C-Area to confirm the occurrence of reductive dechlorination. *Dehalococcoides* were detected in the sites 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 (7). 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 indicated strong genotypic similarity between Bachman *Dehalococcoides* 16S rRNA gene 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* (7).

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. Bioaugmentation has proven to be effective at many sites contaminated with chlorinated ethenes. For example, a pilot scale study completed at Dover Air Force Base, Delaware involved a TCE-contaminated site where dechlorination was stalled at cDCE. An enrichment culture known to dechlorinate both TCE and cDCE to ethene was used in bioaugmentation, and complete reduction to ethene was observed (15). Another study was done at Kelly Air Force Base, Texas. This site faced the problem of incomplete dechlorination from PCE to cDCE. A different mixed culture was used and again there was complete reduction of PCE to ethene (34).

In many situations bioaugmentation has been the most attractive method of enhancing in situ dechlorination, especially in sites that are currently seeing a buildup in daughter products other than ethene. A comparison of biostimulation versus bioaugmentation was done at another site contaminated with PCE. This comparison showed that bioaugmentation was not only more extensive but complete reduction occurred in less than half the time of biostimulation (29). Other studies have offered the same conclusion (9, 15, 34).

The use of a site-developed bioaugmentation culture for SRS has its advantages. There will likely be a financial advantage in using the site-developed culture as opposed to a commercially available culture. In addition, the likelihood of obtaining regulatory approval for using an indigenously derived culture is higher than when using an "imported" commercial culture. Lastly, since the site where the microorganisms were initially discovered and the site that is being investigated for application are similar in geochemistry, the culture may also be better suited to survive in the PBRP.

To investigate the effectiveness of a site-developed culture in bioaugmenting the P-Area, Wood (45) developed a sediment-free enrichment culture from the C-Area microcosms. A microcosm study using samples from the P-Area was then conducted to evaluate the effectiveness of biostimulation with several electron donors and bioaugmentation with the SRS enrichment culture. The results confirmed that dechlorination activity in the bioaugmentation microcosms was significantly higher than that in the biostimulation microcosms. Successful use of the SRS enrichment culture in bioaugmentation of the P-Area microcosms suggests that the SRS enrichment culture may be a feasible option for bioaugmenting the P-Area chlorinated ethene plume. Given the promising results of the bioaugmentation microcosm study using the SRS enrichment culture, the main objectives of the research for this thesis were to scale up the enrichment culture for a field trial in the P-Area groundwater and to further characterize the culture as it relates to other chlorinated ethene bioaugmentation cultures.

The main focus of culture characterization was determining the range of terminal electron acceptors that the SRS culture is capable of using. Various halogenated alkenes,

alkanes, and chlorinated benzenes were tested as potential terminal electron acceptors (TEAs). Other known groups of *Dehalococcoides* (Cornell, Pinellas, and Victoria) respire a variety of halogenated compounds. However, only *Dehalococcoides ethenogenes* strain 195 is known to halorespire chlorinated ethenes and halogenated ethanes as well as chlorinated benzenes. For example, BAV1, VS, and KB-1™ can dechlorinate several of the chlorinated ethenes to ethene (12, 25, 35, 37), while CDBD1 is able to chlororespire chlorinated benzenes but not PCE (8). To quantitatively compare other *Dehalococcoides*-containing cultures and the SRS culture, growth yields were calculated for each TEA tested and compared to yields reported in literature (11).

Investigation of the pathogenicity of the enrichment culture was included in the scope of this thesis, due to health and safety concerns that accompany injection of a microbial culture into a groundwater supply. Pathogenicity data on bioaugmentation cultures is limited, but *Burkholderia cepacia* (used for aerobic cometabolism of TCE) has been identified as an opportunistic human pathogen and EPA has imposed regulations on its use (16). Other cultures, including *Methylosinus trichosporium* OB3b, KB-1™, and mixed cultures from Bioremediation Consulting, Inc. have been reported as being nonpathogenic (16).

Other bioaugmentation cultures have been shown to be inhibited by the presence of low concentrations of 1,1,1-trichloroethane (1,1,1-TCA) and chloroform, which are common co-contaminants with chlorinated ethenes at many hazardous waste sites. Therefore, to be thorough in the comparison, susceptibility of the SRS culture to 1,1,1-TCA was included in the culture characterization. Grostern and Edwards (23)

documented inhibitory effects on the KB-1™ culture amended with 1,1,1-TCA at a concentration of 300 µM and an equimolar concentration of TCE. Inhibition was also documented at a concentration of 30 µM for both 1,1,1-TCA and TCE. Duhamel et al. (13) also reported slowed rates of VC to ethene conversion with KB-1™ at a much lower 1,1,1-TCA concentration of 5.2 µM. Some vendors have reported mixed cultures that are not inhibited by 1,1,1-TCA (6). Thus, there is a need to characterize the SRS enrichment culture in terms of its susceptibility to inhibition by 1,1,1-TCA.

Wood (45) tested the effectiveness of emulsified vegetable oil as an electron donor in a biostimulation study, but did not test its feasibility in the bioaugmentation study. Emulsified oil has the added benefit of slower fermentation and longer retention in a contaminated aquifer. Studies have shown the success of emulsified oil as an electron donor (30, 31, 50). Therefore, as part of characterizing the SRS culture, its ability to use emulsified vegetable oil as an electron donor was evaluated, in comparison to lactate, which is the donor used to maintain the culture. It was necessary to conduct this test in microcosms with soil present, since vegetable oil in groundwater or media alone may result in inhibition, presumptively due to accumulation of inhibitory levels of long chain organic acids (personal communication from Robert Borden to David Freedman). These fermentation products are not a problem in soil microcosms, since they tend to partition to the soil, thereby keeping the aqueous phase concentration low. A soil microcosm test is also more representative of what will occur in situ. The use of emulsified oil as an electron donor as compared to repeat additions of lactate may lower the cost of bioaugmentation (17).

Additional characterization of the SRS culture included testing its tolerance to oxygen exposure and pH extremes. *Dehalococcoides* are strictly anaerobic microorganisms, and the success of bioaugmentation cultures in the field is dependent on preventing oxygen exposure. The sensitivity of anaerobic cultures to oxygen is not necessarily uniform, as not all cultures are maintained under the same conditions. In one study, Seepersad (39) demonstrated a decrease in the TCE degradation rate of microcosms with KB-1 present, when the headspace of the bottles contained approximately 0.3 mg/L O_2. Knowledge of the culture's tolerance to oxygen exposure is important to developing a protocol for field deployment, as well as the methods used to handle the culture while it is being grown in canisters in the laboratory.

In addition to oxygen tolerance, the SRS culture's sensitivity to a range of pH levels is also important for field application of the culture. The culture has been maintained at a pH of 6.5-7.5 in a buffered mineral medium; this is the accepted optimal range for most chlororespiring bacteria (32). KB-1^{TM} has an optimal pH range of 6-8.3 and is reportedly inhibited below pH 5 and above pH 10 (38). Zhuang and Pavlostathis (51) determined that neutral pH was optimum for reductive dechlorination of PCE to VC by a methanogenic mixed culture. *Desulfitobacterium dichloroeliminans* strain DCA1, a non-*Dehalococcoides* dehalorespirator, has an optimal pH range of 7.2-7.8, but has maintained activity at pH levels as low as 5.4 in the field (33). The cost to buffer the pH of groundwater to neutral may become a large portion of the total bioaugmentation cost. Therefore, if the SRS culture is capable of maintaining activity at low pH levels, the cost for bioaugmentation may be lessened. Characterization of the SRS enrichment culture

will shed more light on its potential for application at the P-Area chlorinated ethene plume at SRS, as well as other contaminated sites.

CHAPTER TWO

RESEARCH OBJECTIVES

The main objective of this project was to further characterize the SRS enrichment culture. This was accomplished by investigating potential TEAs and culture pathogenicity, vulnerability to a known chlorinated inhibitor, use of alternate electron donors, and tolerance to oxygen exposure and extreme pH levels. A large volume of culture was also grown in canisters for the purpose of field-scale pilot testing of bioaugmentation in the P-Area.

The specific objectives were:

1) To design and implement a canister for field deployment of the SRS enrichment culture, using the enrichment culture developed from the C-Area Burning/Rubble Pit microcosms;

2) To evaluate the range of TEAs used by the SRS enrichment culture, including PCE, TCE, cDCE, *trans*-DCE (tDCE), 1,1-DCE, VC, 1,2-dichloroethane (1,2-DCA), 1,2-dibromoethane (EDB), and vinyl bromide (VB). In addition to the halogenated ethenes and ethanes, the ability of the culture to reductively dechlorinate several chlorinated benzenes was tested, including hexachlorobenzene (HCB), pentachlorobenzene (PeCB), 1,2,4,5-tetrachlorobenzene (1,2,4,5-TeCB), 1,2,4-trichlorobenzene (1,2,4-TCB), 1,2-dichlorobenzene (1,2-DCB), 1,3-dichlorobenzene (1,3-DCB), 1,4-dichlorobenzene (1,4-DCB), and chlorobenzene (CB);

3) To test the SRS culture for evidence of pathogenicity by assessing its ability to grow at 37°C on a rich substrate (trypticase soy broth, or TSB) and testing for the presence of total coliforms and *E. coli*;

4) To investigate the extent to which 1,1,1-TCA inhibits reductive dechlorination of TCE by the SRS enrichment culture;

5) To compare the culture's performance while utilizing several Emulsified Oil Substrate (EOS®) products to lactate as electron donors in a microcosm study using P-Area sediment and groundwater;

6) To determine the degree to which the enrichment culture can tolerate exposure to oxygen; and

7) To assay the effect of various pH levels (from 5.5 to 8.5) on the SRS culture's ability to reductively dechlorinate PCE and TCE.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemicals and Media

VC (99.5%) was obtained from Fluka. Polymer grade ethene (99.9%), purity grade ethane (99.95%), and chemically pure grade methane (99%) were obtained from Matheson. PCE (99.9%) and chloroethane (CA) (99.7%) were obtained from Sigma-Aldrich, TCE (99.5%) from Fisher, cDCE (99%) and 1,1-dichloroethane (1,1-DCA) (95%) from TCI America, and tDCE (99.5%), 1,1-DCE (99.5%), and 1,1,1-TCA (99.5%) from Chem Services. EDB (99%) was obtained from Acros Organics and 1,2-DCA (99%) from Mallinckrodt. VB (98%) was obtained from Pfaltz & Bauer.

HCB, PeCB, and 1,3,5-TCB were obtained from Chem Services and were all 99.5% purity. 1,2,4,5-TeCB (98%), 1,2,4-TCB (99%), 1,3-DCB (98%), and CB (99%) were obtained from Aldrich, 1,2,3,4-TeCB (98%) and 1,2,3-TCB (99%) from Acros Organics, 1,2,3,5-TeCB (99%) from Ultra Scientific, and 1,4-DCB (99%) from Alfa Aesar. 1,2-DCB (99.5%) and pesticide grade acetone were obtained from Fisher. Dehydrated TSB was obtained from BD Sciences. Colilert® was obtained from Idexx and Readycult® from EMD Chemicals. Sodium lactate syrup (containing 58.8-61.2% sodium lactate; specific gravity = 1.31) was obtained from EM Science. EOS® 450, EOS® 598, and EOS® 598B42 concentrates and EOS® Vitamin B12 solution were obtained from EOS Remediation. All other chemicals used were reagent grade, unless indicated otherwise.

The anaerobic mineral medium used to grow the SRS culture was adapted from Edwards and Grbić-Galić (14). Appendix B provides a description of how media was prepared. Note that to achieve a neutral pH, the media must be in contact with a headspace of 30% CO_2. This was achieved by sparging the headspace of bottles with a gas mixture of 30% CO_2 and 70% N_2.

3.2 Culture Maintenance and Development of Canisters

3.2.1 SRS Enrichment Culture Maintenance

The SRS enrichment culture developed by Wood (45) was maintained for use as inoculum in the characterization experiments. The enrichment was maintained in three 2.6 L glass reagent bottles that were placed in boxes to exclude light. The reagent bottles were sealed with a Teflon-faced rubber septum (35 mm) placed inside a plastic bottle cap, which was modified by drilling 24-33 holes (3 mm each) to provide access for a syringe for sampling. This septum was replaced periodically to prevent diffusive losses of the volatile compounds. The bottles were stored in the anaerobic chamber horizontally to keep liquid in contact with the septa.

These bottles received neat PCE and TCE (resulting in aqueous phase concentrations of approximately 15 mg/L and 38 mg/L, respectively) along with lactate as the electron donor. The amount of PCE and TCE added was determined gravimetrically by weighing the syringe with PCE or TCE present, then reweighing immediately after adding the PCE or TCE to the bottles. Lactate was added each time the enrichment bottles were sampled for headspace analysis and at every PCE and TCE feeding. pH was measured each time PCE and TCE were added and sodium hydroxide (8

M) was added as needed to maintain the pH between 6.7 and 7.2. After every PCE and TCE feeding cycle (approximately two weeks), 300 mL of settled liquid (following 1-4 hours of settling) was decanted from each bottle and replaced with fresh media before the addition of PCE, TCE, and lactate. Addition of fresh media provided nutrients and avoided accumulation of salt (especially NaCl from neutralization) and sulfide from reduction of sulfate in the medium. Periodically, 100-500 mL of completely mixed liquid was removed for use as inoculum in the characterization experiments (see below).

At the time of the second canister's inoculation (see below), 1 L of mixed liquid from each of the three enrichment bottles developed by Wood (45) was combined to provide 3 L of inoculum for canister #2. The remaining mixed liquid was redistributed to two 2.6 L glass reagent bottles, each of which received 830 mL of mixed liquid and 770 mL of media; these cultures were referred to as E-3A and E-3B. The amount of PCE and TCE added were scaled down proportionally to the volume of culture present in the two new enrichment culture bottles. Following reduction of the first addition of PCE and TCE, the amounts added were gradually increased until the target maximum concentrations were reached, i.e., 15 and 38 mg/L, respectively.

3.2.2 Development of Canisters

In preparation for field-scale testing of the enrichment culture in the P-area, the SRS enrichment culture (described above) was transferred to two stainless steel canisters purchased from Sabco Industries. The canisters were modified by adding valves for media addition, liquid wastage, and headspace analysis (Appendix C). Prior to adding the culture, the canisters were leak-tested under pressure and tested for their ability to

retain PCE and TCE (by filling with 18 L of water, adding the compounds, and monitoring PCE and TCE levels over several weeks). No abiotic transformations occurred (Appendix C).

The inoculum source for the canisters was the SRS enrichment culture that actively dechlorinates PCE and TCE to ethene. The canisters were started by adding 15 L of media plus 3 L of culture, using a peristaltic pump. The total liquid volume of 18 L allowed for 1 L of headspace. To maintain anaerobic conditions, the canisters were set up in a Coy® anaerobic chamber. This involved removing the end cap of the chamber, quickly placing the canister, culture, and media into the chamber, and resealing the end cap. Once the oxygen level in the chamber reached zero, the culture and media were pumped into the canister.

After inoculation, neat PCE (30 µL) and TCE (90 µL) were added to the canisters. Taking into account partitioning to the headspace, the initial aqueous concentrations were 3 mg/L of PCE and 7 mg/L of TCE. These concentrations were increased over time to equal the aqueous concentrations added to the enrichment culture maintained in the 2.6 L glass bottles (i.e., 15 mg/L PCE and 38 mg/L TCE). Lactate was added as the sole electron donor, whenever PCE and TCE were added, as well as each time a sample was taken for headspace analysis. Each dose of lactate provided 146 mg/L (5 mL of a stock solution containing approximately 450 g/L of 60% sodium lactate syrup). pH was measured and adjusted as needed by adding approximately 10 mL of 8 M NaOH along with each PCE and TCE addition. The canisters were incubated at room temperature and

on their sides, so that the liquid was in contact with the valves to minimize potential losses of the volatile compounds.

The canisters were vented periodically to release pressure buildup caused by gas production (primarily methane and ethene). A piece of latex tubing with a needle on one end was purged with nitrogen gas to remove air, and then the needle was immediately inserted into the Mininert™ valve on the canister (which was standing upright). The open end of the tubing was simultaneously placed into a beaker of water (located in an exhaust hood to prevent exposure to the vented gases) to ensure that a positive pressure was maintained in the canister, so that room air would not enter. The venting was stopped when gas flow out of the canister nearly stopped.

Settled liquid from the canister was removed every fourth or fifth cycle of PCE and TCE feeding to prevent accumulation of salts and/or development of a nutrient deficiency. The procedure used for liquid wastage is shown in Figure C.2 (Appendix C). The flow of purge gas (30%CO_2-70%N_2) was controlled through two flow meters, which directed gas into the headspaces of the canister and bottle containing fresh media. The canister headspace was purged with the gas mix through the inlet valve on the top of the canister. Simultaneously, a syringe needle connected to tubing was inserted through the Mininert™ septum, and the end of the tubing was placed into a beaker of water. Bubbling of gas through the water provided verification of proper gas flow. While purging the canister, 3 L of settled liquid was drained by gravity from the top valve on the side of the canister; off gases were directed into an exhaust hood, also via tubing ending in a beaker of water. After removing the settled liquid, 3 L of fresh media was fed

by gravity into the canister through the outlet valve on top of the canister. The headspace of the media bottle was purged with gas mix to prevent oxygen contamination during media addition.

3.3 Evaluation of Alternate Terminal Electron Acceptors

The SRS enrichment culture was evaluated for its ability to grow on a variety of halogenated TEAs, falling into two main categories: 1) halogenated alkenes and alkanes; and 2) chlorinated benzenes. The halogenated alkenes and alkanes included PCE, TCE, cDCE, tDCE, 1,1-DCE, VC, EDB, 1,2-DCA, and VB. The chlorinated benzenes included HCB, PeCB, 1,2,4,5-TeCB, 1,2,4-TCB, 1,2-DCB, 1,3-DCB, 1,4-DCB, and CB. Alternate TEA experiments were conducted in 160 mL serum bottles containing media and the SRS enrichment culture (E-3A and E-3B) as inoculum.

3.3.1 Evaluation of Halogenated Alkenes and Alkanes as TEAs

The alternate TEA experiment with halogenated alkenes and alkanes was conducted in two phases. For Phase 1, serum bottles were prepared with 80 mL of media and 20 mL of inoculum from the 2.6 L enrichment culture bottles grown on PCE and TCE. Addition of compounds to the Phase 1 bottles was stopped when 500 µmol of chloride or bromide had been released per bottle. At this point the bottles were shipped (on ice) by an overnight carrier to Dr. Bagwell at SRNL. Phase 2 serum bottles were prepared with 99.9 mL of media and 0.1 mL of inoculum. The sources of inoculum for Phase 2 serum bottles varied and are summarized in Figure 3.1. Addition of compounds to the Phase 2 bottles was stopped when 200 µmol of chloride or bromide was released per bottle. As with the Phase 1 bottles, the Phase 2 bottles were then shipped (on ice) by

an overnight carrier to Dr. Bagwell. The only exception to this experimental protocol was the treatment that received VB as the TEA. There was no Phase 1 step for VB; instead these bottles were started directly with 0.1% inoculum from the Phase 2 bottles that received EDB (Figure 3.1).

Lactate was used as the electron donor, consistent with what the SRS enrichment culture received. It was added at the same time as addition of the test compound and at every headspace analysis. The total amount of lactate added was more than 100 times higher than the electron donor needed for stoichiometric dehalogenation of the TEA. A stock solution of 60% sodium lactate syrup was prepared (approximately 450 g/L) so that the intended amount of donor was added in 0.1 mL of the stock solution.

Live treatments 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. After adding media and inoculum, the bottles were sealed with Teflon-faced red rubber septa and aluminum crimp caps, with the exception of the ones that received VC, which were sealed with slotted gray butyl rubber septa. The bottles were then removed from the anaerobic chamber, sparged with a 30%CO_2-70%N_2 gas mixture, and spiked with the appropriate compound. The initial amounts of TEAs added are shown in Table 3.1. The first addition of PCE and TCE was 20% of the amount fed to the SRS enrichment culture, to account for dilution of the inoculum. The amounts of all other TEAs added was within the range of PCE and TCE added, except for EDB, which was slightly lower.

After a compound was consumed, the subsequent amount added was gradually increased until the target maximum was reached (Table 3.1). The target maxima were similar to the maximum concentrations of PCE and TCE added to the SRS enrichment culture during routine operation (section 3.2.1).

Halogenated alkenes and alkanes were initially added to the serum bottles using saturated solutions. When the amount of saturated water that was needed exceeded 1 mL, neat liquid was used instead (to avoid diluting the contents of the serum bottles). The amounts of neat compound required to reach the maximum concentrations (17-39 mg/L) are also shown in Table 3.1. The only exceptions were for VC and VB, which were always added as neat gases (using a 1.0 mL Pressure-Lok® gas syringe). The live treatments were incubated in an inverted position (i.e., liquid in contact with the septum) at room temperature (21-24°C) and in boxes (to exclude light) in the anaerobic chamber, except during headspace analysis. Neutral pH was maintained by measuring pH and adding NaOH (8 M) at each feeding, corresponding to the amount of acid released by dehalogenation.

For the experiments with halogenated alkenes and alkanes, the concentration of *Dehalococcoides* was determined at the start of the experiment and after repeated cycles of compound consumption to determine if it was used as a TEAs and, if so, to calculate a yield. Quantitative polymerase chain reaction (qPCR) was used to determine the *Dehalococcoides* concentrations. This work was completed by Dr. Bagwell at the SRNL.

In addition to the live treatments, two sets of water controls (WCs) were set up to evaluate the extent of abiotic losses via diffusion through the septa. WCs consisted of

100 mL of distilled, deionized (DDI) water plus the volatile organic compounds. One set received PCE + TCE + cDCE + VC + ethene. A second set of WCs received 1,1-DCE + tDCE + methane. A third set of WCs received 1,2-DCA, EDB and VB. The same initial quantities of compounds added to the live treatments (Table 3.1) were added to the WCs. Ethene and methane were added in the same volume as VC. The WCs were prepared and incubated on the bench top with room air present in the headspace. All bottles were incubated in an inverted position (liquid in contact with the septa), at room temperature (21-24^{o}C), and in boxes (to exclude light).

3.3.2 Reductive Dechlorination of Chlorinated Benzenes

For the experiment to evaluate if the SRS culture is able to reductively dechlorinate chlorinated benzenes, there was only one phase, i.e., only one set of treatments was prepared per compound and no transfers were made. The experiment was performed in serum bottles that received 20 mL of the SRS culture and 80 mL of media. Live treatments were prepared in an anaerobic chamber. Aseptic techniques were used during preparation. After adding media and inoculum, the bottles were sealed with Teflon-faced red rubber septa and aluminum crimp caps. They were then removed from the anaerobic chamber, sparged with a 30%CO_2-70%N_2 gas mixture, and spiked with the appropriate compound. The initial amounts added are shown in Table 3.1.

Chlorinated benzenes were added to the serum bottles in two ways: as a neat liquid (for 1,2-DCB, 1,3-DCB, and CB) and as a stock solution dissolved in acetone (for 1,4-DCB, HCB, PeCB, and 1,2,4,5-TeCB). The latter method was modified from Holliger et al. (27), who delivered chlorinated benzenes dissolved in pentane rather than

acetone. The two-phase liquid method for providing chlorinated benzenes used by Fennell et al. (19) was considered but rejected, due to interference caused by the pentane with headspace analysis on the gas chromatograph (GC).

A concern with delivering 1,4-DCB, HCB, PeCB, and 1,2,4,5-TeCB in acetone was the potential influence of acetone on performance of the SRS enrichment culture. A preliminary experiment with the enrichment culture was conducted to test the effect of acetone on the culture's dehalogenation activity on PCE and TCE. The experiment consisted of two treatments:

1) PCE + TCE; and

2) PCE + TCE + acetone.

Both treatments were prepared in triplicate, in 160 mL serum bottles, with 80 mL of media and 20 mL of enrichment culture. After adding the culture and media in the anaerobic chamber and sealing the bottles, they were removed and sparged with a 30%CO_2-70%N_2 gas mixture. Both treatments received an initial dose of PCE (2.2 µmol) and TCE (7.4 µmol), which was 20% of what the enrichment culture routinely receives, to account for dilution of the inoculum. The treatment without acetone served as a positive control, to confirm the culture's ability to dechlorinate PCE and TCE. All six bottles were initially given PCE, TCE and lactate. When the PCE and TCE were completely consumed, PCE and TCE were added at the maximum concentrations provided to the enrichment culture, along with additional lactate. Three of the bottles also received 70 µL of acetone, which is the same volume used for delivering the stock solution of HCB. In the three bottles with acetone added, reductive dechlorination of

PCE and TCE occurred at the same rate as the bottles without acetone added (Appendix D), indicating that the addition of acetone does not interfere with the dechlorination capability of the SRS culture. The possibility that acetone might benefit the culture by serving as an additional electron donor was not explored.

In addition to the live treatments, WCs and autoclaved controls were prepared. One set of WCs contained DDI water + HCB + PeCB + 1,2,4,5-TeCB; a second set contained DDI water + 1,2,4-TCB + 1,3-DCB; and a third set contained DDI water + 1,4-DCB + 1,2-DCB + CB. Chlorinated benzenes were added in the same quantity as those initially added to the live treatment bottles (Table 3.1). The WCs were prepared and incubated on the bench top with room air present in the headspace. Autoclaved controls were prepared to determine if decreases in the chlorinated benzenes were attributable to adsorption to biomass. Like the live treatments, the bottles were set up in the anaerobic chamber and contained 80 mL of media and 20 mL of enrichment culture. The bottles were then autoclaved for 20 min at 121^{o}C. After the bottles cooled to room temperature, HCB, PeCB, and 1,2,4,5-TeCB were added in the same volumes as the live treatments.

All bottles were sealed with Teflon-faced red rubber septa and aluminum crimp caps and incubated in an inverted position (liquid 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 to minimize the possibility for introduction of oxygen.

3.4 Evaluation of Pathogenicity

The original inoculum source for the SRS culture was wetland sediment, with no known exposure to human pathogens. Enrichment of the culture over several years by

providing high concentrations of PCE and TCE in a defined anaerobic mineral medium further suggests that culture conditions are not conducive to survival of pathogens. Nevertheless, the SRS culture was tested for the presence of pathogens. Two approaches were used.

First, the ability of the culture to grow aerobically on a rich substrate (TSB) was evaluated at 37°C. If growth did not occur at this temperature, it would be reasonable to assume that human pathogens were absent. For this test, three treatments were used:

1) TSB inoculated with activated sludge, to serve as a positive control;

2) TSB inoculated with sterile DDI water, to serve as a negative control; and

3) TSB inoculated with the SRS culture.

All treatments were prepared in triplicate in 500 mL Erlenmeyer flasks containing 250 mL of sterile TSB and stoppered with sterile foam plugs. Inocula were delivered aseptically using a sterile 1.0 mL syringe. Oxygen was provided by agitating the liquid using a magnetic stirrer and stir bar. The treatments were incubated in a Napco 330 incubator for 48 hours at 37°C. Culture growth was indicated by an increase in turbidity.

As the results will show, growth of the SRS culture in TSB did occur at 37°C, so the second approach used was to test for the presence of coliforms and *E. coli*. This was accomplished using two commercial testing kits, Colilert® and ReadyCult®. Colilert® simultaneously detects total coliforms and *E. coli* by utilizing the two nutrient indicators, *ortho*-nitrophenyl-*β*-D-galactopyranoside (ONPG) and 4-methylumbelliferyl-*β*-D-glucuronide (MUG). As coliforms grow in Colilert®, they use *β*-galactosidase to metabolize ONPG into *ortho*-nitrophenol, which results in a color change in the media

from colorless to yellow. If *E. coli* are present, they use *β*-glucuronidase to hydrolize MUG into 4-methylumbelliferone, which fluoresces at 366 nm. ReadyCult® media contains 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal) and MUG. The activity of *β*-galactosidase on X-Gal produces a blue-green color to indicate the presence of total coliforms and hydrolysis of MUG serves as an indicator of *E. coli*, as with Colilert®.

Four treatments were set up with Colilert® media:

1) raw sewage, to serve as a positive control for the presence of coliforms and *E. coli*;

2) sterile DDI water, to serve as a negative control;

3) autoclaved sewage, also to serve as a negative control; and

4) the SRS culture.

All treatments were prepared in triplicate in sterile 125 mL Erlenmeyer flasks containing 45 mL of DDI water and stoppered with foam stoppers. A 5 mL disposable syringe was used to deliver 5 mL of the respective inocula. The Colilert® media was then added by pouring in the contents of a sterile snap pack. The flasks were thoroughly mixed and incubated aerobically in a Napco 330 incubator for 24 hours at 37°C. The flasks were observed for a color change from clear to yellow. Samples that turned yellow (indicating a positive test for total coliforms) were then checked for fluorescence with a 365 nm UV light source to test for presence of *E. coli*.

Three treatments were set up with Readycult® media:

1) a positive control (raw sewage);

2) a negative control (sterile DDI water); and

3) the SRS enrichment culture.

The treatments were set up in triplicate and prepared aseptically in sterile 125 mL Erlenmeyer flasks sealed with foam stoppers, or in 100 mL sterile sample bottles pre-filled with Readycult® media. All flasks and sample bottles received 90 mL of DDI water and 10 mL of the respective inocula to give a total sample volume of 100 mL. After adding the inoculum to the flasks, the Readycult® media was added using a sterile snap pack and then the contents were mixed well. The flasks and sample bottles were incubated aerobically in a Napco 330 incubator for 24 hours at 37°C and were then observed for a color change from clear to blue-green. Water samples that turned blue-green (indicating a positive test for total coliforms) were then illuminated with a 365 nm UV light source to test for fluorescence (indicating the presence of *E. coli*). A Colilert® comparator was used to verify fluorescence of samples.

3.5 Inhibition of TCE Reductive Dechlorination by 1,1,1-TCA

Two sets of experiments were performed to test if reductive dechlorination of TCE by the SRS enrichment culture is inhibited by the presence of 1,1,1-TCA. Phase 1 involved the use of undiluted SRS culture and a high initial concentration of 1,1,1-TCA (300 µM). Three treatments were prepared:

1) SRS culture + 300 µM TCE;

2) SRS culture + 300 µM TCE + 300 µM 1,1,1-TCA; and

3) DDI water + 300 µM TCE + 300 µM 1,1,1-TCA.

Grostern and Edwards (23) documented inhibitory effects on the KB-1™ culture at a similar concentration of 1,1,1-TCA. The TCE-only treatment served as a control to demonstrate the culture's ability to dechlorinate TCE. Both live treatments were set up in 160 mL serum bottles with 100 mL of undiluted SRS culture. Lactate was used as the electron donor, with the amount added at least 100 times greater than the amount needed for stoichiometric reduction of the TCE and 1,1,1-TCA. WCs were prepared with 100 mL of DDI water and both compounds. The WCs were used to estimate initial concentrations of TCE and 1,1,1-TCA in the live bottles, since dechlorination of the TCE was too rapid to permit a time zero measurement (data not shown).

As the results will show, 1,1,1-TCA inhibited the complete reduction of TCE in the Phase 1 experiment. To better define the concentration of 1,1,1-TCA required to cause inhibition, a Phase 2 experiment was performed. In this case, the SRS culture was diluted in order to slow the rate of TCE dechlorination and lower concentrations of 1,1,1-TCA were added. The treatments consisted of:

1) SRS culture (10% v/v) + media + 31 µM TCE;

2) SRS culture (10% v/v) + media + 31 µM TCE + 0.7 µM 1,1,1-TCA;

3) SRS culture (10% v/v) + media + 31 µM TCE + 3.6 µM 1,1,1-TCA;

4) SRS culture (10% v/v) + media + 31 µM TCE + 18 µM 1,1,1-TCA; and

5) Water controls, consisting of DDI water + 31 µM TCE, along with 18 µM each of 1,1,1-TCA, CA, and ethene.

The four live treatments were prepared in 160 mL serum bottles with 90 mL minimal media and 10 mL of SRS enrichment culture; this level of inoculum was

consistent with that used by Duhamel et al. (13) and Grostern and Edwards (23). Lactate was used as the electron donor and the amount added was at least 100 times greater than the amount needed for stoichiometric reduction of the TCE and 1,1,1-TCA for all live treatments. As with the Phase 1 experiments, WCs were set up to evaluate the extent of diffusive losses.

For Phases 1 and 2, live treatments were prepared in an anaerobic chamber with an atmosphere of approximately 98% N_2 and 2% H_2. Aseptic techniques were used. The bottles were sealed with Teflon-faced red rubber septa, removed from the chamber, and the headspaces were sparged with a 30%CO_2-70%N_2 gas mixture. The live treatments were incubated in the anaerobic chamber except during headspace analysis. The WCs were prepared and incubated on the bench top with room air present in the headspace. All bottles were incubated in an inverted position (liquid in contact with the septa), at room temperature (21-24^{o}C), and in boxes (to exclude light).

3.6 Microcosm Evaluation of EOS® as Electron Donor

The only electron donor evaluated thus far for the SRS enrichment culture is lactate. The intent of this experiment was to evaluate several EOS® products, the primary ingredient of which is emulsified vegetable oil. Fermentation of vegetable oil typically yields long chain fatty acids that are potentially inhibitory to anaerobic microbes. To avoid this toxicity issue, the vendor recommended that use of EOS® be evaluated in microcosms with soil present, rather than adding EOS® directly to the enrichment culture in media. Soil provides adsorption sites for the long chain fatty acids, thereby lowering their aqueous solubility and minimizing the risk of inhibition.

Six treatments were prepared using soil samples and groundwater from the P-Area:

1) addition of lactate without bioaugmentation;

2) addition of lactate plus bioaugmentation with 1.0% (v/v) of SRS enrichment culture;

3) addition of EOS® 450 plus bioaugmentation with 1.0% (v/v) of SRS enrichment culture;

4) addition of EOS® 598 plus bioaugmentation with 1.0% (v/v) of SRS enrichment culture;

5) addition of EOS® 598B42 plus bioaugmentation with 1.0% (v/v) of SRS enrichment culture; and

6) addition of EOS® 598 and EOS® Vitamin B_{12} solution plus bioaugmentation with 1.0% (v/v) of SRS enrichment culture.

The groundwater was first amended with nutrients (N, P, Mg^{2+}, Ca^{2+}, Fe^{2+}, and micronutrients) in the same ratio that these compounds are added to the minimal media (i.e., per liter of groundwater: 10 mL phosphate buffer, 10 mL salts solution, 2 mL trace metals solution, 2 mL magnesium sulfate solution, and 10 mL yeast extract solution). The pH of the nutrient-amended groundwater was then adjusted from 6.75 to 7.00 by addition of 15 mL of a sodium bicarbonate solution (16 g/L $NaHCO_3$) and 1 mg/L resazurin was added as a redox indicator. pH adjustment created optimal conditions for chlororespiration of PCE and TCE by the SRS enrichment culture.

Microcosms were prepared in 160 mL serum bottles, and all treatments were set up in triplicate. All of the microcosms were prepared in an anaerobic chamber with an atmosphere of approximately 98% N_2 and 2% H_2; aseptic techniques were used during preparation. The microcosms consisted of 20 g of soil (wet weight) plus 50 mL of nutrient amended and pH adjusted groundwater, minus the liquid volume needed for adding PCE and TCE as saturated water solutions (1.0 mL and 2.5 mL, respectively). The initial aqueous-phase concentrations (taking into account partitioning between the headspace and liquid phases) were approximately 2.5 mg/L PCE and 35 mg/L TCE. These are the same initial concentrations used in the microcosm experiments by Wood (45) to evaluate use of the SRS culture for bioaugmentation. The microcosms were monitored for a change in the color of the groundwater from pink to clear, indicating the establishment of a low redox level. Bioaugmentation with the SRS enrichment culture occurred after low redox levels were reached in all of the microcosms.

The procedures used to prepare EOS® products for addition to the EOS®-amended treatments was performed in accordance with vendor recommendations for microcosm studies. A 1:10 dilution of EOS® 450, EOS® 598, and EOS® 598B42 was prepared by adding 10 mL of EOS® to 90 mL of unadjusted P-Area groundwater. The respective 1:10 EOS® dilution was then further diluted fifty times by adding 0.95 mL to microcosms containing 50 mL of adjusted groundwater and 20 g soil. This process resulted in a 1:500 final dilution for all EOS® products. EOS® Vitamin B_{12} solution (15 µL) was added to treatment #6, in accordance with vendor instructions. The amount of lactate added to treatments #1 and #2 was the same as that added during the nutrient microcosm study by

Wood (i.e., 0.1 mL of a stock solution containing approximately 450 g/L of 60% sodium lactate solution) (45).

3.7 Oxygen Tolerance

A potential issue for successful delivery of the SRS enrichment culture in the field is its sensitivity to oxygen, since some exposure is possible. The effect of oxygen exposure on the culture's ability to dechlorinate PCE and TCE was investigated. This was done in two experiments: Phase 1 consisted of a preliminary evaluation to establish the range of possible times the culture could be exposed to oxygen. Three serum bottles received 100 mL of the SRS culture. One bottle was not exposed to air and thereby, served as a positive control. A second bottle was left open to the atmosphere for 12 hours and a third was left open for 24 hours (without agitation). All bottles were then recapped, spiked with PCE, TCE, and lactate (at the same levels routinely added to the enrichment culture) and monitored.

As the first round of testing will show (Appendix E), exposure to air slowed the onset of reductive dechlorination but did not prevent it. Consequently, a second round of testing (Phase 2) was performed. Two treatments were prepared in 160 mL serum bottles with 80 mL of media and 20 mL of enrichment culture, providing a 20% inoculum; both treatments were prepared in triplicate. The bottles were prepared in an anaerobic chamber with an atmosphere of approximately 98% N_2 and 2% H_2. Aseptic techniques were used. One set was not exposed to air and the other set was exposed to air for 24 hours by leaving the bottles open to the atmosphere (without agitation). After 24 hours, the bottles were recapped, and the pH of all bottles was adjusted to neutral with 8 M

NaOH. PCE-saturated water (2.4 mL) and TCE-saturated water (0.9 mL) were added to both treatments along with lactate as the electron donor. The decrease in percent oxygen in the headspace of the bottles exposed to air was measured using a gas chromatograph equipped with a thermal conductivity detector (see section 3.11). The initial percent oxygen in the headspace of the 24-hour exposure treatment bottles was assumed to be 21%.

3.8 pH Tolerance

The SRS enrichment culture is maintained within a pH range of 6.5 to 7.5, which is comparable to similar chlororespiring cultures. Since field pH levels may not be within this range and the cost of controlling in situ pH may be high, it was of interest to know how the SRS enrichment culture would respond to a range of pH levels. The experiment consisted of six treatments:

1) pH 5.5;

2) pH 6.0;

3) pH 6.5;

4) pH 7.0;

5) pH 8.5; and

6) pH 7.0 with no adjustment.

Each treatment consisted of triplicate 160 mL serum bottles that received 84 mL of media and 21 mL of the SRS enrichment culture. The extra 5 mL of liquid (i.e., 105 mL total rather than 100 mL) was added to allow for removing samples for pH adjustment. After capping the bottles with Teflon-lined septa in the anaerobic chamber,

they were removed and the headspaces were sparged with the 30%CO_2-70%N_2 gas mixture. The serum bottles were returned to the anaerobic chamber where 1 mL samples were removed with a syringe and used to check the pH following additions of 8 M NaOH or 6 M HCl (the pH meter was also moved into the anaerobic chamber); see section 3.10. Once the correct pH level was attained, PCE and TCE were added outside the chamber (2.4 and 0.9 mL of saturated water, respectively) along with lactate as the electron donor.

pH levels were maintained within ±0.25 pH units of the target for the first five treatments by acid or base addition each time the bottles were sampled for headspace analysis. This was accomplished by adding 2 mL of media to the bottles, withdrawing a 1 mL sample to measure the pH, addition of acid or base, and withdrawal of a second 1 mL sample to confirm the pH. Media was added prior to each measurement in order to maintain a constant liquid volume in the bottle and avoid altering the pH after acid or base adjustment. No acid or base was added to treatment #6, i.e., an initial pH of 7.0 with no further pH adjustment, in order to observe the culture's reaction to a gradual decrease in pH.

The serum bottles were incubated in the anaerobic chamber except during headspace analysis. All bottles were incubated in an inverted position (liquid in contact with the septa), at room temperature, (21-24^{o}C), and in boxes (to exclude light). At least once per week, the pH of the bottles was measured and adjusted as described above.

3.9 Volatile Organic Compound Analysis

3.9.1 Halogenated Alkenes and Alkanes

The chlorinated ethenes, 1,1,1-TCA, 1,1-DCA, CA, 1,2-DCA, EDB, bromoethane, VB, 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 (FID) in conjunction with a column packed with 1% SP-1000 on 60/80 Carbopack-B (Supelco, Inc.). The carrier gas used was nitrogen (21).

Two sets of standards were prepared. One set was used for experiments in which the serum bottles contained 100 mL of liquid and no soil. For this type of system, standards were prepared by adding known amounts of each compound to 160 mL serum bottles containing 100 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. A separate stock solution of tDCE was also prepared, as it has the same retention time as cDCE. After adding known amounts of the gases and stock solution to the serum bottles, the bottles were incubated for 1-4 hours. This allowed sufficient time for the compounds to equilibrate but not enough time for significant biotic and abiotic losses to occur. Peak areas obtained from headspace analysis were used to determine response factors (RFs) for each compound, in terms of the total mass per bottle per peak area unit from a 0.5 mL headspace sample. Standards

were prepared in the same manner for the halogenated alkanes. RFs and minimum detection limits are shown in Appendix F.

The second set of standards was used for the microcosm experiment evaluating EOS as an electron donor. For this type of system, standards were prepared by adding 50 mL of DDI water plus several glass beads to the serum bottles. The amount of glass beads added was equivalent to the same volume of liquid displaced as 20 g of soil (approximately 11 mL). The same procedures described above were then used to add the volatile compounds and determine RFs for the microcosms, which are shown in Appendix G.

A separate set of standards was prepared for the canisters, since they contain a different ratio of liquid to headspace volume compared to the serum bottles. The liquid standards (cDCE, TCE, and PCE) were prepared in 70 mL serum bottles, using the same ratio of headspace and liquid as in the canisters (i.e., 1.6 L:18 L), and the gas standards (VC, ethene, and methane) were prepared in 160 mL serum bottles, using the same ratio (i.e., 1.6 L:18 L). The same procedures described above were then used to add the volatile compounds and determine RFs (Appendix H). To obtain RFs for the canisters, the RFs for the 70 mL serum bottles and 160 mL serum bottles were multiplied by the ratio of the total volumes, i.e.

$$\textit{Canister RFs} = \textit{70 mL Serum Bottle RFs} \times (\textit{19600 mL}/\textit{70 mL}) \quad (3.1)$$

$$\textit{Canister RFs} = \textit{160 mL Serum Bottle RFs} \times (\textit{19600 mL}/\textit{160 mL}) \quad (3.2)$$

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 are in

equilibrium, the total mass present was converted to an aqueous phase concentration as follows:

$$C_l = \frac{M}{V_l + H_c V_g} \tag{3.3}$$

where C_l = concentration in the aqueous phase (µM); M = total mass present (µmol/bottle); V_l = volume of the liquid in the bottle (0.1 L for the experimental bottles, 18 L for the canisters, and 0.05 L for the microcosms); V_g = volume of the headspace in the bottle (0.06 L for the experimental bottles, 1.6 L for the canisters, and .099 L for the microcosms); and H_c = Henry's constant (dimensionless) at 23°C (see Table 3.1).

3.9.2 Chlorinated Benzenes

CB, DCB isomers, and 1,2,4-TCB were also quantified by analysis of 0.5 mL headspace samples, using a Hewlett Packard Series II 5890 plus GC equipped with an Rtx 5 column (30m x 0.53 mm x 1.5 µM film) and FID. The injector and detector temperature were set at 250°C and 325°C, respectively. An isothermal oven temperature program was set at 120°C for 9 min. The retention times for CB, 1,3-DCB, 1,4-DCB, 1,2-DCB, and 1,2,4-TCB were 2.0, 3.4, 3.5, 3.9, and 7.4 min, respectively. Helium (5 mL/min) was used as carrier gas, nitrogen (30 mL/min) served as the make-up gas, and hydrogen and air (40 mL/min and 210 mL/min, respectively) were used as the fuel for the FID.

HCB, PeCB, and 1,2,4,5-TeCB were quantified on a ZB 624 capillary column (30 m x 0.53 mm x 3.0 µm film) using an electron capture detector (ECD) in order to improve their detection limits, since these compounds are not sufficiently volatile to

measure by FID at the concentrations tested. An isothermal program was used (240°C), during which 1,2,4,5-TeCB, PeCB, and HCB eluted at 6.3, 9.1, and 13.9 min, respectively. Detection limits for all chlorinated benzenes are shown in Table I.1 (Appendix I).

Standards for the serum bottles were prepared by adding known amounts of each compound to 160 mL serum bottles containing 100 mL of DDI water. Stock solutions of the chlorinated benzenes were prepared gravimetrically in acetone (since the compounds are not adequately soluble in methanol). Separate stock solutions of the dichlorobenzene isomers were prepared due to the overlap of their retention times on the ZB 624 column. After adding a known amount of the stock solution to the serum bottles, they were incubated for 1-4 hours. This allowed sufficient time for the compounds to equilibrate but not enough time for significant biotic and abiotic losses to occur. Peak areas obtained from headspace analysis were used to determine RFs for each compound, in terms of the total mass per bottle per peak area unit from a 0.5 mL headspace sample (Appendix I). Equation 3.2 was used to convert mass per bottle to aqueous phase concentrations.

3.10 pH Analysis and Adjustment

The pH of the enrichment cultures maintained in the 2.6 L glass bottles and canisters was measured in 1 mL samples using a Corning 345 pH meter. The pH meter was calibrated before samples were analyzed using pH 7 and 10 buffer solutions. Using a 1.0 mL gas-tight syringe, a 1 mL sample of mixed liquid was put in a test tube, and initial pH was checked with the pH meter. NaOH (8 M) was added incrementally to the

canisters to adjust pH; after each 8 M NaOH addition, the pH was checked and adjusted until the pH reached neutral.

The pH of the liquid in serum bottles was analyzed using pH test strips. Baker-pHIX strips were used for the pH range of 6.0 to 7.7, in 0.3 pH unit increments. Using a 100 µL gas-tight syringe, a 10 µL drop was placed on the test strip and the color was read approximately 3 sec after contact. The only exception to this pH analysis method was with the pH tolerance experiment; see section 3.8.

3.11 Oxygen Analysis

Oxygen was analyzed by gas chromatographic analysis of 0.1 mL headspace samples. A Hewlett Packard 5890 Series II GC was used, equipped with a thermal conductivity detector set at low sensitivity. The GC was equipped with a Molecular Sieve 5A column (3.175 mm x 1.829 m, Alltech). The oven was held at 70ºC and the injector and detector at 120ºC. Helium served as both the reference (30 mL/min) and carrier gas (30 mL/min). Triplicate room air samples (0.1 mL) were used to develop a response factor (i.e., percent oxygen per GC peak area unit). It was assumed that the detector response was linear over the range tested (i.e., 0-21% O2). The sample peak area was multiplied by the response factor to determine the percent oxygen in the headspace of the bottles.

To minimize contamination of the sample with room air, samples were taken inside the anaerobic chamber. The tip of the syringe needle was then pushed into a gray butyl rubber septum so that the tip of the needle was covered. The syringe was removed

from the chamber and taken to the GC, where the needle was pushed through the gray butyl rubber septum directly into the injection port of the GC.

CHAPTER FOUR

RESULTS

4.1 Canister Development

Two canisters were set up as described in section 3.2.2. The canisters were prepared identically with inoculum from the 2.6 L enrichment bottles. Canisters #1 and #2 took 20 and 31 days to completely dechlorinate the PCE and TCE from the first addition, respectively, after no apparent lag (Figures 4.1 and 4.2). cDCE and VC accumulated transiently and then decreased as ethene increased. Ethene was the major terminal product. PCE and TCE concentrations were increased at every addition until the target PCE (15 mg/L) and TCE (38 mg/L) concentrations were reached. The same trend of dechlorination continued over subsequent additions with little accumulation of cDCE and VC. When the canisters were vented or media was wasted, the rate of PCE and TCE dechlorination did not decrease. The cumulative amount of ethene produced was larger than the cumulative amount of PCE and TCE added to the canisters (Figures 4.3 and 4.4). The average molar ratio of ethene to PCE + TCE was 1.16 for canister #1 and 1.48 for canister #2. Standards were not prepared using the canisters; therefore, error associated with the ethene response factor for the canisters is most likely the cause of the higher than expected molar ratios. Results for the leak tests of both canisters showed no abiotic losses of PCE or TCE (Appendix C) when incubated with only DDI water and no live enrichment culture. Canister #1 and canister #2 were successfully maintained for 513 and 329 days, respectively.

4.2 Yield Experiment: Chlorinated Ethenes and Halogenated Ethanes

As explained in section 3.3, the experiment to determine if various halogenated compounds are used as terminal electron acceptors by the SRS enrichment culture was conducted in two phases. Phase 1 was started with a 20% (v/v) inoculum from the enrichment that was being maintained with PCE and TCE, while Phase 2 was started with a 0.1% (v/v) inoculum. Results for Phase 1 are presented first, followed by Phase 2 and then a description of the yield coefficients.

4.2.1 Phase 1

Results for the triplicate bottles that received PCE as the sole chlorinated terminal electron acceptor are shown in Figures 4.5-4.7. Following a brief lag of six days, all of the bottles started rapidly consuming PCE. The amount of PCE added was increased and the rate of utilization responded accordingly. An increasing rate of utilization over time is consistent with cultures that use a compound as a terminal electron acceptor. Over the first 56 days of incubation, there was no apparent accumulation of TCE. Relatively low amounts of cDCE and VC appeared and then declined with each cycle of PCE consumption. Ethene was the predominant daughter product. This pattern of activity was expected, since the SRS culture had been maintained on PCE and TCE as electron acceptors for several years.

However, with the fifth addition of PCE, there was a notable decline in the performance of the culture. The rate of PCE dechlorination slowed considerably. After reviewing the manner in which the cultures were being maintained, one oversight in particular became apparent: No action was being taken to neutralize the HCl released

during PCE dechlorination. On day 95, the pH of all three bottles was found to be less than 6.0, which is generally considered too low for most chlororespiring cultures. The pH was raised to 6.7-7.0 by adding 0.25-0.30 mL of 8 M NaOH. Lactate additions continued during this interval, and despite the pH being less than 6.0, methanogenesis continued. This led to a considerable build up of pressure in the bottles. To alleviate this, the bottles were vented on day 120. This explains the vertical drop in methane, PCE, and ethene on day 120. Thereafter, there was only a limited improvement in the rate of PCE reduction. Apparently, the culture was unable to recover from the low pH episode.

Table 4.1 summarizes the performance of the Phase 1 cultures in terms of the stoichiometry of ethene formation and the percentage of lactate added (based on electron equivalents) that was used for the respective TEA's reduction to ethene. For PCE, an average of 89% of the amount consumed was recovered as ethene and only 0.32% of electron equivalents of the lactate added was consumed for reductive dechlorination.

Figures 4.8-4.10 present the results for triplicate bottles that received TCE as the sole chlorinated terminal electron acceptor. As with PCE, rapid utilization of TCE started after a brief lag period. The amount of TCE added was increased, and the rate of utilization responded accordingly through day 40. Ethene was the predominant daughter product. Relatively low amounts of cDCE and VC appeared and then declined with each cycle of PCE consumption. This pattern of activity was expected, since the SRS culture had been maintained on PCE and TCE as electron acceptors for several years.

Beginning with the fourth addition of TCE, the rate of utilization started to slow down. As with the PCE bottles, a problem with pH was suspected, since no attempt was made to neutralize the HCl released from TCE dechlorination. On day 95, the pH of the bottles was found to be less than 6.0. The pH was raised to 7.0 by adding 0.25 mL of 8 M NaOH. Lactate additions continued during this interval, and despite the pH being less than 6.0, methanogenesis continued. This led to a considerable build up in pressure in the bottles. To alleviate this, the bottles were vented on day 120. This explains the vertical drop in methane, TCE, and ethene on day 120. Thereafter, there was only a limited improvement in the rate of TCE reduction. Apparently, the TCE culture was also unable to recover from the low pH episode. During the full incubation period of 172 days, all of the TCE consumed was accounted for as ethene, with 0.32% of the lactate equivalents added consumed for reductive dechlorination (Table 4.1).

Figures 4.11-4.13 present the results for triplicate bottles that received cDCE as the sole chlorinated terminal electron acceptor. There was no apparent lag in the onset of cDCE utilization. The amount of cDCE added was increased, and the rate of utilization responded accordingly through day 45. VC accumulated transiently during each cycle of cDCE dechlorination, although ethene was the predominant terminal product. This provided the first direct evidence that the SRS culture is capable of using cDCE as a sole electron acceptor.

Beginning with the fourth addition of cDCE, the rate of utilization started to slow down. As with the PCE and TCE bottles, a problem with pH was suspected, since no attempt was made to neutralize the HCl released from cDCE dechlorination. On day 98,

the pH of the bottles was found to be in the range of 6.0-6.4. The pH was raised to 7.0 by adding 0.12 mL of 8 M NaOH. Lactate additions continued during this interval, and despite the pH being less than 6.4, methanogenesis continued. This led to a considerable build up in pressure in the bottles. To alleviate this, the bottles were vented on day 120. This explains the vertical drop in methane, cDCE, and ethene on day 120. Thereafter, there was only a limited improvement in the rate of cDCE reduction. Apparently, the culture was also unable to recover from the low pH episode. During the full incubation period of 173 days, all of the cDCE consumed was accounted for as ethene and only 0.42% of the lactate equivalents added was consumed for reductive dechlorination (Table 4.1).

Figures 4.14-4.16 present the results for triplicate bottles that received tDCE as the sole chlorinated terminal electron acceptor. Following a lag phase of approximately 20 days, reduction of tDCE to ethene was observed. The rate of dechlorination with the second addition of tDCE was initially similar but started to slow down considerably after day 79. The reason for this is unclear. It seemed unlikely to be a problem with pH because not as much tDCE had been dechlorinated compared to PCE, TCE and cDCE. However, on day 109, the pH was lower than desirable (i.e., 6.0-6.4), so 0.12 mL of 8 M NaOH was added to raise the pH to 7.0. Lactate additions continued during this interval, and despite the pH being less than 6.4, methanogenesis continued. This led to a considerable build up in pressure in the bottles. To alleviate this, the bottles were vented on day 120. This explains the vertical drop in methane, tDCE, and ethene on day 120. Thereafter, there was a short period of recovery, but then the rate of dechlorination

slowed again. It became necessary to vent the bottles a second time on day 217. Following the fourth addition of tDCE to bottles #2 and #3, reductive dechlorination of tDCE was inconsistent (Figures 4.15 and 4.16). During the full incubation period of 179 days, less than half of the tDCE consumed was accounted for as ethene (Table 4.1), with the balance primarily VC; only 0.12% of the lactate equivalents added was consumed for reductive dechlorination (Table 4.1). The tDCE results for Phase 1 suggested that tDCE is not used as a terminal electron acceptor by the SRS culture.

Results for the triplicate bottles that received 1,1-DCE as the sole chlorinated terminal electron acceptor are shown in Figures 4.17-4.19. There was no apparent lag in the onset of 1,1-DCE utilization. The amount of 1,1-DCE added was increased, and the rate of utilization responded accordingly through day 120. VC accumulated transiently during each cycle of 1,1-DCE dechlorination, although ethene was the predominant terminal product. This provided the first direct evidence that the SRS culture is capable of using 1,1-DCE as a sole terminal electron acceptor.

Beginning with the seventh addition of 1,1-DCE, the rate of utilization started to slow down. As with the PCE, TCE, and cDCE bottles, a problem with pH was suspected, since no attempt was made to neutralize the HCl released from 1,1-DCE dechlorination. On day 109, the pH of the bottles was found to be less than 6.0. The pH was raised to 6.7-7.0 by adding 0.22-0.28 mL of 8 M NaOH. Methanogenesis was very active in these bottles, leading to a considerable build up in pressure. To alleviate this, the bottles were vented on days 120, 171, and 219 (bottle #1) or 232 (bottles #2 and #3). This explains the vertical drop in methane, 1,1-DCE, and ethene on these days. Three more additions

of 1,1-DCE were made after the pH adjustment. Unlike the situation with the other chlorinated ethenes described so far, the bottles that received 1,1-DCE improved in performance after pH adjustment and venting. The final addition of 1,1-DCE was consumed as fast as or faster than any of the previous additions. During the full incubation period of 252 days, all of the 1,1-DCE consumed was accounted for as ethene and only 0.42% of the lactate equivalents added was consumed for reductive dechlorination (Table 4.1).

Results for the triplicate bottles that received VC as the sole chlorinated terminal electron acceptor are shown in Figures 4.20-4.22. There was no apparent lag in the onset of VC utilization. The amount of VC added was increased, and the rate of utilization responded accordingly. Ethene was the only product observed. This provided the first direct evidence that the SRS culture is capable of using VC as a sole terminal electron acceptor. On day 109, the pH of the bottles was found to be approximately 6.0. The pH was raised to 6.7-7.0 by adding 0.13-0.16 mL of 8 M NaOH. Methanogenesis was very active in these bottles, leading to a considerable build up in pressure. To alleviate this, the bottles were vented on days 120, 171, and 199. This explains the vertical drop in methane, VC, and ethene on these days. Unlike the situation with the other chlorinated ethenes, the rate of VC utilization was quite consistent through the full incubation period, both before and after pH adjustment and venting. All of the VC consumed was accounted for as ethene and only 0.56% of the lactate equivalents added was consumed for reductive dechlorination (Table 4.1).

Figures 4.23-4.25 present the results for triplicate bottles that received 1,2-DCA as the sole chlorinated terminal electron acceptor. Following a lag phase of approximately 18 days, reduction of 1,2-DCA to ethene began at a high rate. Ethene was the only significant product observed; trace amounts of chloroethane and VC were detected occasionally. After eight additions of approximately 7.5 µmol per bottle, the amount of 1,2-DCA added was increased to approximately 25 µmol per bottle (24 mg/L), and the rate of reduction increased correspondingly. This provided the first direct evidence that the SRS culture is capable of using 1,2-DCA as a sole terminal electron acceptor. The Phase 1 bottles that received 1,2-DCA were started after the bottles that received chlorinated ethenes, so the potential problem with pH control was known. To avoid this issue, the pH was monitored periodically, and 8 M NaOH was added as needed to return the pH to neutral. At no time was the pH allowed to drop below 6.4. Consequently, there was no interruption in the rate of 1,2-DCA utilization. This is in contrast to what occurred with the PCE, TCE, cDCE, and 1,1-DCE bottles, in which the pH decreased below 6.0 and a high rate of reduction could not be restored even after adjusting the pH back to neutral. Methanogenesis was very active in these bottles, leading to a considerable build up in pressure. To alleviate this, the bottles were vented on days 62, 141, 158, and 170. After 194-227 days of incubation, 94% of the 1,2-DCA consumed was recovered as ethene and only 0.44% of the lactate equivalents added was consumed for dihaloelimination (Table 4.1).

Results for the triplicate bottles that received EDB as the sole brominated terminal electron acceptor are shown in Figures 4.26-4.28. Although this culture had never before

been exposed to a brominated organic compound, the lag phase prior to the onset of EDB utilization was only three days. The amount of EDB added was gradually increased, and the rate of debromination responded accordingly. Ethene was the only significant product observed; trace amounts of bromoethane and VB were detected occasionally. The highest amount of EDB added reached 24 μmol per bottle (22 mg/L). This provided the first direct evidence that the SRS culture is capable of using EDB as a sole terminal electron acceptor. As with the 1,2-DCA bottles, the Phase 1 bottles that received EDB were started after the bottles that received chlorinated ethenes, so the potential problem with pH control was known. To avoid this issue, the pH was monitored periodically, and 8 M NaOH was added as needed to return the pH to neutral. At no time was the pH allowed to drop below 6.4. Consequently, there was no interruption in the rate of EDB utilization. Methanogenesis was very active in these bottles, leading to a considerable build up in pressure. To alleviate this, the bottles were vented on days 62 and 134. After 173 days of incubation, all of the EDB consumed was accounted for as ethene and only 0.56% of the lactate equivalents added was consumed for dihaloelimination (Table 4.1).

Water controls were prepared for all of the volatile organic compounds that were quantified. Average results for triplicate bottles that contained the chlorinated ethenes and ethene are shown in Figure 4.29. Over 208 days of incubation, losses of PCE were highest, averaging 50%, although this was minor in comparison to the amount of PCE consumed in the live bottles (Figures 4.5-4.7). Losses of TCE and VC averaged 20% and 12%, respectively, and there was no loss of cDCE or ethene. Average results for triplicate water controls that contained 1,2-DCA, EDB and VB indicate that only minor

losses occurred during 212 days of incubation (Figure 4.30), especially in comparison to the amount of 1,2-DCA and EDB consumed by the live bottles over a similar time period.

The pattern by which PCE, TCE, cDCE, 1,1-DCE, VC, 1,2-DCA and EDB were consumed during the Phase 1 experiment suggested that all are used as TEAs by the SRS culture. Results for tDCE were inconclusive. At the end of the incubation period, samples from all bottles were sent to Dr. Christopher Bagwell at SRS to quantify *Dehalococcoides* levels using qPCR. Given the standard error in the measurement, it was difficult to discern a significant increase in *Dehalococcoides* between the start and end of the incubation period (data not shown). A second experiment (referred to as Phase 2) was, therefore, performed to establish more definitively that consumption of the halogenated compounds is linked to an increase in the *Dehalococcoides* population. A key difference between Phase 1 and Phase 2 was that Phase 2 was started with a much lower inoculum level (0.01%) in order to facilitate detection of a significant increase in the *Dehalococcoides* population.

Another major difference between Phases 1 and 2 was pH control. Unlike Phase 1, the pH in Phase 2 bottles was not allowed to deviate significantly from neutral. This was accomplished by adding stoichiometric amounts of 8 M NaOH along with the halogenated compounds.

4.2.2 Phase 2

The source of inoculum for the Phase 2 experiment is presented in Figure 3.1. Because the Phase 1 cultures with PCE, TCE, cDCE, and tDCE faltered as a result of problems with pH, the Phase 2 inoculum source for these compounds was the stock

enrichment culture maintained in 2.6 L glass bottles. For 1,1-DCE, VC and 1,2-DCA, the sources of inocula were the respective Phase 1 bottles, since they performed consistently, and use of Phase 1 inocula offered the possibility of a reduced lag period, since the culture had already been acclimated to these compounds. For EDB, all of the Phase 1 culture was used to prepare a single large source of culture for a different project. This EDB "mother bottle" was fed EDB repeatedly and performed similarly to the Phase 1 bottles. A 0.1% inoculum from the "EDB mother" was used for the Phase 2 EDB experiment. Lastly, Phase 2 included an evaluation of VB as a terminal electron acceptor, even though it had not been tested during Phase 1. The source of inoculum was the Phase 2 EDB bottles, once those had reached the targeted amount of Br^- released (200 μmol/bottle).

Results for the Phase 2 PCE bottles are shown in Figures 4.31-4.33. Following a lag period of 19-31 days, all of the bottles started rapidly consuming PCE. The longer lag period compared to Phase 1 (Figures 4.5-4.7) was not surprising, given the much lower inoculum level used for Phase 2. cDCE accumulated and then started to decline, followed by a transient accumulation of VC. A second addition of PCE was made on day 67. More VC accumulated, although ethene started to overtake VC. By the time the third addition of PCE was consumed, ethene was the only significant daughter product, and this trend continued over subsequent additions. pH problems did not arise (due to routine additions of NaOH and monitoring), and it was, therefore, possible to sustain a rapid rate of PCE dechlorination. Following 112 days of incubation, 83% of the PCE consumed was attributable to ethene, and 0.74% of the lactate equivalents added was

consumed for reductive dechlorination (Table 4.2). Methane output was approximately tenfold lower compared to the Phase 1 PCE bottles.

Results for the Phase 2 TCE bottles are shown in Figures 4.34-4.36. Similarly to the PCE bottles, following a lag period of 19 days, all of the bottles started rapidly consuming TCE. The longer lag period compared to Phase 1 (Figures 4.8-4.10) was not surprising, given the much lower inoculum level used for Phase 2. cDCE accumulated and then started to decline, followed by a transient accumulation of VC. A second addition of TCE was made on day 48. VC and smaller amounts of cDCE accumulated transiently, but they were consumed and replaced by ethene as the major terminal product. This trend continued over subsequent additions. pH problems did not arise, and it was, therefore, possible to sustain a rapid rate of TCE dechlorination. Following 103 days of incubation, 91% of the TCE consumed was attributable to ethene, and 0.79% of the lactate equivalents added was consumed for reductive dechlorination (Table 4.2). Methane output was approximately eightfold lower compared to the Phase 1 TCE bottles.

Results for the Phase 2 cDCE bottles are shown in Figures 4.37-4.39. Following a lag period of 31 days, all of the bottles started rapidly consuming cDCE. The longer lag period compared to Phase 1 (Figures 4.11-4.13) was not surprising, given the much lower inoculum level used for Phase 2. VC accumulated and then declined as ethene was produced. A second addition of cDCE was made on day 54. VC and ethene were produced simultaneously as the cDCE was consumed.

On the fourth addition of cDCE, dechlorination began to slow down, and by the fifth addition, dechlorination had slowed significantly. pH problems did not appear to be

the cause of the decrease in dechlorination activity, as the pH was not allowed to drop below 6.7 during the entire incubation. The only difference in the fourth and fifth additions as compared to the previous additions was the manner in which cDCE was added to the bottles. cDCE was added as saturated water prior to the fourth addition, at which time cDCE was added as a neat compound. The same neat cDCE was used to make the saturated water; therefore, the decrease in activity did not appear to be caused by inhibitors in the neat cDCE (99% purity). The Phase 1 cDCE bottles never received neat cDCE, so it is unclear if this was the cause of the inhibition. Bottle #1 eventually consumed all of the cDCE from the fifth addition, as well as the VC produced during the cycle, to produce ethene as the primary terminal product. Bottles #2 and #3 did not completely consume the cDCE and VC from the fifth addition but were expected to follow the same trend as bottle #1. Following 174 days of incubation, 80% of the cDCE consumed was attributable to ethene and 6% to VC; 0.58% of the lactate equivalents added was consumed for reductive dechlorination (Table 4.2). Methane output was approximately half the output of the Phase 1 cDCE bottles.

Results for the Phase 2 tDCE bottles are shown in Figures 4.40-4.42. Following a lag period of 31 days, all of the bottles started rapidly consuming tDCE. The longer lag period compared to Phase 1 (Figures 4.14-4.16) was not surprising, given the much lower inoculum level used for Phase 2. VC accumulated transiently and then declined as ethene was produced. A second addition of tDCE was made on day 54. The same trend continued over subsequent additions with ethene as the major terminal product. Although results from the Phase 1 tDCE bottles were inconclusive, the Phase 2 results

provided the first direct evidence that the SRS culture is also capable of using tDCE as a sole terminal electron acceptor. pH problems did not arise, and it was, therefore, possible to sustain a rapid rate of tDCE dechlorination. Following 127 days of incubation, 93% of the tDCE consumed was attributable to ethene, and 0.66% of the lactate equivalents added was consumed for reductive dechlorination (Table 4.2). Methane output was approximately twenty times lower compared to the Phase 1 tDCE bottles.

Results for the Phase 2 1,1-DCE bottles are shown in Figures 4.43-4.45. Following a lag period of 34-48 days, all of the bottles started rapidly consuming 1,1-DCE. The longer lag period compared to Phase 1 (Figures 4.17-4.19) was not surprising, given the much lower inoculum level used for Phase 2. VC accumulated transiently and then declined as ethene was produced. A second addition of 1,1-DCE was made on day 57. VC and ethene were produced simultaneously with the major terminal product being ethene. The same trend continued over subsequent additions. pH problems did not arise, and it was, therefore, possible to sustain a rapid rate of 1,1-DCE dechlorination. Following 138 days of incubation, 72% of the 1,1-DCE consumed was attributable to ethene, and 0.60% of the lactate equivalents added was consumed for reductive dechlorination (Table 4.2). Methane output was more than tenfold lower compared to the Phase 1 1,1-DCE bottles.

Results for the Phase 2 VC bottles are shown in Figures 4.46-4.48. Following a lag period of 34-48 days, all of the bottles started rapidly consuming VC. The longer lag period compared to Phase 1 (Figures 4.20-4.22) was not surprising, given the much lower inoculum level used for Phase 2. Ethene was the only product observed. A second

addition of VC was made on day 57. pH problems did not arise, and it was, therefore, possible to sustain a rapid rate of VC dechlorination. Following 138 days of incubation, 91% of the VC consumed was attributable to ethene, and 0.79% of the lactate equivalents added was consumed for reductive dechlorination (Table 4.2). Methane output was six times lower than the methane production in the Phase 1 VC bottles.

Results for the Phase 2 1,2-DCA bottles are shown in Figures 4.49-4.51. Following a lag period of 36-48 days, all of the bottles started rapidly consuming 1,2-DCA. The longer lag period compared to Phase 1 (Figures 4.23-4.25) was not surprising, given the much lower inoculum level used for Phase 2. Ethene was the only significant product observed; trace amounts of VC were detected occasionally. A second addition of 1,2-DCA was made on day 60, and the same trend continued over subsequent additions. pH problems did not arise, and it was, therefore, possible to sustain a rapid rate of 1,2-DCA dechlorination. Following 105 days of incubation, 94% of the 1,2-DCA consumed was attributable to ethene, and 0.41% of the lactate equivalents added was consumed for dihaloelimination (Table 4.2). Methane output was threefold less than the Phase 1 1,2-DCA bottles.

Results for the Phase 2 EDB bottles are shown in Figures 4.52-4.54. Following a lag period of 20-28 days, all of the bottles started rapidly consuming EDB. The longer lag period compared to Phase 1 (Figures 4.26-4.28) was not surprising, given the much lower inoculum level used for Phase 2. Ethene was the only significant product observed; trace amounts of bromoethane were detected occasionally. A second addition of EDB was made on day 28 (bottle #3) or day 36 (bottles #1 and #2), and the same trend

continued over subsequent additions. EDB was consumed the fastest of all tested compounds. pH problems did not arise, and it was, therefore, possible to sustain a rapid rate of EDB debromination. Following 90 days of incubation, 84% of the EDB consumed was attributable to ethene, and 0.60% of the lactate equivalents added was consumed for dihaloelimination (Table 4.2). Methane output was more than tenfold lower than the Phase 1 EDB bottles.

Results for the Phase 2 triplicate bottles that received VB as the sole brominated terminal electron acceptor are shown in Figures 4.55-4.57. Following a lag period of 26 days, all of the bottles started rapidly consuming VB. The amount of VB added was gradually increased, and the rate of debromination responded accordingly. Ethene was the only significant product observed. The highest amount of VB added reached 62 µmol per bottle (51 mg/L). This provided the first direct evidence that the SRS culture is capable of using VB as a sole terminal electron acceptor. As these bottles were started after Phase 1, the potential problem with pH control was known. To avoid this, the pH was monitored at every VB addition, and 8 M NaOH was added along with VB to return the pH to neutral. At no time was the pH allowed to drop below 6.6. Consequently, there was no interruption in the rate of VB utilization. It was, therefore, possible to sustain a rapid rate of VB debromination. Following 122 days of incubation, 95% of the VB consumed was attributable to ethene, and 0.81% of the lactate equivalents added was consumed for reductive debromination (Table 4.2). Methanogenesis occurred during the incubation but methane did not reach high enough levels to require venting.

It is apparent from the consumption patterns that all of the halogenated alkenes and alkanes tested are used rapidly by the SRS culture, suggesting that they are used as terminal electron acceptors. A more robust indication of use as a terminal electron acceptor is a direct association between dehalogenation and an increase in *Dehalococcoides*. The levels of *Dehalococcoides* in all treatments were quantified at the start and end of the Phase 2 incubation period by Dr. Christopher Bagwell, using qPCR. Results are shown in Table 4.3. All treatments consumed enough of the test compound to release at least 200 µmol of Cl^- or Br^- per bottle. By comparing the initial and final *Dehalococcoides* concentrations, it is readily apparent that there was an increase in *Dehalococcoides* of at least 2 to 3 orders of magnitude, i.e., the concentrations increased from an initial concentration of approximately 10^6 gene copies per mL to a final concentration of 10^8-10^9 genes copies per mL. For 1,1-DCE, VC, EDB, and VB, the initial concentrations were even lower; recall that the source of inoculum for these treatments was not the stock enrichment culture (Figure 3.1). Nevertheless, these treatments finished with similar concentrations of *Dehalococcoides* compared to the other compounds (i.e., 10^8-10^9 genes per mL). The exception was EDB, which had a considerably lower final concentration of *Dehalococcoides*.

Based on the net increase in *Dehalococcoides* and the average release of chloride or bromide (calculated based on the amount of parent compound consumed and daughter products formed, rather than direct measurement of the anions), yield values were calculated in terms of gene copies per µmol of Cl^- or Br^- released. Yields ranged from 7.9×10^7 for VC to 1.8×10^9 gene copies per µmol of Cl^- for 1,2-DCA. The only outlier

was for EDB, which had an unexpectedly low yield of $6.8x10^5$ gene copies per µmol of Br^-. A comparison of these values to yields reported for other cultures will be presented in the Discussion.

4.3 Reductive Dechlorination of Chlorinated Benzenes

Results for the triplicate bottles that received HCB as the sole chlorinated terminal electron acceptor are shown in Figures 4.58-4.60. After 19 days of incubation, almost all of the HCB was consumed. PeCB and 1,2,4,5-TeCB were detected, which indicated that HCB was dechlorinated to PeCB and 1,2,4,5-TeCB. However, the levels of PeCB and 1,2,4,5-TeCB were much higher than the molar amount of HCB that was added to the bottles. The same amount of HCB was added during repeat additions (Table 3.1). Rapid consumption of HCB was observed with formation of PeCB and 1,2,4,5-TeCB. On day 27, 1,2,4-TCB was also detected as a dechlorination product. 1,2,4-TCB was present in even larger amounts than the PeCB or 1,2,4,5-TeCB, which was also inconsistent with the molar amount of HCB added to the bottles. The percent recoveries of HCB dechlorination products are shown in Table 4.4, as well as the ratio of electron equivalents used for dechlorination to the electron equivalents of lactate added. To further support disappearance of HCB as a result of biotic dechlorination, the autoclaved controls (Figure 4.61) showed no loss of HCB over 36 days of incubation. Regardless of the poor mass balance for the daughter products, the observed disappearance of HCB and appearance of 1,2,4-TCB suggests that the SRS culture may be capable of using HCB as a sole chlorinated electron acceptor. No dichlorobenzene isomers or CB were detected over the course of incubation. However, the lack of DCBs and CB is insufficient

evidence to conclude that 1,2,4-TCB cannot be reductively dechlorinated by the SRS culture. The amounts of DCBs and CB that could have formed based on the total amount of HCB consumed (i.e., an average of 9.31×10^3 µmol/bottle) were too low to be detected. Results for treatments started with DCBs and CB at higher levels are presented below. For the triplicate bottles with HCB added, methane production was very active, which indicates that the concentrations of HCB used, and its subsequent daughter products, were not inhibitory (Table 4.4).

Results for the triplicate bottles that received PeCB as the sole chlorinated terminal electron acceptor are shown in Figures 4.62-4.64. After 20 days of incubation, all of the PeCB was consumed. 1,2,4-TCB and 1,3,5-TCB were detected as dechlorination products; only a trace amount of 1,2,4,5-TeCB was detected. The presence of 1,3,5-TCB indicated that 1,2,3,5-TeCB must also be a dechlorination product, as 1,3,5-TCB is only formed from dechlorination of 1,2,3,5-TeCB; however, both 1,2,3,5-TeCB and 1,2,4,5-TeCB co-elute on the column and could not be distinguished as two separate compounds. Therefore, it was assumed that the TeCB isomers eluting at 6.3 min was all 1,2,4,5-TeCB. The percent recoveries of PeCB dechlorination products are shown in Table 4.4, as well as the ratio of electron equivalents used for dechlorination to the electron equivalents of lactate added. Subsequent additions of PeCB were made at the same concentrations initially added to the bottles (Table 3.1). Initially, the bottles were only being analyzed on the ECD. On days 67 (bottles #1 and #2) and 82 (bottle #3), the bottles were analyzed for the first time using the FID, and 1,2-DCB and 1,4-DCB were also detected as daughter products. This

explains why no data for 1,2-DCB or 1,4-DCB is shown on Figures 4.62-4.64 prior to these days. No 1,3-DCB or CB were detected over the course of incubation. These results suggested that the SRS culture may be capable of using PeCB as a sole electron acceptor. There was an 85% recovery of the PeCB as 1,2,4-TCB, 1,3,5-TCB, 1,2-DCB, and 1,4-DCB. For the triplicate bottles with PeCB added, methane production was very active, which indicates that the concentrations of PeCB used, and its subsequent daughter products, were not inhibitory (Table 4.4).

Results for the triplicate bottles that received 1,2,4,5-TeCB as the sole chlorinated terminal electron acceptor are shown in Figures 4.65-4.67. After 20 days of incubation, all of the 1,2,4,5-TeCB was consumed. 1,2,4-TCB was the only dechlorination product detected. The percent recoveries of 1,2,4,5-TeCB dechlorination products are shown in Table 4.4, as well as the ratio of electron equivalents used for dechlorination to the electron equivalents of lactate added. Subsequent additions of 1,2,4,5-TeCB were made at the same concentrations initially added to the bottles (Table 3.1) and rapid consumption continued. Like the PeCB bottles, the 1,2,4,5-TeCB bottles were not analyzed on the FID prior to day 67. On day 67, the bottles were analyzed using the FID for the first time, and 1,2-DCB and 1,4-DCB were also detected as daughter products. This explains the absence of data for 1,2-DCB and 1,4-DCB on Figures 4.65-4.67 prior to day 67. 1,3-DCB and CB were not detected over the course of incubation. There was a 91% recovery of the PeCB as 1,2,4-TCB, 1,3,5-TCB, 1,2-DCB, and 1,4-DCB. These results suggested that the SRS culture may be capable of using PeCB as a sole electron acceptor. For the triplicate bottles with 1,2,4,5-TeCB added, methane production was

very active, which indicates that the concentrations of 1,2,4,5-TeCB used, and its subsequent daughter products, were not inhibitory (Table 4.4).

Results for the triplicate bottles that received 1,2,4-TCB as the sole chlorinated terminal electron acceptor are shown in Figures 4.68-4.70. By day 20, there was a significant decrease in the concentration of 1,2,4-TCB, but no significant increase in daughter products. This may have been caused by an error in the initial measurements or abiotic losses. After day 20, further decreases in 1,2,4-TCB were accompanied by an increase in 1,2-DCB and 1,4-DCB. A trace amount of CB also accumulated, which was the first detection of CB as an end product of chlorinated benzene dechlorination by the SRS enrichment culture. On day 82, a second addition of 1,2,4-TCB was consumed with no apparent lag. There was a 58% recovery of the 1,2,4-TCB as 1,2-DCB, 1,4-DCB, and CB. The average percent recoveries of 1,2,4-TCB dechlorination products are shown in Table 4.4, as well as the ratio of electron equivalents used for dechlorination to the electron equivalents of lactate added. These results suggested that the SRS culture may be capable of using 1,2,4-TCB as a sole electron acceptor. For the triplicate bottles with 1,2,4-TCB added, methane production was very active, which indicates that the concentrations of 1,2,4-TCB used, and its subsequent daughter products, were not inhibitory (Table 4.4).

Results for the triplicate bottles that received 1,2-DCB as the sole chlorinated terminal electron acceptor are shown in Figures 4.71-4.73. After 18 days of incubation, the amount of 1,2-DCB present decreased, although CB was not detected as a dechlorination product. This may have been caused by an error in the initial

measurement or abiotic losses. Thereafter, the concentration of 1,2-DCB remained constant, with no formation of CB or benzene. These results demonstrated that the SRS enrichment culture does not utilize 1,2-DCB as an electron acceptor. Methane production was active in these bottles, although no dechlorination occurred (Table 4.4).

Results for the triplicate bottles that received 1,3-DCB as the sole chlorinated terminal electron acceptor are shown in Figures 4.74-4.76. After 18 days of incubation, there was a decrease in the amount of 1,3-DCB present, although CB was not detected as a dechlorination product. This may have been caused by an error in the initial measurements or abiotic losses. Thereafter, the concentration of 1,3-DCB remained approximately constant, with no formation of CB or benzene. These results demonstrated that the SRS enrichment culture does not utilize 1,3-DCB as an electron acceptor. Methane production was active in these bottles, although no dechlorination occurred (Table 4.4).

Results for the triplicate bottles that received 1,4-DCB as the sole chlorinated terminal electron acceptor are shown in Figures 4.77-4.79. After 18 days of incubation, there was an approximately 20% decrease in 1,4-DCB, although CB was not detected as a dechlorination product. After day 18, the concentration of 1,4-DCB fluctuated, with no apparent decreasing trend. These results demonstrated that the SRS enrichment culture does not utilize 1,4-DCB as an electron acceptor. Methane production was active in these bottles, although no dechlorination occurred (Table 4.4).

Results for the triplicate bottles that received CB as the sole chlorinated terminal electron acceptor are shown in Figures 4.80-4.82. After 18 days of incubation, there was

a notable drop in CB, although benzene was not detected as a dechlorination product. After day 18, the concentration of CB remained approximately constant, with no formation of benzene. These results demonstrated that the SRS enrichment culture does not utilize CB as an electron acceptor. Methane production was active in these bottles although, no dechlorination occurred (Table 4.4).

In summary, HCB, PeCB, 1,2,4,5-TeCB, and 1,2,4-TCB were reductively dechlorinated at increasing rates over time, a pattern of consumption that suggests these compounds may be utilized as TEAs by the SRS enrichment culture. The results for 1,2-DCB, 1,3-DCB, 1,4-DCB, and CB demonstrate that the SRS enrichment culture does not reductively dechlorinate these compounds, and therefore, they are not useable as TEAs. The large decreases in the initial concentrations of 1,2-DCB, 1,3-DCB, and CB were similar to the decreases in the concentrations of these compounds in the WCs (Figures 4.83-4.84). As mentioned above, this may be due to an error in the initial measurements or abiotic losses. The percent losses of all of the compounds from the WCs and live bottles were similar and are summarized in Table 4.5.

4.4 Evaluation of Potential Pathogens in the SRS Enrichment Culture

4.4.1 Growth in TSB

A preliminary test for the presence of pathogens in the SRS enrichment culture was conducted by determining if the culture grows at the temperature of the human body (37°C) in a rich medium (TSB) under aerobic conditions. Growth was evaluated based on an increase in turbidity. The positive control, inoculated with activated sludge from the City of Clemson wastewater treatment plant, performed as expected, i.e., the medium

became turbid within 24 hours of incubation. The negative control, inoculated with sterile water, also performed as expected, i.e., there was no increase in turbidity after 48 hours of incubation. This indicated that the aseptic technique used to prepare the experiment was adequate. The flasks inoculated with the SRS culture became turbid within 24 hours, indicating that the culture does contain microbes that are capable of growing aerobically at 37°C. Had this result been negative, it would have indicated that human pathogens are very likely absent from the SRS culture. The fact that the result with TSB was positive did not demonstrate the presence of pathogens; however, it did suggest that further testing was necessary.

4.4.2 Evaluation of Total Coliforms and *E. coli*

A subsequent experiment was conducted to determine the presence or absence of coliforms (i.e., without quantification) and *E. coli* in the SRS enrichment culture. Both positive controls set up with Colilert® and Readycult®, inoculated with raw sewage from the City of Clemson wastewater treatment plant, performed as expected. All of the positive controls grown in Colilert® turned yellow and those grown in the Readycult® turned blue/green within 24 hours of incubation. These color changes indicated the presence of coliforms. Positive samples were then illuminated with a 365 nm UV light, and all of them fluoresced, indicating the presence of *E. coli*.

One set of negative controls were inoculated with sterile water and performed as expected. The Colilert® samples remained clear and the Readycult® samples turned yellow after 24 hours of incubation. A second set of negative controls, using only Colilert® media, were inoculated with autoclaved sewage. These also performed as

expected; i.e., the samples remained clear. These results indicated that the aseptic technique used to prepare the experiment was adequate.

The SRS culture tested positive for coliforms with both types of media. All of these samples, however, tested negative for *E. coli*.

4.5 Evaluation of Chlorinated Ethene Inhibition by 1,1,1-TCA

As described in Section 3.5, a preliminary inhibition experiment was performed with a high concentration of 1,1,1-TCA (300 µM), modeled after a previous study by Grostern and Edwards (23). Based on the results of this first phase, a second set of experiments was performed at lower concentrations of 1,1,1-TCA as well as a more dilute initial culture, to better simulate conditions that may exist during bioaugmentation. The results for the Phase 1 experiment are presented first, followed by Phase 2.

4.5.1 Phase 1

Results for the triplicate bottles provided with 300 µM TCE (30.11 µmol/bottle) are shown in Figures 4.85-4.87. In the absence of 1,1,1-TCA, TCE dechlorination occurred rapidly, partly due to the use of undiluted SRS enrichment culture. After only one day of incubation, less than five µmol TCE remained in the bottles, and a stoichiometric increase in ethene was observed. Complete consumption of TCE was observed when the next measurement was made on day three, although it is likely that dechlorination was completed even earlier. Only transient amounts of cDCE and VC were detected during the first three days. Methane production was immediate and continued through day 12.

Figures 4.88-4.90 present the results for the triplicate bottles that were amended with 300 µM TCE + 300 µM 1,1,1-TCA (30.11 µmol/bottle TCE and 48.25 µmol/bottle 1,1,1-TCA). Although there was no apparent lag in dechlorination of TCE, the rate of consumption was slower than in the TCE-only bottles. The presence of 1,1,1-TCA approximately doubled the average time required for TCE dechlorination (Figure 4.91). The maximum amount of cDCE that accumulated also increased in the presence of 1,1,1-TCA (Figure 4.92). Most significantly, the presence of 1,1,1-TCA severely inhibited reductive dechlorination of VC to ethene (Figure 4.93). A decrease in VC and stoichiometric increase in ethene occurred on day 7 and continued until VC and ethene stabilized at approximately 10 and 23 µmol per bottle, respectively, on day 18. The bottles were monitored for 19 more days, and VC reduction to ethene slowed considerably (Figure 4.94). Methane production was completely inhibited by the presence of 1,1,1-TCA (Figure 4.95). The amount of 1,1,1-TCA present decreased by approximately 35% during the first seven days of incubation and then remained relatively constant for the duration of the experiment. No significant increase in 1,1-DCA, CA, or ethane was detected to account for this decrease. It is not yet clear what may have caused the initial decrease in 1,1,1-TCA.

4.5.2 Phase 2

Results for the triplicate bottles provided with 31 µM TCE (2.2 µmol/bottle) are shown in Figures 4.96-4.98. There was no apparent lag in the onset of TCE dechlorination. TCE was completely consumed by day 15 (bottles #1 and #3) or day 22 (bottle #2). Small amounts of cDCE and VC accumulated transiently, but all of the TCE

was accounted for as ethene after 26 days of incubation. Due to the much lower inoculum level used for the Phase 2 experiment, TCE was consumed approximately six times slower than in the Phase 1 TCE-only bottles, even though the initial TCE concentration was an order of magnitude lower with Phase 2.

Results for the triplicate bottles provided with 31 µM TCE + 0.7 µM 1,1,1-TCA (2.3 µmol/bottle TCE and 0.1 µmol/bottle 1,1,1-TCA) are shown in Figures 4.99-4.101. As with the TCE-only treatment, there was no apparent lag in the onset of TCE dechlorination. TCE was completely consumed by day 15 (bottles #1 and #2) or day 22 (bottle #3). Small amounts of cDCE and VC accumulated transiently, but all of the TCE was accounted for as ethene after 27 days of incubation. These results indicated that 0.7 µM 1,1,1-TCA is not an inhibitory concentration for the SRS culture, at least under the conditions of this experiment. There was no significant decrease in the initial concentration of 1,1,1-TCA, and 1,1-DCA, CA, and ethane were not detected as daughter products.

Results for the triplicate bottles provided with 31 µM TCE + 3.6 µM 1,1,1-TCA (2.2 µmol/bottle TCE and 0.5 µmol/bottle 1,1,1-TCA) are shown in Figures 4.102-4.104. TCE dechlorination began after a lag of 13 days in bottles #1 and #2; however, TCE dechlorination started immediately in bottle #3. Despite the lag in bottles #1 and #2, TCE was completely consumed by day 22 in all bottles. Bottle #3 performed similarly to the first two treatments, with only transient accumulation of small amounts of cDCE and VC; all TCE was accounted for as ethene by day 22. cDCE and VC accumulated to higher amounts in bottles #1 and #2, reaching a maximum on day 22. After day 22,

cDCE and VC declined and ethene production increased. These results indicated that 3.6 µM 1,1,1-TCA slows, but does not prevent, the complete dechlorination of TCE to ethene by the SRS enrichment culture. There was no significant decrease in the initial concentration of 1,1,1-TCA, and 1,1-DCA, CA, and ethane were not detected as daughter products.

Results for the triplicate bottles amended with 31 µM TCE + 18 µM 1,1,1-TCA (2.1 µmol/bottle TCE and 2.5 µmol/bottle 1,1,1-TCA) are shown in Figures 4.105-4.107. There was no apparent lag in the onset of TCE dechlorination. TCE was completely consumed by day 27 or day 36 for bottles #1 and #2, respectively. After 42 days of incubation, 0.22 µmol/bottle TCE remained in bottle #3. cDCE and VC increased as TCE declined, along with some ethene production. As cDCE declined, VC and ethene increased further. On day 42, a slight decrease in VC was observed in bottles #1 and #2, but it was unclear whether the VC would be completely consumed or if it would stall. These results indicated that 18 µM 1,1,1-TCA slows dechlorination of TCE and may permanently inhibit conversion of VC to ethene. There was no significant decrease in the initial concentration of 1,1,1-TCA, and 1,1-DCA, CA, and ethane were not detected as daughter products.

Figure 4.108 summarizes the effect of 1,1,1-TCA at all levels tested during Phase 2 on TCE reductive dechlorination. The lowest concentration of 1,1,1-TCA (0.7 µM) did not appear to be inhibitory. However, higher concentrations of 1,1,1-TCA (3.6 and 18 µM) slowed the rate of TCE reduction. At a 1,1,1-TCA concentration of 3.6 µM, there was an increase in the maximum amount of cDCE that accumulated and the length of

time it took to reach the maximum (Figure 4.109). At 18 µM 1,1,1-TCA, reduction of cDCE appeared to stall after reaching approximately 0.25 µmol/bottle but after 36 days, decreased to less than 0.1 µmol/bottle. Increasing concentrations of 1,1,1-TCA increased the maximum amount of VC that accumulated (Figure 4.110). Most importantly, increasing concentrations of 1,1,1-TCA slowed the rate of ethene formation and, at 18 µM 1,1,1-TCA, prevented complete reduction even after 42 days of incubation (Figure 4.111). At the concentrations tested during Phase 2, 1,1,1-TCA inhibited methanogenesis (Figure 4.112), although to a lesser extent than during Phase 1. A decrease in 1,1,1-TCA was observed in all treatments amended with 1,1,1-TCA as well as the WCs (Figure 4.113). A 21% decrease in 1,1,1-TCA occurred in the WCs which was similar to the 18 µM 1,1,1-TCA treatment, which decreased by 27%. The other treatments lost an average of 34%-48% of the 1,1,1-TCA added to the bottles.

4.6 Microcosm Evaluation of EOS® as an Electron Donor

During the microcosm experiment to evaluate EOS as an electron donor, all of the treatments reached a low redox level by day eight, based on a change in the color of the resazurin added to the groundwater. The treatment with lactate added and no bioaugmentation was used as a control.

Results for the lactate-only microcosms are presented in Figures 4.114-4.116. As expected, no dechlorination of PCE or TCE was observed, and there was no formation of methane. The fluctuation in TCE levels over the first several weeks may be attributable to abiotic processes such as adsorption. The average losses of PCE and TCE during 85 days of incubation were 23% and 15%, respectively.

Results for the triplicate microcosms amended with lactate and bioaugmented with the SRS enrichment culture on day 10 are presented in Figures 4.117-4.119. After a lag of 18 days following bioaugmentation, consumption of PCE and TCE started. cDCE and VC accumulated during PCE and TCE dechlorination. cDCE increased to a maximum on day 49 and then declined as VC and ethene increased. Methanogenesis was minimal, with an average accumulation of 1.4 µmol/bottle.

Results for the triplicate microcosms amended with EOS® 450 and bioaugmented with the SRS enrichment culture on day 10 are presented in Figures 4.120-4.122. After a lag of 18 days following bioaugmentation, rapid consumption of PCE and TCE started and was complete by day 42. cDCE accumulated, along with lesser amounts of VC, followed by an increasing rate of ethene formation. More methane was produced in this treatment than in the lactate-fed microcosms, averaging 8.5 µmol/bottle.

Results for the triplicate microcosms amended with EOS® 598 and bioaugmented with the SRS enrichment culture on day 10 are presented in Figures 4.123-4.125. After a lag of 18 days following bioaugmentation, rapid consumption of PCE and TCE started and was complete by days 42-65. cDCE accumulated, along with lesser amounts of VC, followed by an increasing rate of ethene formation. An average of 8.5 µmol/bottle of methane was produced in this treatment, which was comparable to the EOS® 450 treatment.

Results for the triplicate microcosms amended with EOS® 598B42 and bioaugmented with the SRS enrichment culture on day 10 are presented in Figures 4.126-4.128. After a lag of 18 days following bioaugmentation, consumption of PCE and TCE

started. cDCE accumulated, along with lesser amounts of VC, followed by an increasing rate of ethene formation. An average of 2.8 µmol/bottle of methane was produced in this treatment, which was approximately four times lower than in the EOS® 450 and EOS® 598 treatments.

Results for the triplicate microcosms amended with EOS® 598B42 + EOS® Vitamin B_{12} solution and bioaugmented with the SRS enrichment culture on day 10 are presented in Figures 4.129-4.131. After a lag of 17-26 days following bioaugmentation, consumption of PCE and TCE started and was complete after 43-65 days of incubation. cDCE, VC, and ethene increased simultaneously in microcosms #1 and #2, followed by a decrease in cDCE and VC and further increase in ethene. Microcosm #3 accumulated more cDCE initially, followed by an increase in VC, and then ethene. An average of 7.5 µmol/bottle of methane was produced in this treatment, which was similar to the EOS® 450 and EOS® 598 treatments.

A comparison of the microcosm treatments shows that the rate of PCE dechlorination was approximately equivalent with all of the EOS® products, and somewhat slower with lactate (Figure 4.132). A similar trend occurred with TCE, although the rates for EOS® 450 and EOS® 598 were slightly faster than for EOS® 598B42 and EOS® 598B42 + EOS® Vitamin B_{12} solution (Figure 4.133). Larger amounts of cDCE and VC accumulated and disappeared more rapidly when EOS® 450 and EOS® 598 were used as electron donors (Figures 4.134-4.135). Most importantly, ethene production was initially faster in microcosms amended with EOS® 450 and EOS® 598, although the final level of ethene was reached at approximately the same time in all

EOS®-amended treatments (Figures 4.136). The addition of the Vitamin B_{12} solution did not appear to greatly improve the effectiveness of the EOS® 598B42. Ethene accumulation was notably slower with lactate, in spite of the fact that the enrichment culture is routinely grown with lactate as the electron donor.

4.7 Oxygen Tolerance Experiment

Results for the preliminary oxygen tolerance experiment conducted with single bottles are not included in this section but are shown in Figures E.1-E.3 (Appendix E). In order to evaluate the effect of exposing the SRS enrichment culture to air, a treatment was included with no air exposure, serving as a positive control. Results for PCE and TCE dechlorination were as expected (Figures 4.137-4.139). Consumption of PCE and TCE began immediately without a lag. On day six, small amounts of cDCE and VC were detected. Most of the PCE and TCE consumed was converted directly to ethene. Stoichiometric reduction of the PCE and TCE to ethene was complete by day 13. By day 44, an average of 5.0% of the electron equivalents of lactate added was recovered as methane.

Results for the triplicate bottles that were quiescently exposed to air for 24 hours are shown in Figures 4.140-4.142. Reductive dechlorination of the PCE and TCE in bottles #1 and #2 started without a lag but was comparatively slow, with accumulation of cDCE by day 20. In bottle #3, there was a definite lag period prior to the onset of PCE and TCE dechlorination. By day 27, the PCE and TCE were consumed in all three bottles. This was accompanied by a stoichiometric increase in cDCE. On day 44, disappearance of the majority of cDCE and a concurrent increase in VC and ethene was

observed, followed by stoichiometric accumulation of ethene. This indicated that the culture's ability to completely dechlorinate PCE and TCE is slowed but not completely inhibited by exposure of the headspace to air for at least 24 hours. There was a lag in methane production of six to 13 days, after which methane increased steadily, although not at the same rate as in the control bottles that were not exposed to the air.

The percentage of oxygen in the headspace of the bottles was also monitored and decreased by approximately half by day two (Figure 4.143). The next time an oxygen measurement was made, on day seven, no oxygen was detected in bottles #2 and #3, suggesting it may have been consumed even sooner. A small amount of oxygen remained in bottle #1 but was no longer detectable by day nine.

While the bottles were exposed to the air, the liquid turned pink and the top layer of settled iron sulfides turned from black to a gray color, indicating the media had become oxidized. After 24 hours, the bottles were sealed and mixed, at which point all of the sulfides became a grayish color. By day seven, the media had become clear and the iron sulfides returned to a black color, indicating the reestablishment of a low redox level in the bottles. The rapid reduction in headspace oxygen and visual observation of the change in media color indicated that the media in which the SRS enrichment culture is maintained is quite resilient to oxygen exposure.

4.8 Effect of pH on Reductive Dechlorination of PCE and TCE

In order to evaluate the effect of pH on the SRS enrichment culture's ability to reductively dechlorinate PCE and TCE, a treatment was included in which the pH was maintained at 7.0±0.25. Results are presented in Figures 4.144-4.146. Bottle #1 broke

on day 48, which is why no data are shown after that point. In the other two bottles, the culture performed as expected, with rapid and repeatable reduction of PCE and TCE to ethene, with only low and transient levels of cDCE and VC observed. Methane increased steadily. pH levels were adjusted each time that headspace samples were taken. Except for a brief excursion on days 48 and 55, the pH was maintained between 6.75 and 7.25 (Figure 4.147). At no time did the pH drop below 6.5.

The second treatment evaluated was also started at close to neutral pH, although no adjustments were made, and the pH was allowed to decrease as HCl produced from dechlorination accumulated. As shown in Figures 4.148-4.150, these bottles behaved similarly to the treatment described above, since all were started under the same conditions. There was only a transient increase in cDCE and VC during the first PCE and TCE dechlorination cycle. Repeat additions of PCE and TCE were made at higher concentrations. Gradually, with increasing amounts of dechlorination, the pH level started to decline (Figure 4.151), with the pH dropping below 6.0 near days 60, 70, and 75 for bottles #1-#3. The effect on the rate of ethene accumulation became apparent around day 63 for bottle #1 and day 70 for bottles #2 and #3. The dechlorination of PCE and TCE began to slow, and accumulation of cDCE and VC increased with less accumulation of ethene.

Results for the triplicate bottles maintained at pH 6.5±0.25 are presented in Figures 4.152-4.154. There was no apparent lag in PCE dechlorination, which was complete on day 14, however, a significant decrease in TCE did not occur until most of the PCE was consumed. A large decrease in TCE occurred by day 14 and was complete

by day 20. cDCE and VC accumulated transiently during the first cycle of PCE and TCE dechlorination, although ethene was the predominant terminal product. Three more additions of PCE and TCE were made at increasing amounts. PCE continued to be dechlorinated first, followed by TCE, with low and transient levels of cDCE and VC observed. Ethene was consistently the predominant daughter product. Methane also increased steadily. As shown in Figure 4.155, the pH was successfully maintained within the target range.

Figures 4.156-4.158 present the results for the triplicate bottles that were maintained at pH 6.0±0.25. There was no apparent lag in PCE dechlorination which was complete on day 14; however, a significant decrease in TCE did not occur until most of the PCE was consumed. A significant decrease in TCE occurred by day 14 and was complete on day 20. The predominant product from the first addition of PCE and TCE was cDCE in bottle #1, whereas a mixture of cDCE, VC, and ethene was observed for bottles #2 and #3. A second PCE and TCE addition was made on day 20. Bottle #1 accumulated a large amount of cDCE, reaching a maximum on day 35. VC accumulated in bottle #1 to a lesser degree, and reduction to ethene was nearly complete by day 63. Bottles #2 and #3 responded similarly to the second PCE and TCE addition. A small amount of cDCE and VC accumulated simultaneously, with subsequent reduction to ethene. Methane production occurred with no lag, but slowed considerably after day 28. As shown in Figure 4.159, the pH was successfully maintained within the target range.

Results for the triplicate bottles that were maintained at pH 5.5±0.25 are shown in Figures 4.160-4.162. There was a lag of 6-13 days before any significant dechlorination

of PCE and TCE began and was then complete by day 20. cDCE began to accumulate by day 14. No VC or ethene production was detected. After 48 days of incubation, reductive dechlorination stalled at cDCE. A second addition of PCE and TCE was made on day 48. This addition is not shown on Figure 4.160, as bottle #1 was broken and discarded prior to being respiked. PCE and TCE were dechlorinated with no apparent lag accompanied by an increase in cDCE in bottle #2. PCE and TCE were also dechlorinated in bottle #3, however, cDCE decreased and activity on all three chlorinated ethenes appeared to stall at day 70. Methanogenesis began without lag and continued over the duration of the experiment, despite the low pH. As shown in Figure 4.163, the pH was successfully maintained within the target range.

On the other side of the pH spectrum, one of the treatments for this experiment was maintained at a pH of 8.5±0.25. Results for the triplicate bottles are shown in Figures 4.164-4.166. Reductive dechlorination of PCE and TCE at this high pH was severely inhibited. In bottles #1 and #2, there was only a modest decrease in PCE and TCE and a correspondingly minor accumulation of cDCE and VC. In bottle #3, PCE was dechlorinated by day 48, although there was no evidence of TCE reduction and only minor amounts of cDCE and VC accumulation. None of the pH 8.5 bottles produced ethene. Methane production began with no lag and continued over the duration of the experiment. As shown in Figure 4.167, the pH was successfully maintained within the target range, except for a slight dip below 8.25 on day 14.

A comparison of the microcosm treatments based on the first addition of PCE and TCE shows that a pH of 5.5 slowed the rate of PCE consumption by 4-5 days, while a pH

of 8.5 proved to be the most inhibitory (Figure 4.168). pH levels of 6.5 and 6.0 did not significantly delay PCE consumption. pH levels below 7.0 slowed the rate of TCE consumption, and a pH of 8.5 severely inhibited TCE consumption (Figure 4.169). pH levels below 7.0 also increased the amount of cDCE that accumulated (Figures 4.170). VC accumulation was greatest at a pH of 6.0 (Figure 4.171). Most importantly, complete reduction to ethene was significantly slowed at pH 6.5 and severely inhibited at pH 5.5, 6.0 and 8.5 (Figure 4.172).

CHAPTER FIVE

DISCUSSION

Given the promising results of Wood's (45) previous work with the SRS enrichment culture, it was beneficial to further characterize the culture as it relates to other bioaugmentation cultures that chlororespire chlorinated ethenes. The scope of this project included characterization of the SRS enrichment culture based on the range of TEAs used by the culture, potential pathogenicity, susceptibility to 1,1,1-TCA, use of EOS® as an alternative electron donor, and sensitivity to oxygen exposure and a range of pH levels. Through this characterization, it was discovered that the SRS enrichment culture is capable of using a wide range of halogenated alkenes and alkanes including all of the chlorinated ethenes, 1,2-DCA, EDB, and VB. The culture reductively dechlorinates HCB, PeCB, 1,2,4,5-TeCB, and 1,2,4-TCB in a manner that is suggestive of their use as TEAs. The culture's ability to dechlorinate the aromatic compounds in addition to the halogenated alkenes and alkanes, sets it apart from other mixed cultures that are able to dehalogenate alkenes and alkanes but not aromatics, and vice versa.

With respect to pathogenicity, the SRS enrichment culture does contain coliforms, but does not contain *E. coli*, which will aid in gaining regulatory approval for use of this culture in bioaugmentation. Use of the SRS enrichment culture will be limited at sites co-contaminated with 1,1,1-TCA, which is inhibitory to complete reduction of PCE and TCE at 1,1,1-TCA concentrations of 3.7 μM and higher. Although the SRS culture was enriched on lactate as the electron donor, a microcosm evaluation of EOS® as an alternate electron donor suggested that the culture performs better with EOS®. This is

advantageous from the perspective that emulsified oils ferment more slowly and thereby provide donor over a longer period of time. Lastly, the SRS enrichment culture proved to be quite resilient to oxygen exposure, but sensitive to pH levels below 6 and above 8.5. By completing this characterization, the properties of the SRS enrichment culture are better known with respect to other mixed cultures that are available for bioaugmentation. Further discussion of characteristics of the SRS culture is presented below.

5.1 Types of Compounds Used as TEAs

The SRS enrichment culture is capable of utilizing the following halogenated alkenes and alkanes as TEAs: PCE, TCE, cDCE, tDCE, 1,1-DCE, VC, EDB, 1,2-DCA, and VB. The SRS culture contains several types of *Dehalococcoides* species; therefore, it is unclear which *Dehalococcoides* populations are responsible for the different dechlorination steps. However, the findings of this study show that the SRS culture contains a *Dehalococcoides* consortium capable of chlororespiring all of the chlorinated ethenes, including the three dichloroethene isomers. Table 5.1 compares the compounds used as TEAs by the SRS enrichment culture to other mixed cultures containing *Dehalococcoides* (VS, CBDB1, and KB-1^{TM}), as well as several pure cultures of *Dehalococcoides* (strains 195, BAV1, GT, and FL2). Like strain 195, BAV1, VS, and KB-1^{TM}, the SRS enrichment culture can dechlorinate PCE to ethene. However, the SRS enrichment culture utilizes all of the chlorinated ethenes as TEAs; a quality which none of the other cultures listed in Table 5.1 possesses (25, 35, 37). The SRS enrichment culture is also unique from the perspective that its ability to use EDB and 1,2-DCA as TEAs has been confirmed. The SRS culture's ability to degrade these compounds has

potential application for bioaugmentation of sites contaminated with leaded gasoline. EDB and 1,2-DCA were once added to leaded gasoline, and evidence that these compounds continue to persist in the environment is mounting (18). The SRS culture's ability to use VB as a TEA is also of interest, as VB is a potential abiotic transformation product from dehydrohalogenation of EDB.

A quantitative comparison of the SRS enrichment culture to other cultures that use halogenated ethenes and ethanes as TEAs was achieved by calculating yield values in terms of gene copies per µmol of Cl^- or Br^- released. Yields based on the Phase 2 TEA experiment are summarized in Table 5.2 and compared to previously reported values for other cultures. Previous yield values for PCE, TCE, cDCE, VC, and 1,2-DCA were compiled by Duhamel and Edwards (11) based on a variety of *Dehalococcoides* cultures, including strain 195, KB-1™, BAV1, and VS. Most of the yields calculated for the SRS enrichment culture are on the same order of magnitude or slightly higher than the reported yields for other cultures. The yield obtained for the SRS culture while using VB as the TEA was the highest reported, while the yield obtained for EDB was the lowest for this study. Because the yield for EDB is so much lower than the other compounds, it is recommended that this result be verified through additional experimentation. A lower yield on EDB versus 1,2-DCA is not expected based on thermodynamics, since dehalogenation of EDB is more energetically favorable than for 1,2-DCA (26).

The SRS enrichment culture is also capable of reductively dechlorinating several types of chlorinated aromatic compounds, including HCB, PeCB, 1,2,4,5-TeCB, and 1,2,4-TCB. Measurement of yields for these compounds was beyond the scope of this

research. The pattern of reductive dechlorination suggests that these compounds are used as TEAs, i.e., a lag phase followed by increasing rates of consumption. Nevertheless, in order to confirm their use as TEAs, additional experiments need to be run, including quantification of an increase in *Dehalococcoides* numbers using qPCR.

Reductive dechlorination of most of the chlorinated benzenes tested came as somewhat of a surprise. The wetland area from which the SRS enrichment culture was developed actively dechlorinates chlorinated ethenes, and two previously described cultures that chlororespire chlorinated ethenes also show activity on at least one halogenated ethane (Table 5.1). However, the wetland area at SRS does not appear to be contaminated with chlorinated benzenes, so activity on these compounds was not expected. Most of the cultures that chlororespire chlorinated ethenes have not yet been tested for their ability to respire chlorinated benzenes. The fact that the SRS culture contains a diverse population of *Dehalococcoides* with the ability to reductively dechlorinate chlorinated benzenes suggests these compounds may have been disposed of in the CBRP, and the ability to dechlorinate these compounds has been maintained.

Among the cultures listed in Table 5.1, the ability to use chlorinated benzenes as TEAs has only been reported for strain 195 and CBDB1 (2, 19, 28). CBDB1 is capable of using HCB, PeCB, all TeCB isomers, 1,2,3-TCB, and 1,2,4-TCB as TEAs (2, 28). Fennell et al. (19) demonstrated the ability of strain 195 to use HCB, PeCB, 1,2,4,5-TeCB, and 1,2,3,4-TeCB as TEAs. Strain 195 can also cometabolically dechlorinate 1,2,3,5-TeCB and the TCB isomers. The SRS culture more closely resembles the dechlorination ability of CBDB1. Although 1,2,3,5-TeCB was not directly tested, it was

apparent that the SRS culture can dechlorinate this isomer, as 1,3,5-TCB was detected in the PeCB treatment bottles as a dechlorination product, and 1,3,5-TCB can only be produced from dechlorination of 1,2,3,5-TeCB. It is not clear if this activity is metabolic or cometabolic, but this result warrants further testing of the other TeCB and TCB isomers as sole electron acceptors.

The most frequently reported pathway for reductive dechlorination of HCB is (27, 49):

$$\text{HCB} \rightarrow \text{PeCB} \rightarrow \text{1,2,3,5-TeCB} \rightarrow \text{1,3,5-TCB}$$

This also happens to be the most energetically profitable pathway (5). The proposed pathway for reductive dechlorination of chlorinated benzenes by the SRS culture is shown in Figure 5.1. Pathways for chlorobenzene reductive dechlorination used by other cultures are also shown (2, 5, 19, 28, 49). To further investigate the SRS culture's use of the chlorinated benzenes as TEAs and better quantify the results, an improved method of chlorobenzene analysis should be implemented, such as a liquid extraction analysis using internal standards.

5.2 Pathogenicity of the SRS Culture

The source of inoculum for the SRS enrichment culture was soil from the Twin Lakes wetland at SRS. This, in addition to the manner in which the culture was enriched, suggested that it was unlikely that the culture contains pathogenic microorganisms. The presence of coliforms was confirmed. Since various types of coliforms are commonly found in soil, this was not a surprising outcome. More importantly, *E. coli* is not present in the SRS enrichment culture.

Investigation of pathogenicity of the enrichment culture was included in the scope of this thesis, due to health and safety concerns that accompany injection of a microbial culture into a groundwater supply. Pathogenicity data on bioaugmentation cultures is limited. Use of some cultures has been restricted while others have been permitted. For example, *B. cepacia* has a broad range of biodegradation capabilities, although it has also been identified as an opportunistic human pathogen implicated in severe infections in immuno-compromised populations (3). *B. cepacia* has a high potential for adaptability and genetic exchange as well as a multidrug resistant phenotype (22, 44). Due to this information, the U.S. Environmental Protection Agency has applied strict regulations on the use of *B. cepacia*.

A number of pure and mixed cultures have been permitted for use in bioaugmentation. *Methylosinus trichosporium* OB3b, KB-1™, and mixed cultures from Bioremediation Consulting, Inc. have been reported as nonpathogenic (16, 34). However, the testing methods and frequency of testing were not reported and may vary. The MSDS that accompanies the KB-1™ culture reports that the culture tested negative for a variety of pathogenic organisms including: *Salmonella* sp., *Listeria monocytogenes*, *Vibrio* sp., *Camplyobacter* sp., *Clostridia* sp., *Bacillus anthracis*, *Pseudomonas aeruginosa*, *Yersinia* sp., yeast and mold, fecal coliforms, and *Enterococci*. In contrast, the MSDS for the SDC-9 bioaugmentation culture from the Shaw Group, Inc. does not include information on pathogenicity testing. Molecular testing of the SRS culture for specific pathogenic microorganisms other than *E. coli* is being conducted by Dr. Christopher Bagwell at SRNL to further evaluate pathogenicity of the SRS culture.

5.3 Inhibition of Chloroethene Respiration by 1,1,1-TCA

Other bioaugmentation cultures have been shown to be inhibited by the presence of low concentrations of 1,1,1-TCA and chloroform (13, 23). Grostern and Edwards (23) documented inhibitory effects on the KB-1™ culture amended with 1,1,1-TCA at a concentration of 300 µM, an equimolar concentration of TCE, and a 25% inoculum (v/v). TCE degradation was halted at cDCE and VC with no ethene production, and no 1,1,1-TCA conversion was observed. The same inhibition pattern was also documented at a concentration of 30 µM for both 1,1,1-TCA and TCE and a 10% inoculum. In this study, the SRS culture was also inhibited by 300 µM 1,1,1-TCA. Reductive dechlorination of TCE to ethene was slowed and conversion of VC to ethene was inhibited; there was complete inhibition of methanogenesis. Unlike KB-1™, the SRS culture produced some ethene. A major difference between the Grostern and Edwards (23) study and this study was the percent inocula. In this study, an undiluted inoculum was used to test the high 1,1,1-TCA concentration. The percent inoculum may have an effect on the extent of inhibition, as KB-1™ accumulated more cDCE and the SRS culture accumulated more VC and ethene as terminal products.

Duhamel et al. (13) also reported slowed rates of VC to ethene conversion with KB-1™ at a much lower 1,1,1-TCA concentration of 5.2 µM and a 10% inoculum. During Phase 2 of the inhibition experiment, the SRS enrichment culture was uninhibited at 5 µM 1,1,1-TCA, suggesting that the SRS culture has a higher tolerance to 1,1,1-TCA than KB-1™. In response to the problem presented by 1,1,1-TCA, SiREM (the company that markets KB-1™) offers a culture (referred to as MS) that contains *Dehalobacter* spp.

that reductively dechlorinate 1,1,1-TCA. Co-inoculation of KB-1™ and MS allows cDCE and VC degradation to ethene to proceed after MS has dechlorinated all of the 1,1,1-TCA to 1,1-DCA, which is not inhibitory (23). A similar approach may be necessary if the SRS culture is used in the presence of 1,1,1-TCA. Other vendors have reported mixed cultures capable of dechlorinating TCE that are resistant to inhibition by 1,1,1-TCA (1, 6). Adamson and Parkin (1) reported transformation of 1,1,1-TCA to 1,1-DCA and partially to CA by a lactate- and PCE-enriched culture. It is conceivable that the SRS culture could be grown with a 1,1,1-TCA dechlorinator so that a single source could be used in bioaugmentation.

5.4 Use of Emulsified Vegetable Oil as an Electron Donor

Wood (45) tested the effectiveness of emulsified vegetable oil as an electron donor for biostimulation of microcosms prepared with soil and groundwater from the P-Area at SRS. As with the other biostimulation treatments, emulsified vegetable oil was not effective. This was in contrast to a number of studies that have demonstrated the effectiveness of vegetable oil for biostimulation (30, 31, 50). However, Wood did not test the feasibility of vegetable oil as a substrate for bioaugmentation with the SRS culture. For this research, the use of emulsified vegetable oil was compared to lactate as an electron donor for the SRS enrichment culture. Results of the experiment with EOS® suggested that EOS® is a better electron donor than lactate, as the reductive dechlorination of PCE and TCE occurred faster in treatments amended with EOS® and with less accumulation of daughter products.

The initial concentration of TCE in some of the EOS®-amended bottles was lower than that in the lactate-amended bottles. The large drop in TCE concentration was most likely a result of partitioning of the TCE into the oil phase, as well as a lack of equilibrium, as the lactate-amended bottles also experienced an initial drop in TCE concentration. The tailing effect observed with TCE in the EOS®-amended bottles also suggests that some of the TCE partitioned into the oil phase. This apparently led to slow diffusion of cDCE and VC into the liquid phase, producing a steady level of VC during the last portion of incubation. Although partitioning of the chlorinated ethenes into the oil may extend the time needed for complete dechlorination to ethene, emulsified oil has the added benefit of slower fermentation and longer retention in a contaminated aquifer. These characteristics of emulsified oil may lower operating and maintenance costs typically associated with repeat injections of soluble carbon sources such as lactate (17).

5.5 Tolerance of the SRS Culture to Oxygen

Additional characterization of the SRS culture included its tolerance to oxygen exposure. *Dehalococcoides* are strictly anaerobic microorganisms, and the success of bioaugmentation cultures in the field depends in part on preventing oxygen exposure. The sensitivity of bioaugmentation cultures to oxygen likely varies, as not all cultures are maintained under the same conditions. In one study, Seepersad (39) demonstrated a decrease in TCE degradation rate in microcosms containing approximately 0.3 mg/L oxygen in the headspace of the bottles. The SRS enrichment culture is capable of sustaining at least 24 hours of exposure to air (21% oxygen) without any permanent effect on reductive dechlorination of PCE and TCE to ethene. Like the culture in

Seepersad's experiment, the SRS culture experienced slowed dechlorination rates of PCE, TCE, cDCE, and VC, but complete dechlorination to ethene did eventually occur following a return to anaerobic conditions. The SRS enrichment culture's resilience to oxygen exposure is a positive characteristic which will make field deployment of the culture easier.

It is unlikely that the SRS culture will be exposed to aerobic conditions for as long as 24 hours. However, if oxygen exposure did occur, the SRS culture would most likely be able to retain its dechlorinating activity after anaerobic conditions are established. Given the results of the oxygen tolerance experiment, the success of the SRS enrichment culture will not be greatly affected by potential oxygen exposure during injection of the culture into groundwater.

It should be noted that the oxygen tolerance experiment was performed in the anaerobic media used to maintain the SRS culture. This media has a substantial quantity of iron sulfides present to serve as a redox buffer. Although there were indications that the media in contact with the air became oxidized during the quiescent incubation period (i.e., the liquid turned pink and the black iron sulfides turned grey), it is possible that the same experiment conducted with groundwater and a low inoculum of the SRS culture might yield a less favorable result. The experiment does indicate, however, that brief exposure of the culture to air during addition to groundwater should not present a problem, as long as a low redox level exists in the groundwater.

5.6 Effect of pH on Reductive Dechlorination of PCE and TCE

In addition to the importance of the culture's sensitivity to oxygen exposure, its sensitivity to various pH conditions is also relevant. The SRS enrichment culture has been maintained at a pH of 6.5-7.5 in a buffered mineral medium, as that is the accepted optimum pH range for chlororespiring bacteria (32). However, the cost to buffer the pH of groundwater to neutral may become a large portion of the total cost of bioaugmentation. If the culture can sustain activity at lower pH levels, the operating and maintenance costs associated with adjusting the groundwater pH could be significantly lowered. When exposed to a pH of 6.0, the dechlorinating activity of the SRS enrichment culture was slowed and increased accumulation of cDCE and VC occurred. At pH 5.5, reductive dechlorination stopped at cDCE and no VC or ethene was produced. This strong inhibition is evidence that the SRS enrichment culture cannot tolerate pH levels less than 6.0, and groundwater will have to be adjusted to pH 6.5 or higher prior to bioaugmentation. Also, the SRS culture was unable to dechlorinate PCE and TCE at a pH of 8.5.

KB-1^{TM} has been reported to be completely inhibited below pH 5.0 and above pH 10; the optimal range given for the KB-1^{TM} culture is between 6.0 and 8.3 (38). Zhuang and Pavlostathis (51) conducted a pH assay similar to the one in this study and found that neutral pH was optimum for reductive dechlorination by a methanogenic mixed culture capable of dechlorinating PCE to VC. The mixed culture was exposed to pH levels of 4, 6, 7, 8, and 9.5. PCE to TCE dechlorination was similar in the pH 7 and 8 treatments, but the end products were VC and cDCE, respectively. Significant accumulation of cDCE

was also observed at pH 6 with less PCE dechlorination than the pH 7 and 8 treatments. At pH 4 and 9.5, there was limited dechlorination of PCE, along with accumulation of cDCE and no VC.

The SRS culture's response to a gradual decrease in pH was evaluated by not adjusting pH over the course of several PCE and TCE additions. The pH was allowed to decrease below 7.0 due only to acid generation from reductive dechlorination of the PCE and TCE. The expectation was that at pH levels below 6.5, reductive dechlorination would be inhibited. After the pH dropped below 6.0, these bottles began to show signs of decreased activity; e.g., VC began to accumulate. This is similar to the findings of Fogel et al. (20). *Desulfitobacterium dichloroeliminans* strain DCA1, a non-*Dehalococcoides* dehalorespirator, has an optimal pH range of 7.2-7.8 but maintained activity at pH levels as low as 5.4, caused by release of HCl (33). Although many investigators report the need to maintain dechlorinating cultures at a neutral pH, these claims are not substantiated extensively in the literature.

With that in mind, it is important to note that the SRS enrichment culture was developed from C-Area sediment where natural attenuation of PCE and TCE to ethene is occurring. The same C-Area sediment and groundwater were used to set up microcosms for an experiment not included in this thesis. The initial pH of the C-Area groundwater was 5.5 and was not adjusted to neutral at any time during the experiment. Despite this low pH, the microcosms successfully dechlorinated VC to ethene and ethane over several additions of VC. This indicated that the *Dehalococcoides* present at the C-Area site are capable of tolerating pH levels below 6.0 and that it may be possible to develop a

bioaugmentation culture that is able to grow at a low pH. This is of considerable interest, as it would eliminate the need to adjust groundwater pH for bioaugmentation.

CHAPTER SIX

CONCLUSIONS

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

1. The SRS enrichment culture is capable of utilizing the following halogenated ethenes and ethanes as TEAs: PCE, TCE, cDCE, tDCE, 1,1-DCE, VC, EDB, 1,2-DCA, and VB. The SRS culture can reductively dehalogenate all of these compounds at concentrations similar to the highest concentrations of PCE and TCE (15 and 38 mg/L, respectively) found in groundwater in the P-Area at SRS. Growth yields calculated for the SRS enrichment culture, with the exception of EDB, are within the same order of magnitude reported for other *Dehalococcoides*-containing cultures, ranging from 7.9×10^7 cells/µmol Cl^- for VC to 1.8×10^9 cells/µmol Cl^- for 1,2-DCA. The culture also reductively dechlorinates the chlorinated aromatic compounds HCB, PeCB, 1,2,4,5-TeCB, and 1,2,4-TCB. Although the pattern and rate of dechlorination is suggestive of growth, use of these aromatics as TEAs has not yet been established due to a lack of qPCR data correlating an increase in the population of *Dehalococcoides* to chloride release.

2. The SRS enrichment culture tested positive for coliforms using two types of commercial media (Colilert® and ReadyCult®). More importantly, however, it tested negative for the presence of *E. coli*. This confirms the absence of at least one important type of potential pathogen in the culture. Further evaluation is warranted to demonstrate that other potential pathogens are absent from the culture. Demonstrating the absence of

pathogenic microbes in the SRS culture will facilitate receiving approval for use of the culture at SRS, by assuring regulators that no health threat is posed to the groundwater by introduction of the culture.

3. The SRS enrichment culture is inhibited by the presence of 1,1,1-TCA at concentrations of 3.6 μM (0.48 mg/L) and higher. The inhibition is most significant for the critical step of VC reduction to ethene. At a concentration of 300 μM 1,1,1-TCA (40 mg/L), the SRS culture was strongly inhibited in its ability to dechlorinate VC to ethene after 42 days of incubation. This susceptibility to 1,1,1-TCA will limit the SRS culture's use at sites co-contaminated with chlorinated ethenes and 1,1,1-TCA. Such a limitation may be overcome by first bioaugmenting with a culture that is capable of dechlorinating 1,1,1-TCA, thereby eliminating its inhibitory effect.

4. The SRS enrichment culture utilizes EOS®, a commercial form of emulsified vegetable oil, as an electron donor for reductive dechlorination of PCE and TCE. Reductive dechlorination occurs at faster rates than when lactate is used as the electron donor. Although the SRS culture was enriched and maintained with lactate serving as the electron donor, microcosms amended with EOS® accumulated less daughter products and produced ethene at a faster rate. Bioaugmentation with EOS® may be beneficial as emulsified oil is a much longer lasting donor than lactate and may reduce the costs associated with repeat feedings of lactate. Among the four varieties of EOS® tested (450, 598, 598B42, 598B42 + Vitamin B_{12}), the rate of ethene production was initially faster in microcosms amended with EOS® 450 and EOS® 598, although the final level of ethene was reached at approximately the same time for all varieties.

5. The SRS enrichment culture is resilient to at least 24 hours of quiescent exposure to air (21% oxygen). The culture's ability to dechlorinate PCE and TCE is slowed after oxygen exposure, taking approximately 4 times longer to completely dechlorinate PCE and TCE. In spite of this effect on the rate, the culture does retain its ability to completely reduce PCE and TCE to ethene. The presence of iron sulfides in the mineral media likely played a role in reducing oxidative damage to the culture during oxygen exposure. The resilience of the culture implies it will survive a brief period of exposure to oxygen during field application, assuming low redox conditions have been pre-established in the groundwater.

6. The SRS enrichment culture is inhibited by pH levels below 6.0 and above 8.5. At pH 6.0, reductive dechlorination of PCE and TCE slowed and cDCE and VC accumulated. At pH 5.5, dechlorination stalled at cDCE, with no production of VC or ethene. Reductive dechlorination of PCE and TCE was severely inhibited at a pH level of 8.5. When the pH of the culture was allowed to gradually decrease from neutral as repeated additions of PCE and TCE were dechlorinated (by release of HCl and no addition of a base), ethene formation slowed considerably once the pH dropped below 6.0. Given the inhibitory effect of pH levels below 6.0, the pH of the P-Area groundwater should be adjusted within the range of 6.5-7.5 for successful bioaugmentation to occur. Sufficient buffer must also be available to prevent the pH from decreasing below 6.0 as HCl is released during PCE and TCE dechlorination.

TABLES

Table 3.1 Initial and maximum amounts of TEAs added and Henry's constants for the TEAs, ethene, ethane, and methane.

	Initial			Maximum				
	Concentration		Volume Added	Concentration		Volume Added	H_c	
TEA	µmol/bottle	(mg/L)	(mL sat. H_2O)	µmol/bottle	(mg/L)	(µL neat)	dimensionless	Reference[a]
PCE	3.0	3.6	2.6	12.0	17.0	1.5	0.64	A
TCE	7.5	8.2	0.9	36.0	39.0	3.5	0.35	A
cDCE	9.5	8.5	0.2	36.0	32.0	2.7	0.14	A
tDCE	6.5	2.4	0.1	36.0	29.0	2.7	0.35	A
1,1-DCE	7.0	4.3	0.3	36.0	22.0	2.9	0.97	A
VC	7.0	2.8	– [b]	48.0	19.0	– [b]	1.01	A
1,2-DCA	5.0	4.7	0.06	23.0	20.0	2.0	0.05	B
EDB	2.7	5.0	0.125	24.0	23.0	2.0	0.03	B
VB	7.0	5.8	– [b]	48.0	39.0	– [b]	0.50	B
HCB	0.0011	0.0031	70[c]	0.0011	0.0031	70[c]	0.024	B
PeCB	0.4	1.0	75[c]	0.4	1.0	75[c]	0.029	B
1,2,4,5-TeCB	0.2	0.4	150[c]	0.2	0.4	150[c]	0.041	B
1,2,4-TCB	8.0	13.9	1.0[d]	16.0	27.7	2.0	0.058	B
1,2-DCB	9.0	12.7	1.0[d]	17.8	25.0	2.0	0.077	B
1,3-DCB	9.0	12.3	1.0[d]	17.5	23.9	2.0	0.11	B
1,4-DCB	0.10	0.13	100[c]	0.19	0.26	200[c]	0.10	B
CB	9.9	10.2	1.0[d]	29.6	30.5	3.0	0.15	B
Ethene	N/A[e]	N/A	N/A	N/A	N/A	N/A	7.24	B
Ethane	N/A	N/A	N/A	N/A	N/A	N/A	17	B
Methane	N/A	N/A	N/A	N/A	N/A	N/A	1188	C

[a] A = ref (21), B = ref (43), and C = ref (41); [b] Added as neat gas; 0.175 mL initially; 1.5 mL maximum; [c] Added dissolved in acetone (µL); [d] Added as neat compound (µL); [e] N/A = Compound was not added as a TEA

Table 4.1 TEA Experiment (Phase 1) percent recoveries of TEAs as ethene and percentage of lactate added that was used for dechlorination of the respective TEA and methanogenesis (based on electron equivalents).

Treatment	% Recovered as Ethene	% lactate added used for dechlorination	% lactate added used for methanogenesis
PCE	89%	0.32%	6.6%
TCE	104%	0.32%	3.2%
cDCE	111%	0.42%	0.50%
tDCE	47%	0.12%	12%
1,1-DCE	110%	0.42%	6.4%
VC	109%	0.56%	4.6%
1,2-DCA	94%	0.44%	2.0%
EDB	112%	0.56%	0.53%

Table 4.2 TEA Experiment (Phase 2) percent recoveries of TEAs as ethene and percentage of lactate added that was used for dechlorination of the respective TEA and methanogenesis (based on electron equivalents).

Treatment	% Recovered as Ethene	% lactate added used for dechlorination	% lactate added used for methanogenesis
PCE	83%	0.74%	1.5%
TCE	91%	0.79%	0.83%
cDCE	80%	0.58%	0.39%
tDCE	93%	0.66%	1.1%
1,1-DCE	72%	0.60%	1.6%
VC	91%	0.79%	1.7%
1,2-DCA	94%	0.41%	1.3%
EDB	84%	0.60%	0.086%
VB	95%	0.81%	0.66%

Table 4.3 qPCR data and calculated yield values for TEA Experiment Phase 2.

	Cl^- or Br^- released[a] (µmol/bottle)		**Final gene copies (DHC/mL)**		**Initial gene copies (DHC/mL)**		**Net gene copies (DHC/mL)**		**Yield (gene copies/µmol Cl^- or Br^-)**	
Compound	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev	Ave	Stdev
PCE	210	4.81	$3.5x10^9$	$2.8x10^8$	$1.1x10^6$	$2.0x10^5$	$3.5x10^9$	$2.83x10^8$	$1.7x10^9$	$1.4x10^8$
TCE	306	5.23	$5.2x10^8$	$3.4x10^8$	$1.1x10^6$	$2.0x10^5$	$5.2x10^8$	$3.37x10^8$	$1.7x10^8$	$1.1x10^8$
cDCE	222	18.1	$2.8x10^9$	$7.7x10^7$	$1.1x10^6$	$2.0x10^5$	$2.8x10^9$	$7.67x10^7$	$1.3x10^9$	$1.1x10^8$
tDCE	216	9.27	$8.3x10^8$	$6.7x10^7$	$1.1x10^6$	$2.0x10^5$	$8.3x10^8$	$6.67x10^7$	$3.8x10^8$	$3.5x10^7$
1,1-DCE	207	28.7	$1.2x10^9$	$8.7x10^7$	$1.1x10^3$	$7.6x10^1$	$1.2x10^9$	$8.67x10^7$	$5.9x10^8$	$9.3x10^7$
VC	210	35.1	$1.7x10^8$	$1.6x10^7$	$2.2x10^5$	$1.4x10^4$	$1.7x10^8$	$1.60x10^7$	$7.9x10^7$	$1.5x10^7$
1,2-DCA	218	2.75	$4.0x10^9$	$1.1x10^8$	$1.2x10^6$	$2.2x10^4$	$4.0x10^9$	$1.10x10^8$	$1.8x10^9$	$5.6x10^7$
EDB	275	10.3	$1.9x10^6$	$7.7x10^4$	$1.8x10^2$	$6.0x10^0$	$1.9x10^6$	$7.67x10^4$	$6.8x10^5$	$3.8x10^4$
VB	232	0.142	$4.3x10^9$	$2.7x10^8$	$4.5x10^2$	$6.0x10^1$	$4.2x10^9$	$2.67x10^8$	$1.8x10^9$	$1.2x10^8$

[a] Calculated based on the amount of parent compound consumed and daughter products formed; chloride and bromide were not directly measured.

Table 4.4 Ratios of electron equivalents used for dechlorination and methanogenesis to electron equivalents of lactate added.

Treatment	% Daughter Product Formed										% lactate used for dechlorination[a]	% lactate used for methanogenesis
	PeCB	**1,2,4,5-TeCB**	**1,3,5-TCB**	**1,2,4-TCB**	**1,4-DCB**	**1,3-DCB**	**1,2-DCB**	**CB**	**Benzene**	**Total**		
HCB	3%	**58%**	0%	32%	0%	0%	0%	0%	0%	93%	0.00021%	12.1%
PeCB	--	2%	**33%**	7%	32%	0%	11%	0%	0%	85%	0.076%	8.7%
1,2,4,5-TeCB	--	--	0%	27%	**38%**	0%	18%	0%	0%	83%	0.038%	9.6%
1,2,4-TCB	--	--	--	--	**40%**	0%	16%	2%	0%	58%	0.76%	24%
1,4-DCB	--	--	--	--	--	--	--	0%	0%	0%	N/A[b]	74%
1,3-DCB	--	--	--	--	--	--	--	0%	0%	0%	N/A	69%
1,2-DCB	--	--	--	--	--	--	--	0%	0%	0%	N/A	61%
CB	--	--	--	--	--	--	--	--	0%	0%	N/A	69%

[a] Ratio calculated based on electron equivalents required to produce major end product.
[b] N/A = no significant dechlorination

Table 4.5 Comparison of average percent loss of chlorinated benzenes in water controls and live bottles.

Treatment	Average % Loss	
	WCs	Live Bottles
HCB	0%	100%
PeCB	0%	100%
1,2,4,5-TeCB	0%	100%
1,2,4-TCB	69%	100%
1,4-DCB	29%	0%
1,3-DCB	53%	51%
1,2-DCB	49%	52%
CB	45%	33%

Table 5.1 Comparison of SRS enrichment culture and other bioaugmentation cultures.[a]

Compound	strain 195 (Cornell)	BAV1 (Pinellas)	GT (Pinellas)	VS (Victoria)	FL2 (Pinellas)	CBDB1 (Pinellas)	KB-1 (Pinellas)	SRS Enrichment Culture
PCE	+ (35)	● (25)	- (40)	● (37)	● (24)	- (8)	+	+
TCE	+ (35)	● (25)	+ (40)	+ (37)	+ (24)	?	+ (12)	+
cDCE	+ (35)	+ (25)	+ (40)	+ (37)	+ (24)	?	+ (12)	+
tDCE	● (35)	+ (25)	- (40)	+ (37)	+ (24)	?	?	+
1,1-DCE	+ (35)	+ (25)	+ (40)	+ (37)	- (24)	?	+ (13)	+
VC	● (35)	+ (25)	+ (40)	+ (37)	● (24)	?	+ (12)	+
1,2-DCA	+ (35)	+ (25)	- (40)	?	- (24)	?	?	+
EDB	+ (36)	?	?	?	?	?	?	+
VB	?	+ (25)	- (40)	?	?	?	?	+
CB	- (19)	?	?	?	?	- (2)	?	-
1,2-DCB	- (19)	?	?	?	?	?	?	-
1,3-DCB	- (19)	?	?	?	?	?	?	-
1,4-DCB	- (19)	?	?	?	?	?	?	-
1,2,3-TCB	● (19)	?	?	?	?	+ (2)	?	?
1,2,4-TCB	● (19)	?	?	?	?	+ (2)	?	✓
1,3,5-TCB	● (19)	?	?	?	?	- (2)	?	?
1,2,3,4-TeCB	+ (19)	?	?	?	?	+ (2)	?	?
1,2,3,5-TeCB	● (19)	?	?	?	?	+ (2)	?	?
1,2,4,5-TeCB	+ (19)	?	?	?	?	+ (2)	?	✓
PeCB	+ (19)	?	?	?	?	+ (28)	?	✓
HCB	+ (19)	?	?	?	?	+ (28)	?	✓

[a] + = used as a TEA, - = not used as a TEA, ● = used cometabolically, ✓ = reductively dechlorinated, not evaluated for chlororespiration, ? = not tested as a TEA

Table 5.2. Summary of reported yields for *Dehalococcoides* cultures.

TEA	Average[a]	Standard Deviation[a]	Terminal Product	Reference[b]
PCE	$6.9x10^8$	---	ethene	D[c]
	$1.7x10^9$	$1.4x10^8$	ethene	A
TCE	$3.6x10^8$	$1.3x10^8$	ethene	C
	$7.1x10^8$	$3.6x10^8$	cDCE	B[c]
	$1.7x10^8$	$1.1x10^8$	ethene	A
cDCE	$2.7x10^8$	$5.6x10^7$	ethene	B
	$1.3x10^9$	$1.1x10^8$	ethene	A
tDCE	$3.8x10^8$	$3.5x10^7$	ethene	A
1,1-DCE	$5.9x10^8$	$9.3x10^7$	ethene	A
VC	$1.2x10^8$	$1.4x10^7$	ethene	F[c]
	$6.3x10^7$	$1.0x10^7$	ethene	F
	$5.2x10^8$	$1.5x10^8$	ethene	E
	$5.6x10^8$	$1.4x10^8$	ethene	C
	$2.7x10^8$	$5.6x10^7$	ethene	B[c]
	$7.9x10^7$	$1.5x10^7$	ethene	A
1,2-DCA	$3.7x10^6$	$3.9x10^5$	ethene	G[c]
	$1.4x10^8$	$2.8x10^7$	ethene	B[c]
	$1.8x10^9$	$5.6x10^7$	ethene	A
EDB	$6.8x10^5$	$3.8x10^4$	ethene	A
VB	$1.8x10^9$	$1.2x10^8$	ethene	A

[a] Yields and standard deviations are in reported in units of copies/µmol Cl^- released.

[b] A = This study, B = ref (11), C = ref (12), D = ref (4), E = ref (10), F = ref (25), G = ref (35)

[c] Original units of reported yields were converted using assumptions used by Duhamel and Edwards (11).

FIGURES

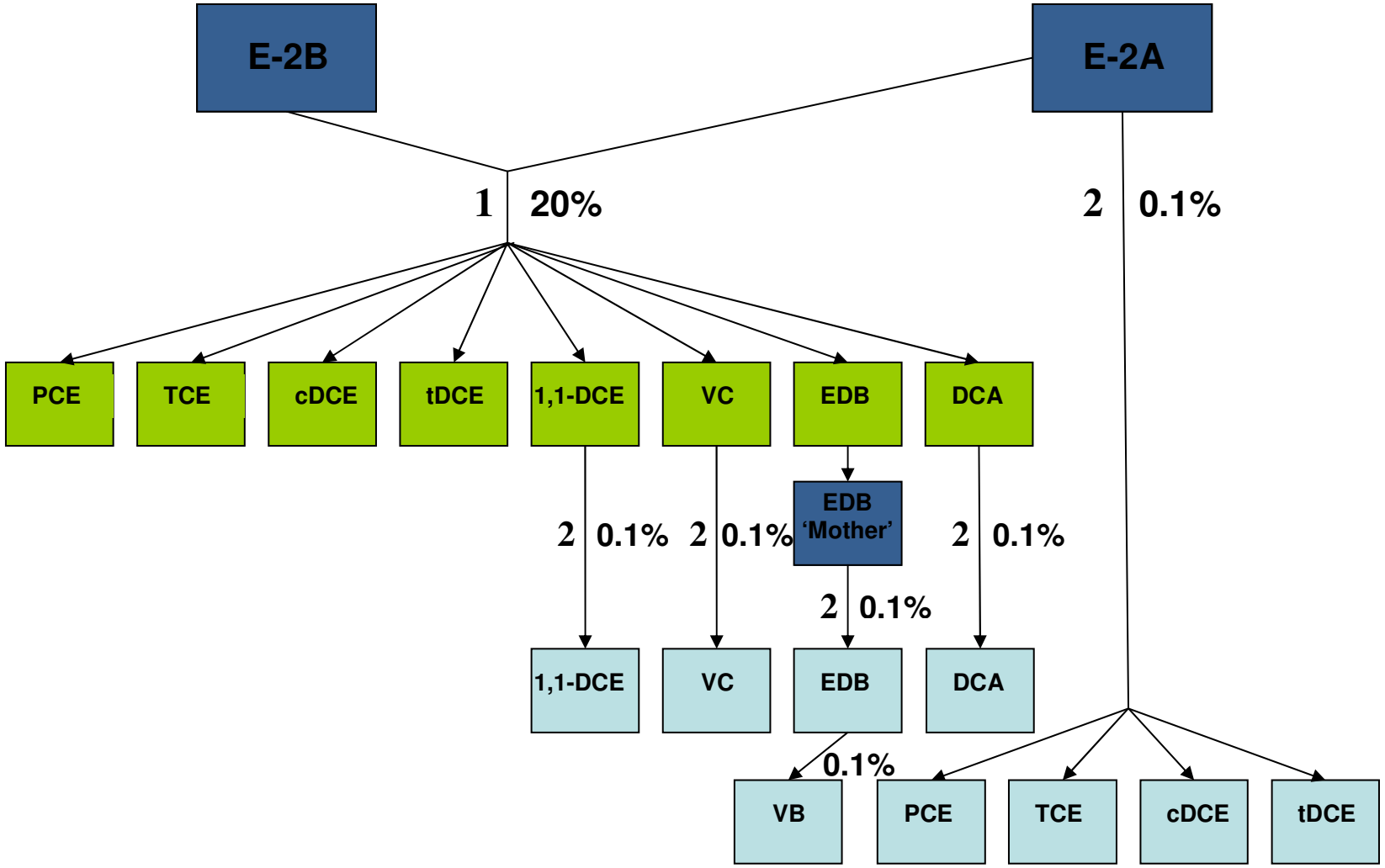

Figure 3.1 Diagram of inocula sources for Phase 1 and Phase 2 of the Alternate TEA Experiment.

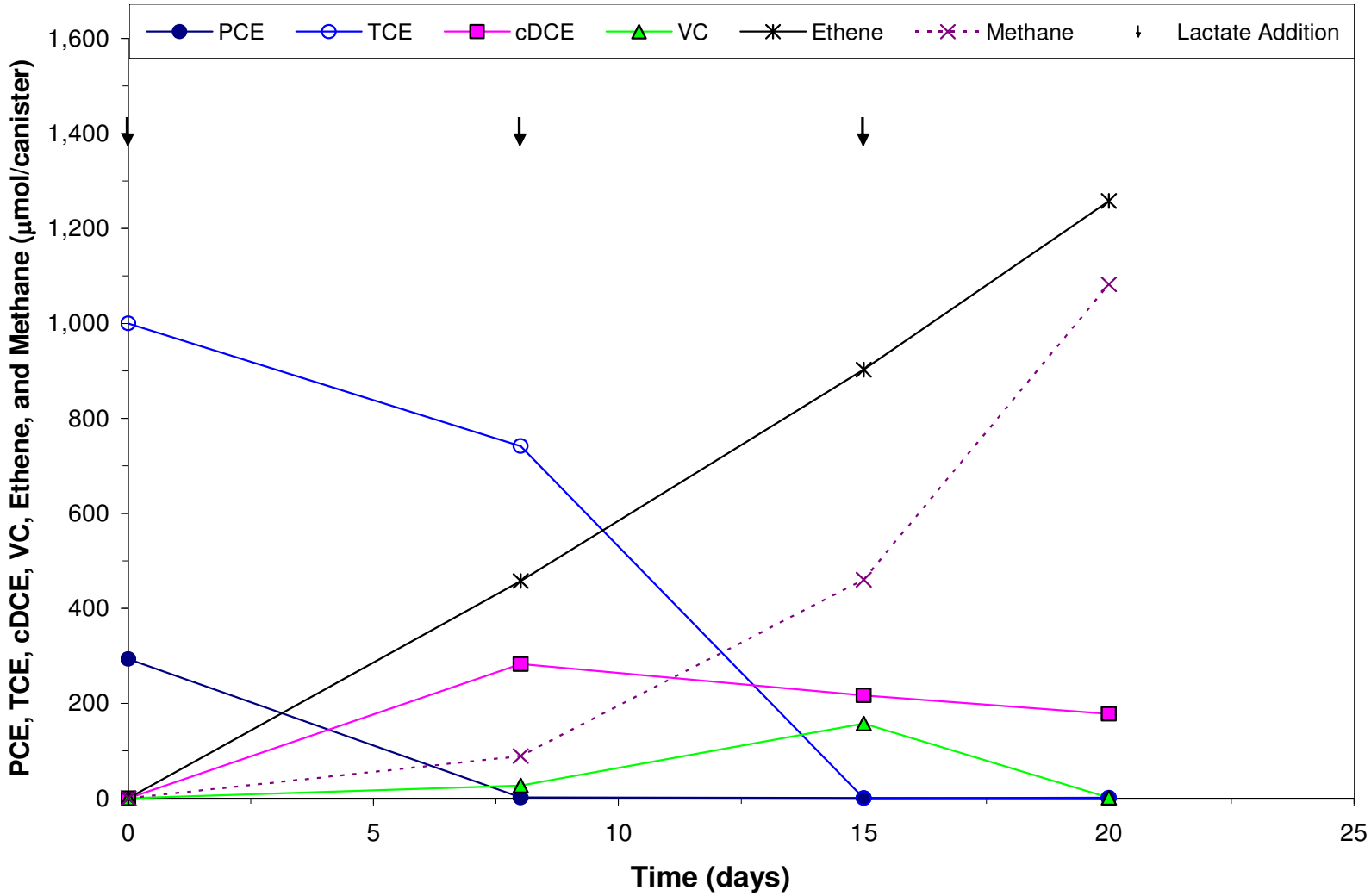

Figure 4.1 First cycle of PCE and TCE dechlorination in Canister #1.

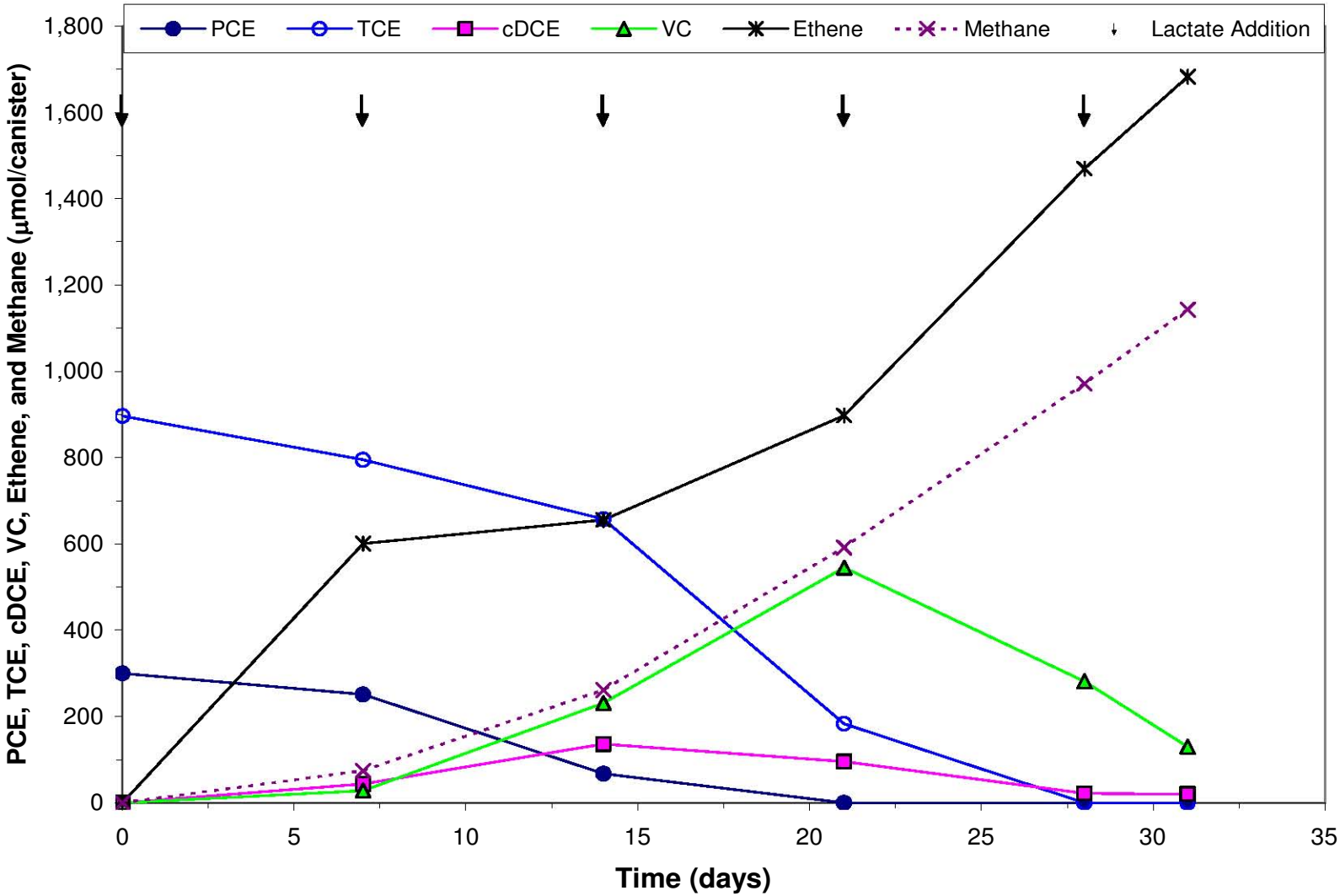

Figure 4.2 First cycle of PCE and TCE dechlorination in Canister #2.

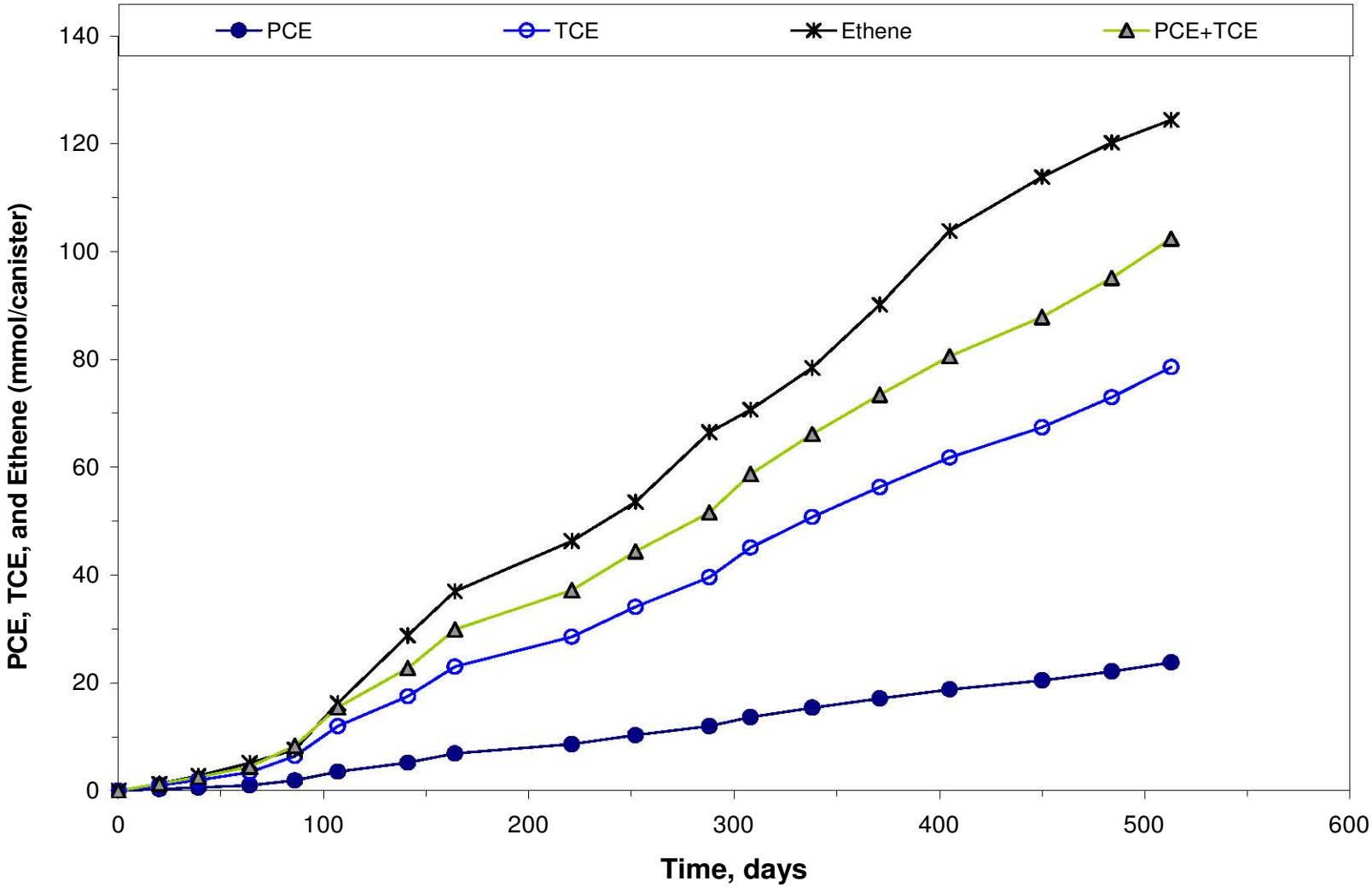

Figure 4.3 Cumulative PCE and TCE dechlorinated and ethene produced in Canister #1.

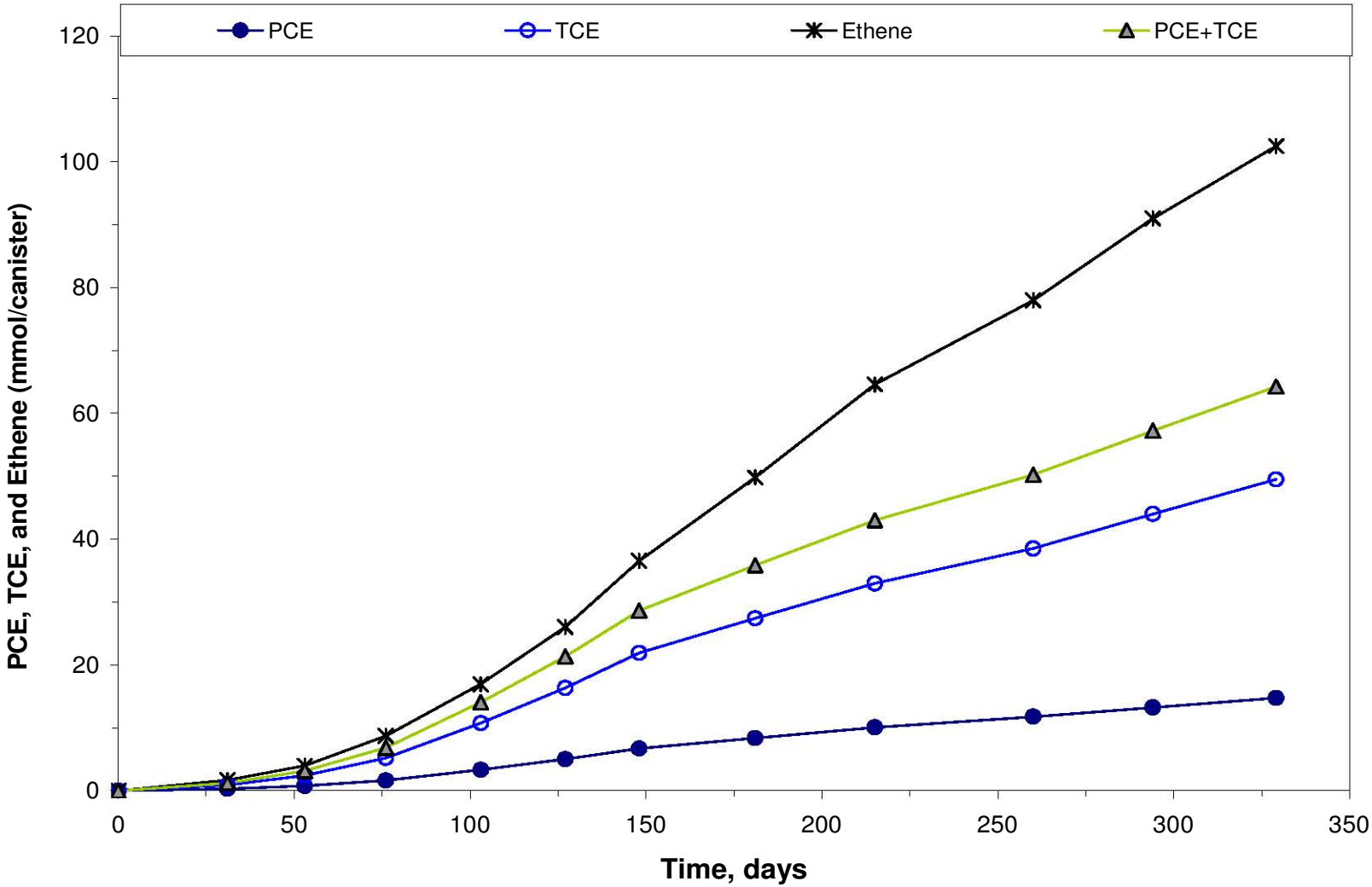

Figure 4.4 Cumulative PCE and TCE dechlorinated and ethene produced in Canister #2.

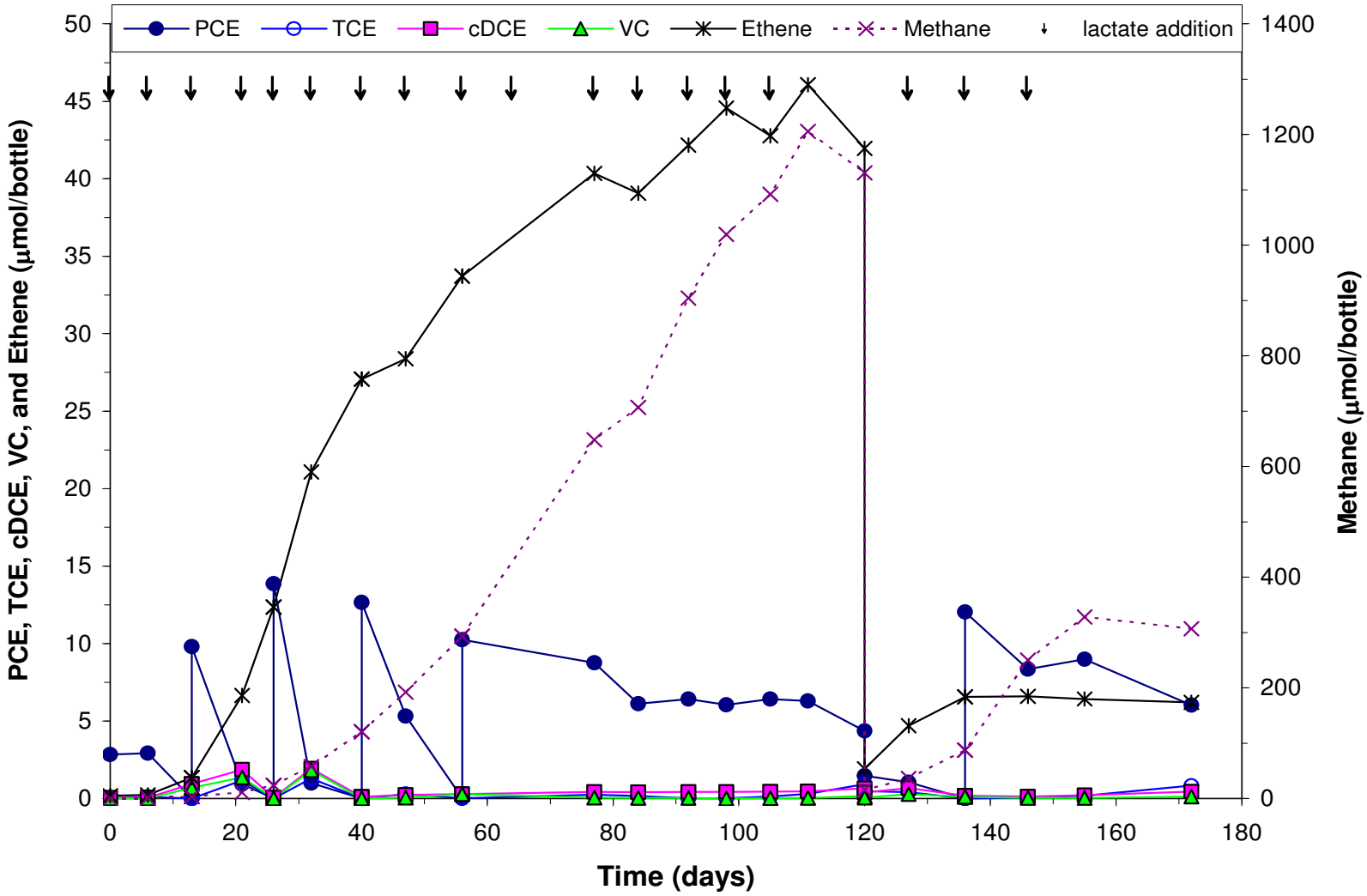

Figure 4.5 Results for one of the Yield Experiment Phase 1 PCE bottles (PCE-1).

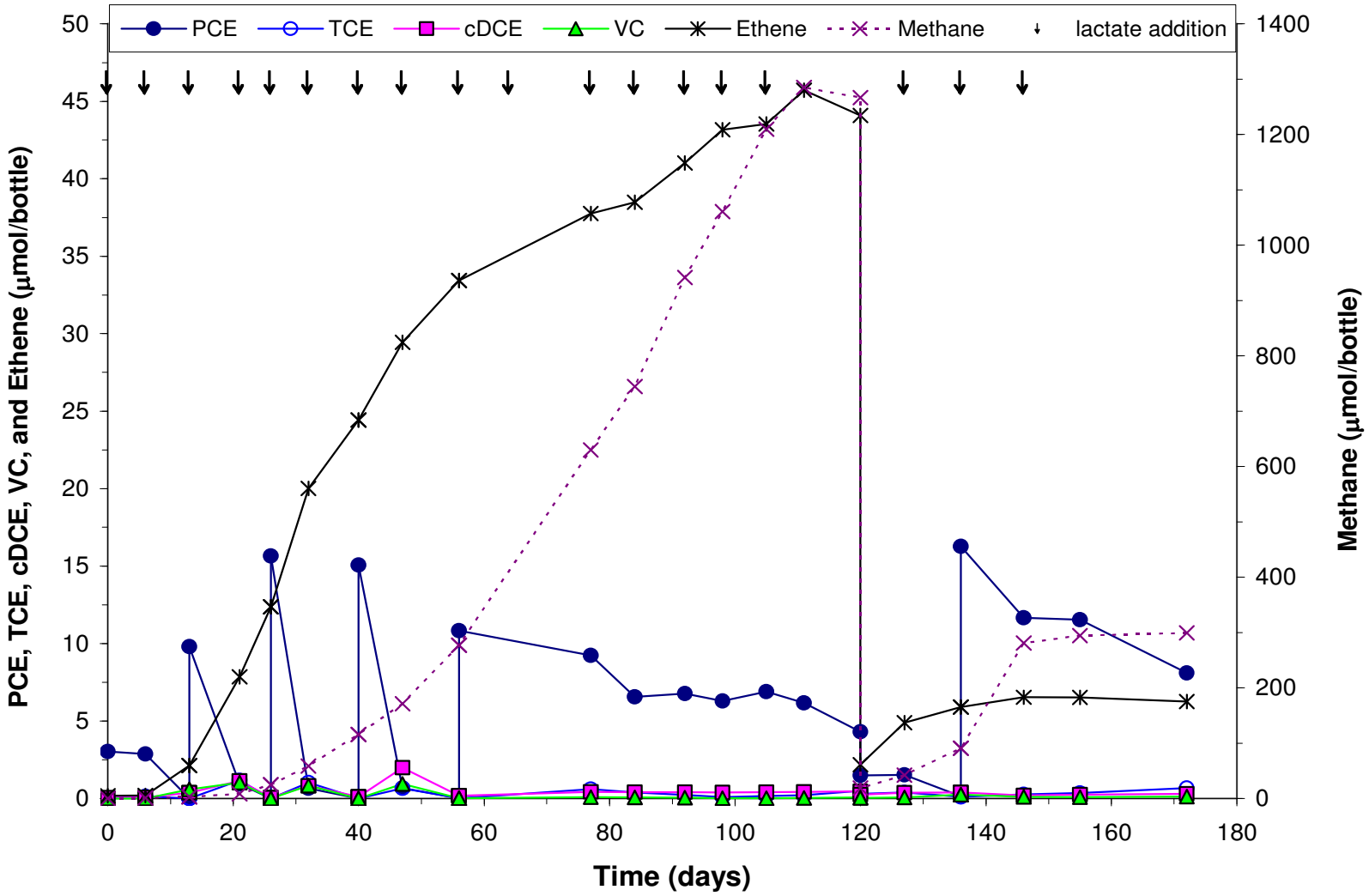

Figure 4.6 Results for one of the Yield Experiment Phase 1 PCE bottles (PCE-2).

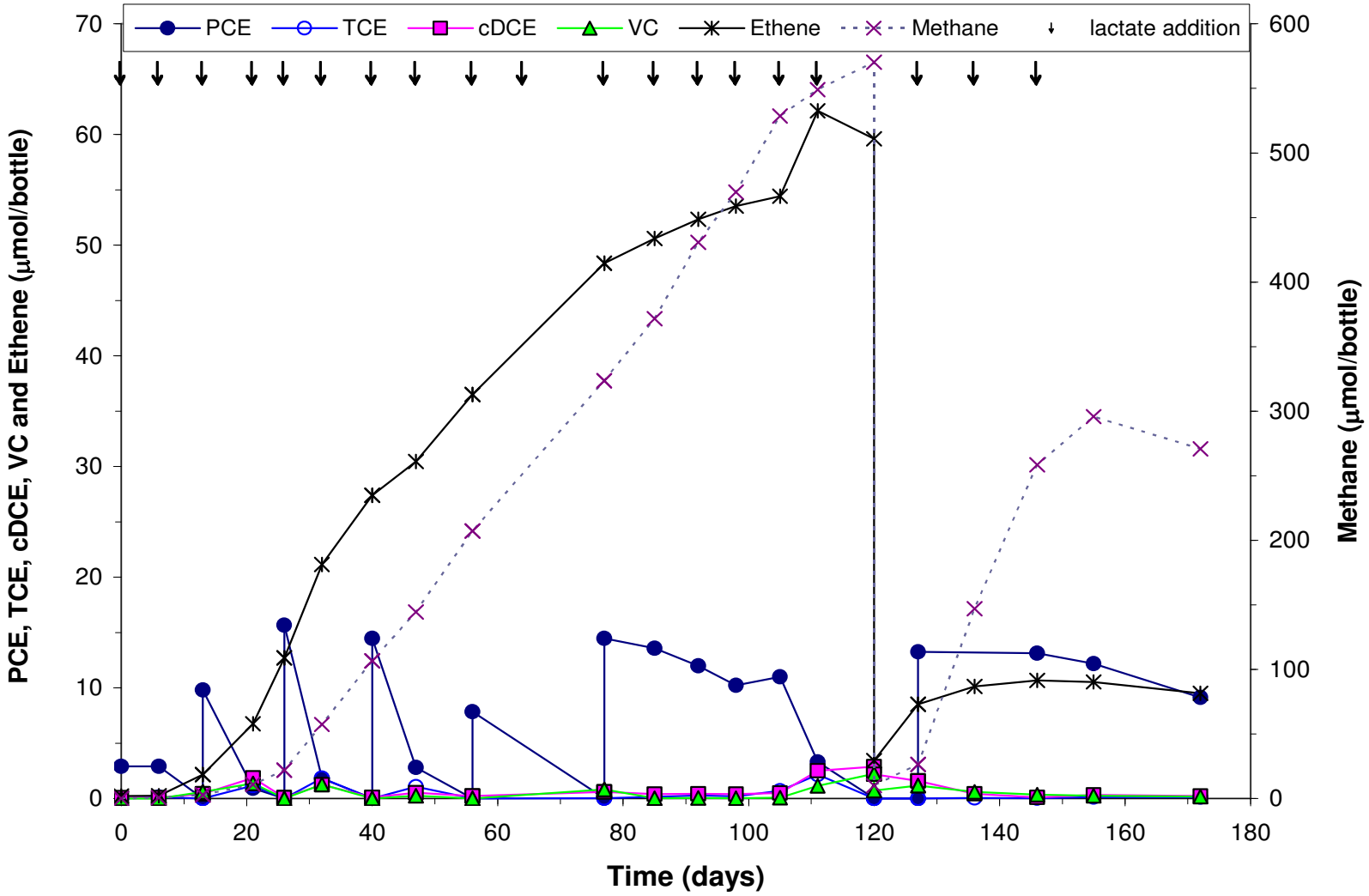

Figure 4.7 Results for one of the Yield Experiment Phase 1 PCE bottles (PCE-3).

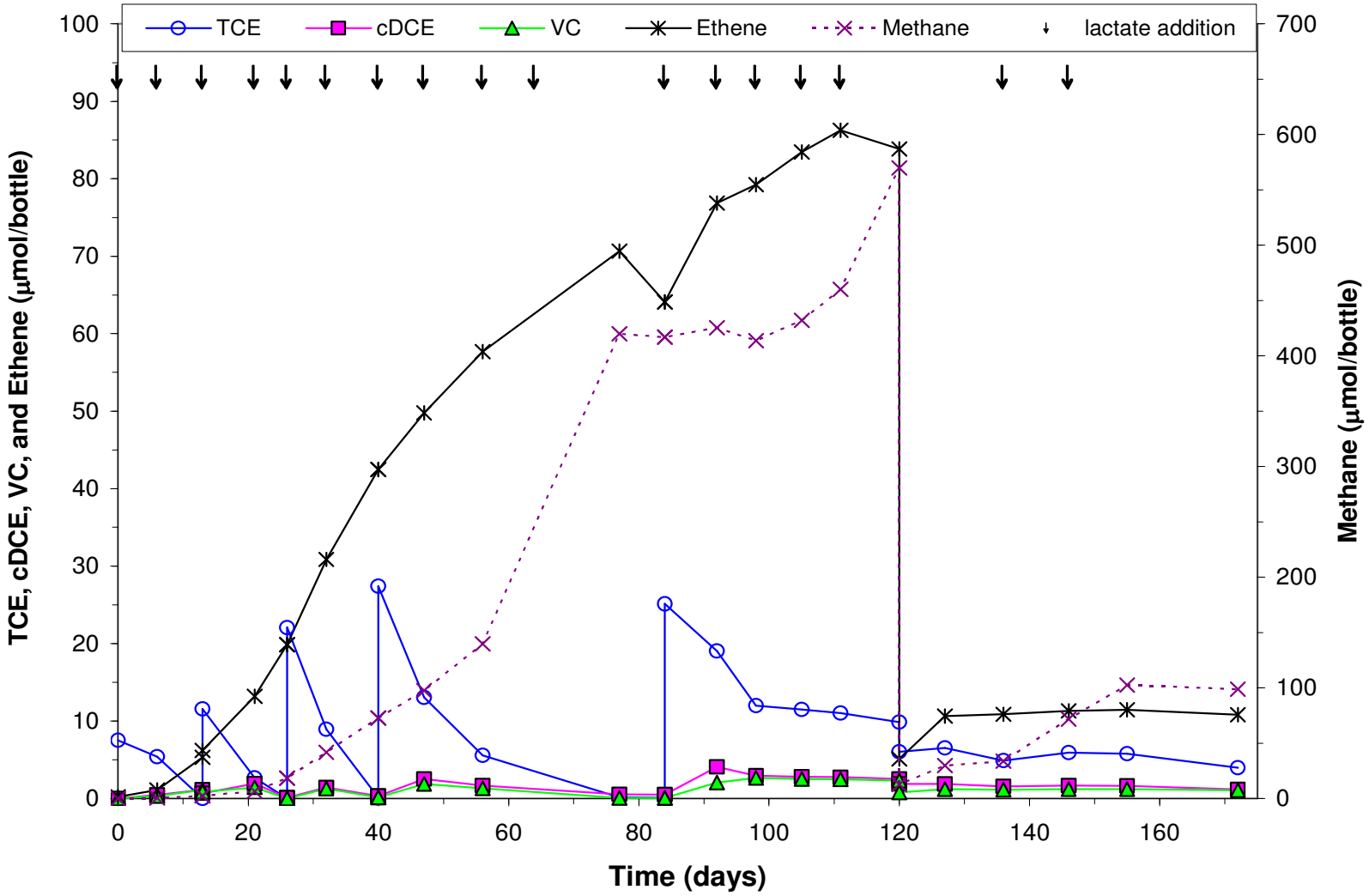

Figure 4.8 Results for one of the Yield Experiment Phase 1 TCE bottles (TCE-1).

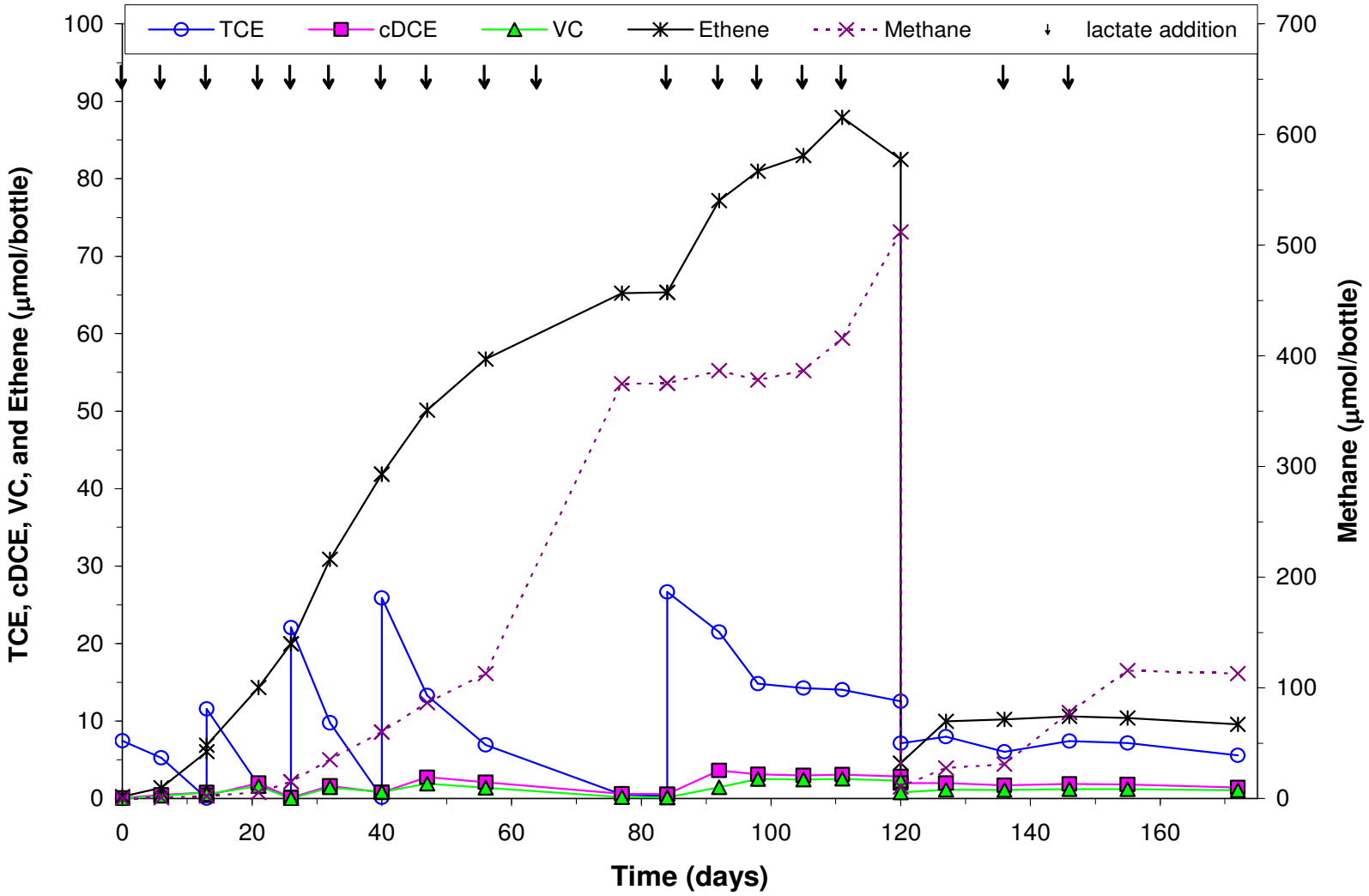

Figure 4.9 Results for one of the Yield Experiment Phase 1 TCE bottles (TCE-2).

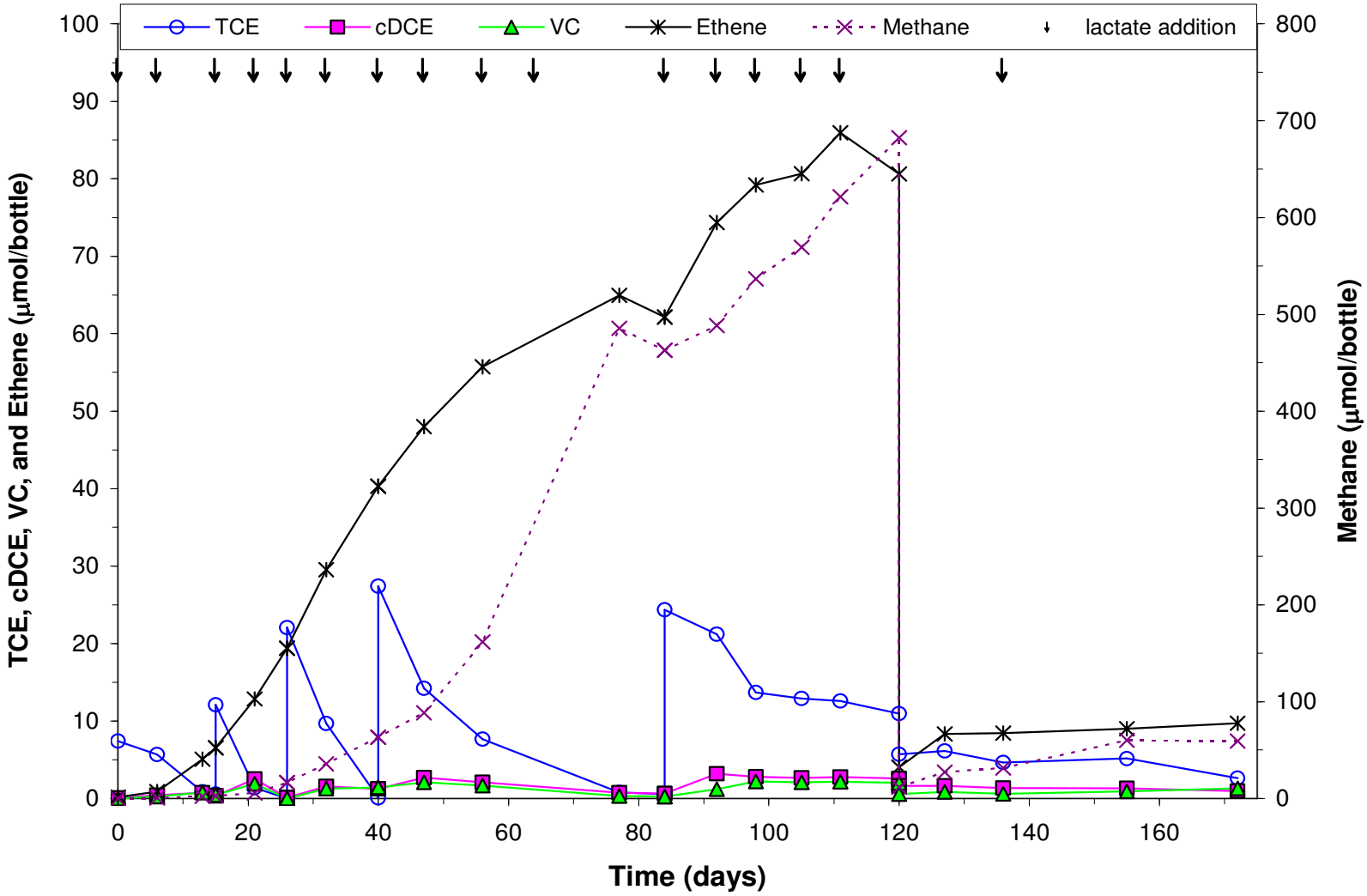

Figure 4.10 Results for one of the Yield Experiment Phase 1 TCE bottles (TCE-3).

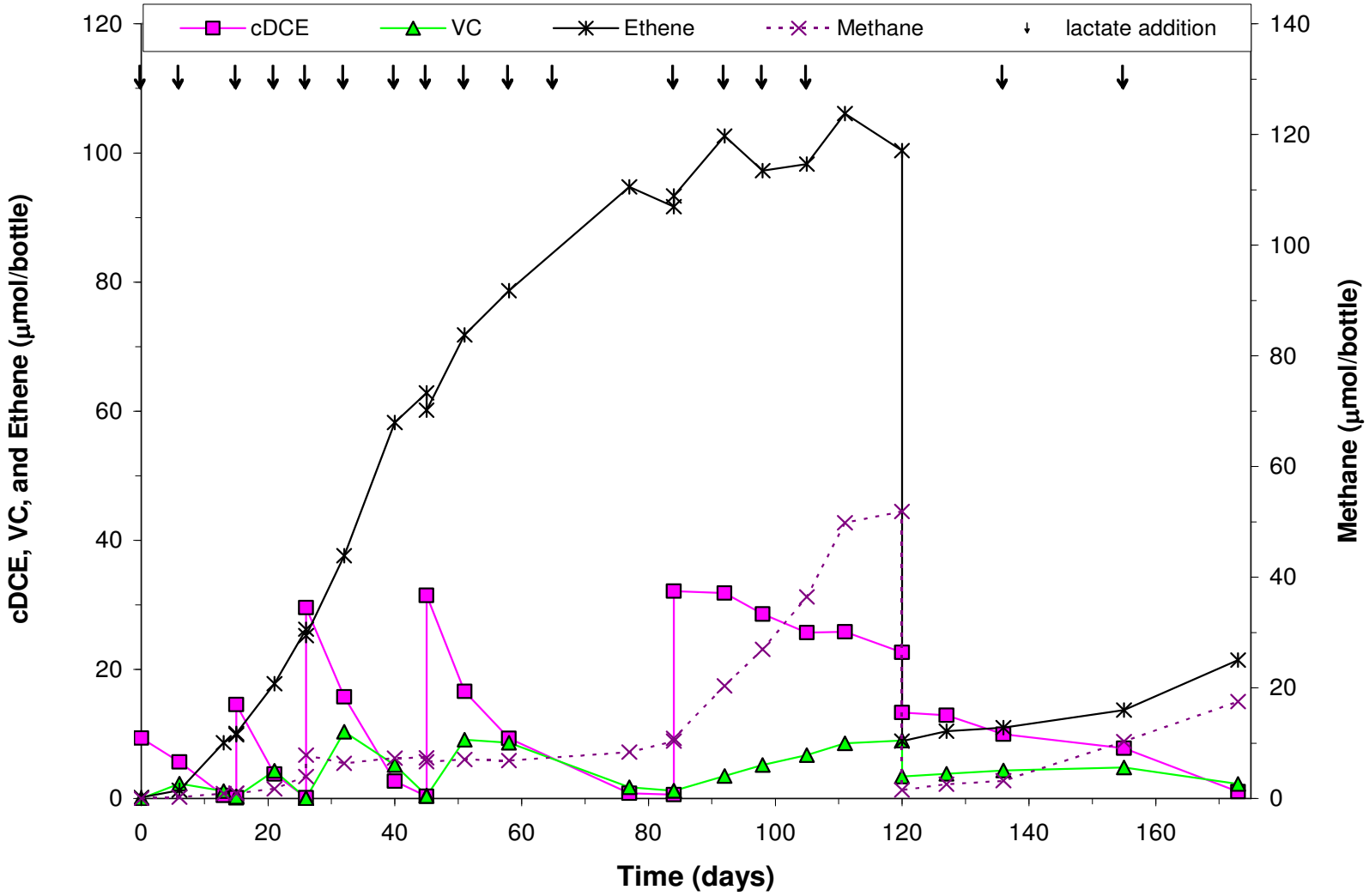

Figure 4.11 Results for one of the Yield Experiment Phase 1 cDCE bottles (cDCE-1).

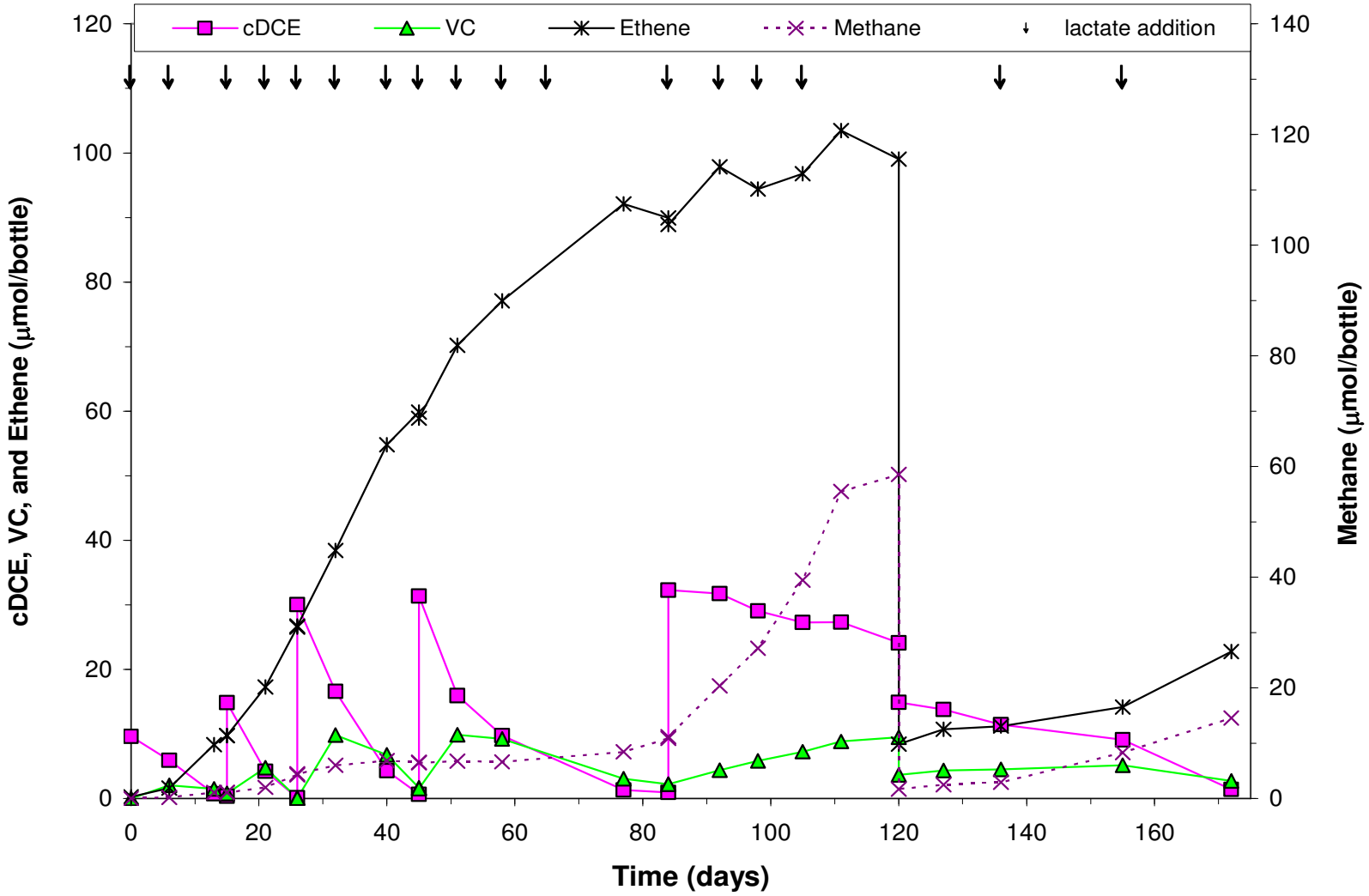

Figure 4.12 Results for one of the Yield Experiment Phase 1 cDCE bottles (cDCE-2).

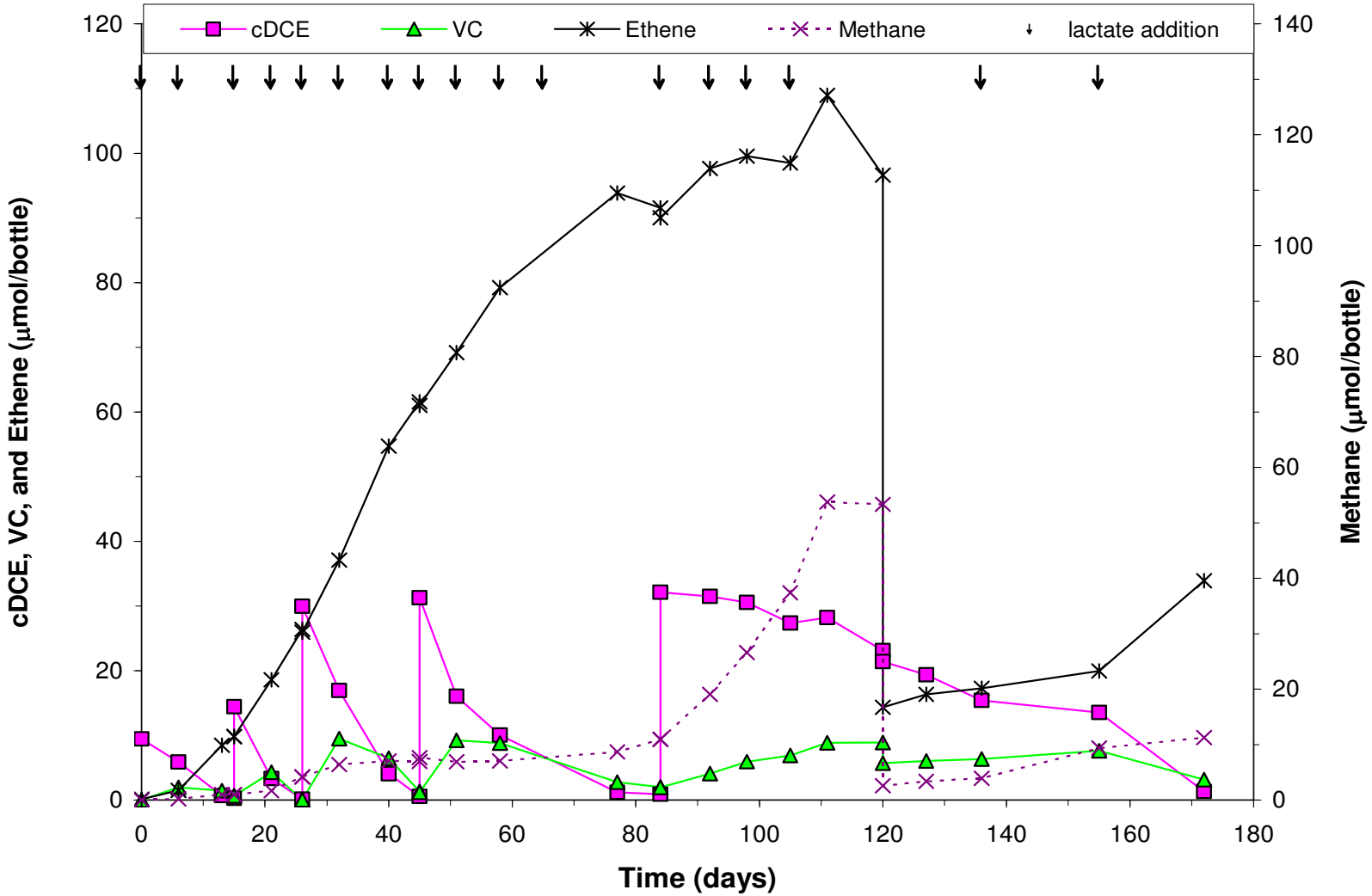

Figure 4.13 Results for one of the Yield Experiment Phase 1 cDCE bottles (cDCE-3).

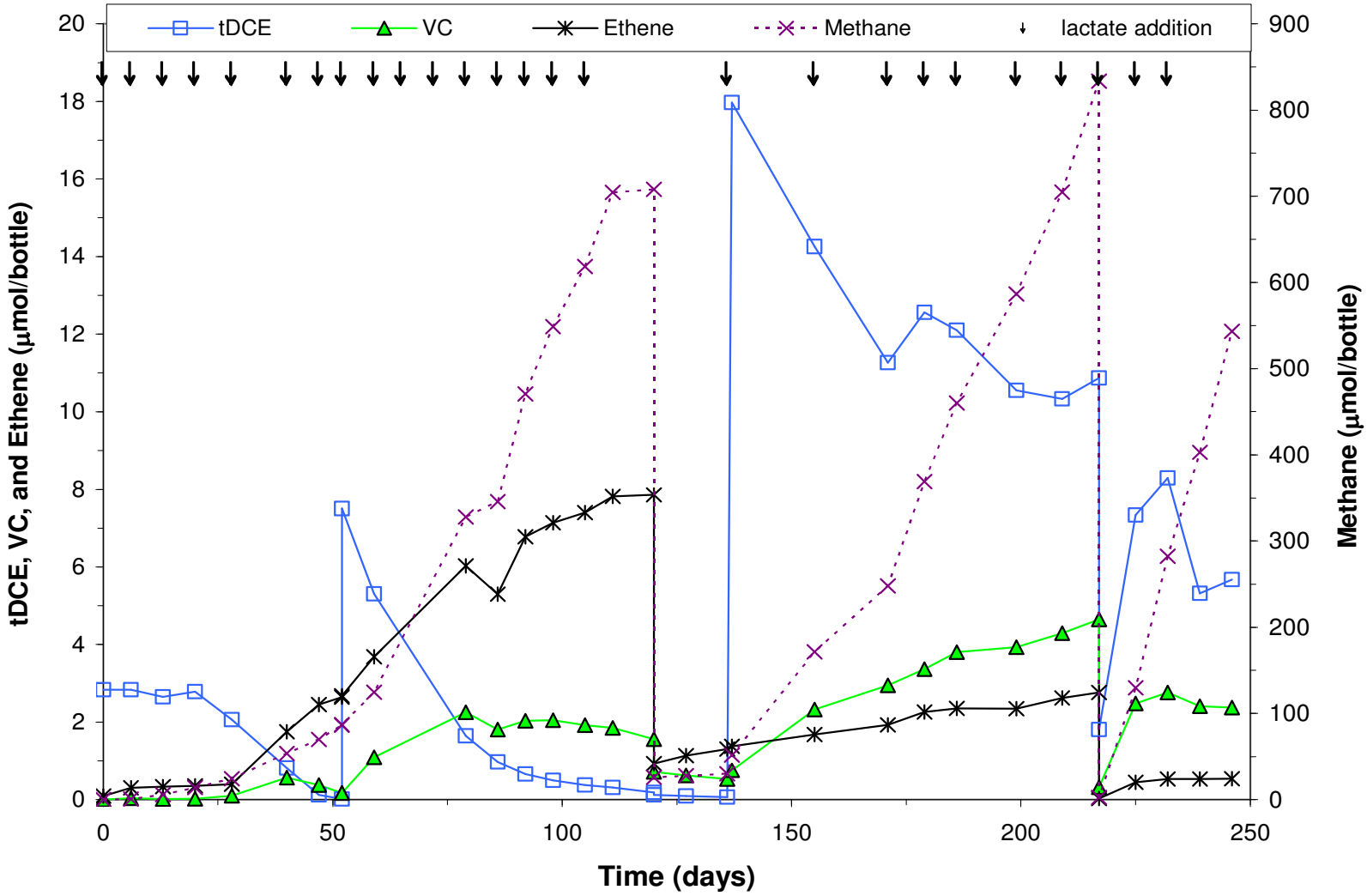

Figure 4.14 Results for one of the Yield Experiment Phase 1 tDCE bottles (tDCE-1).

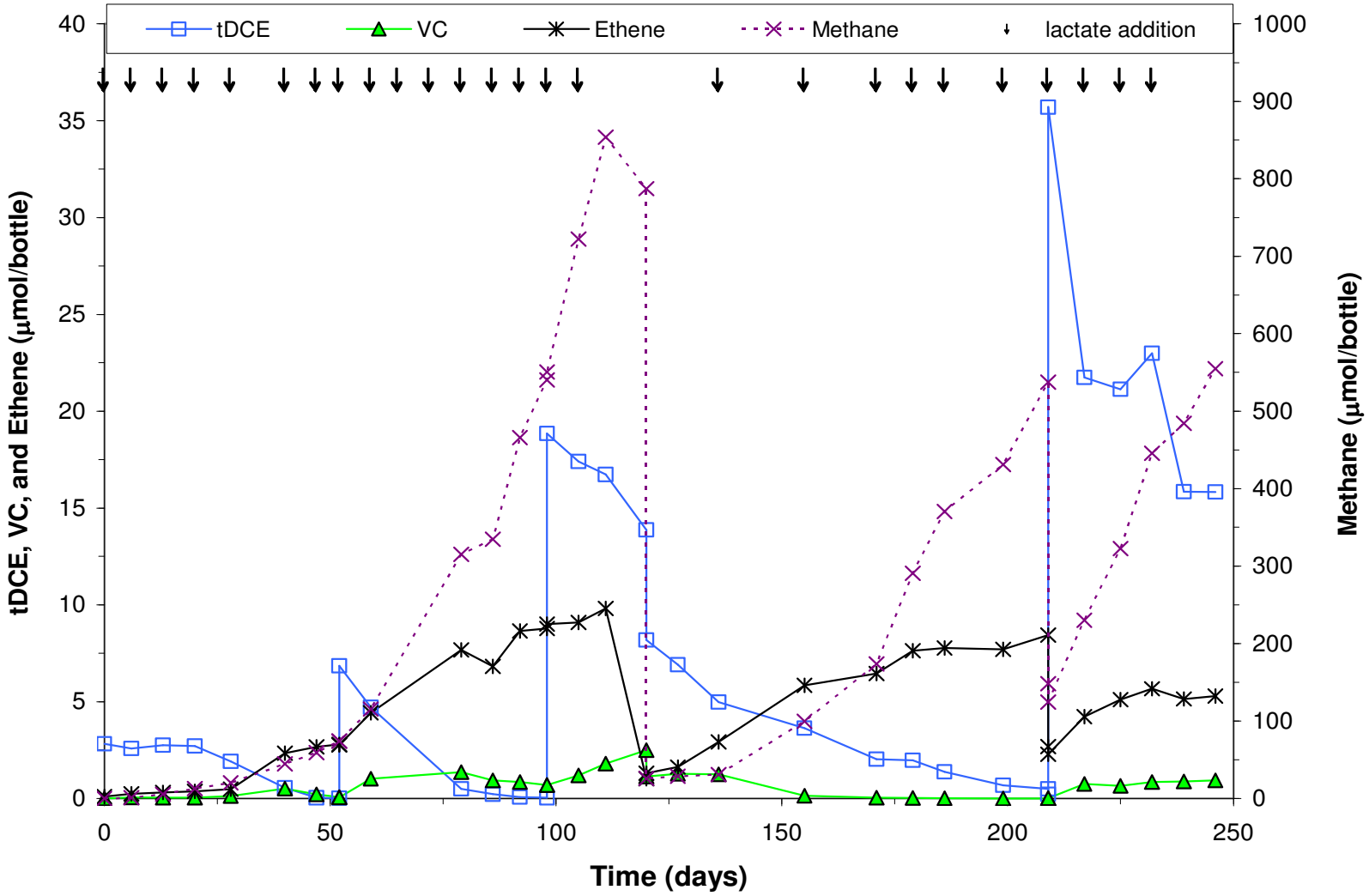

Figure 4.15 Results for one of the Yield Experiment Phase 1 tDCE bottles (tDCE-2).

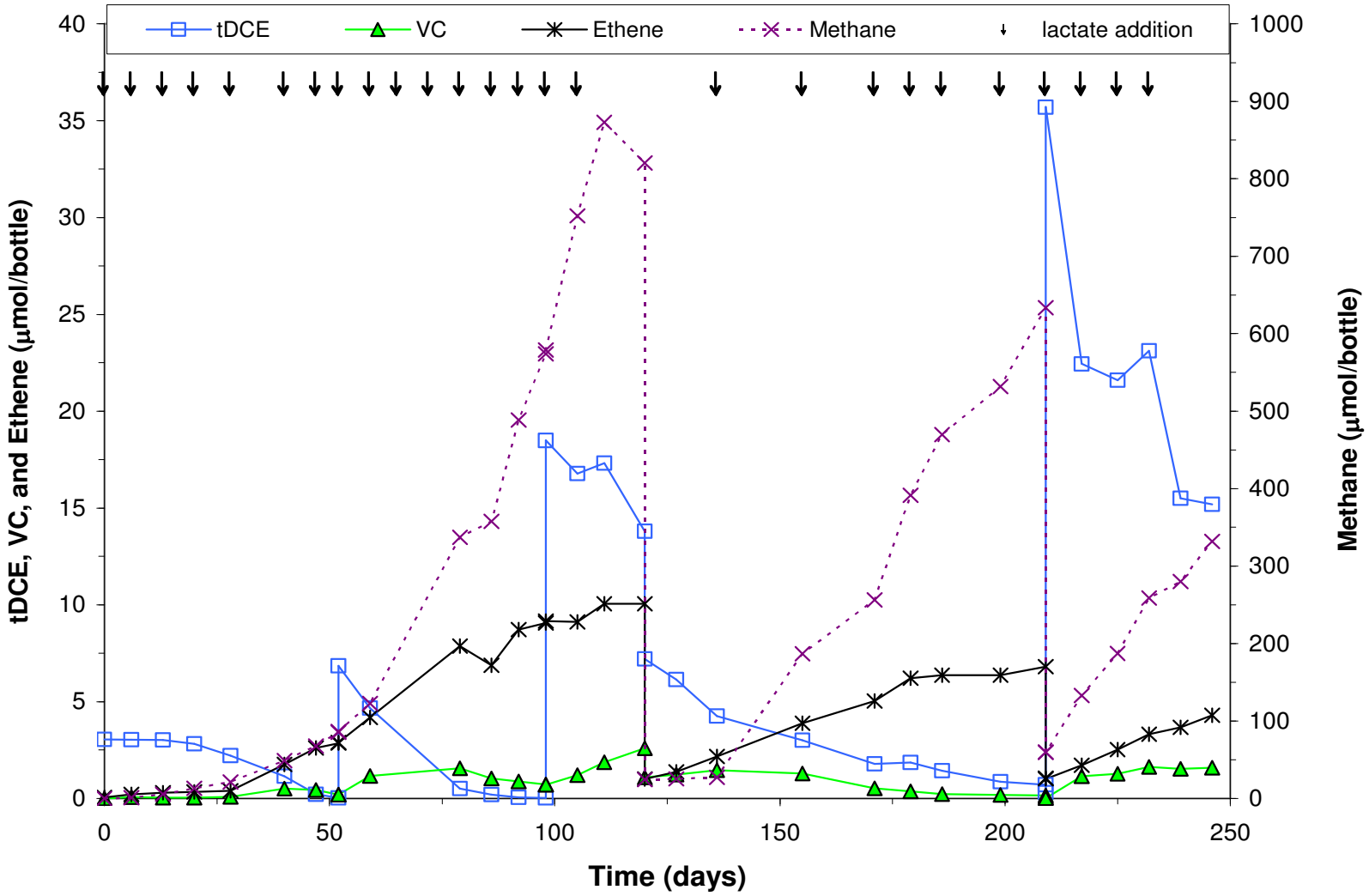

Figure 4.16 Results for one of the Yield Experiment Phase 1 tDCE bottles (tDCE-3).

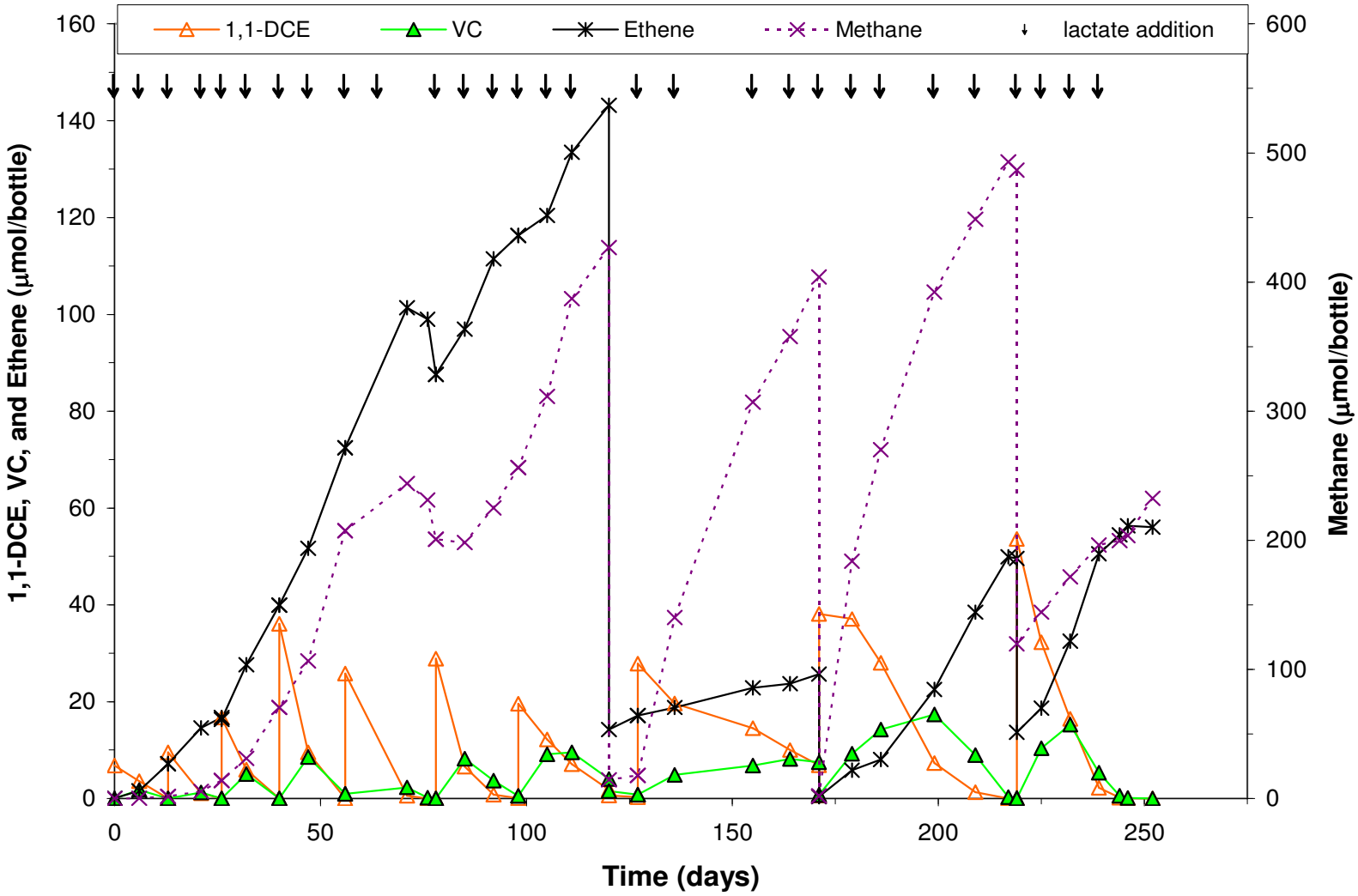

Figure 4.17 Results for one of the Yield Experiment Phase 1 1,1-DCE bottles (1,1-DCE-1).

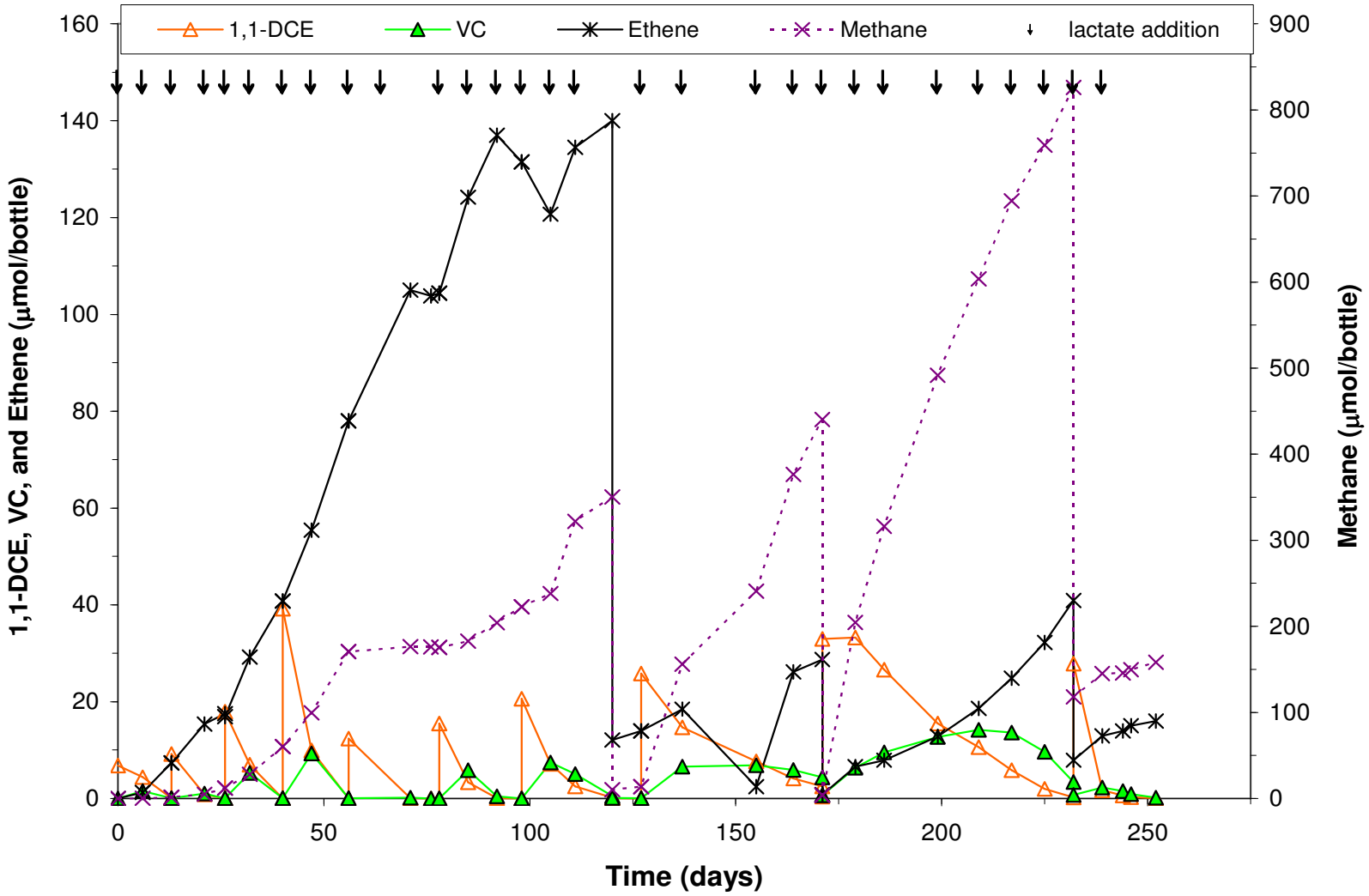

Figure 4.18 Results for one of the Yield Experiment Phase 1 1,1-DCE bottles (1,1-DCE-2).

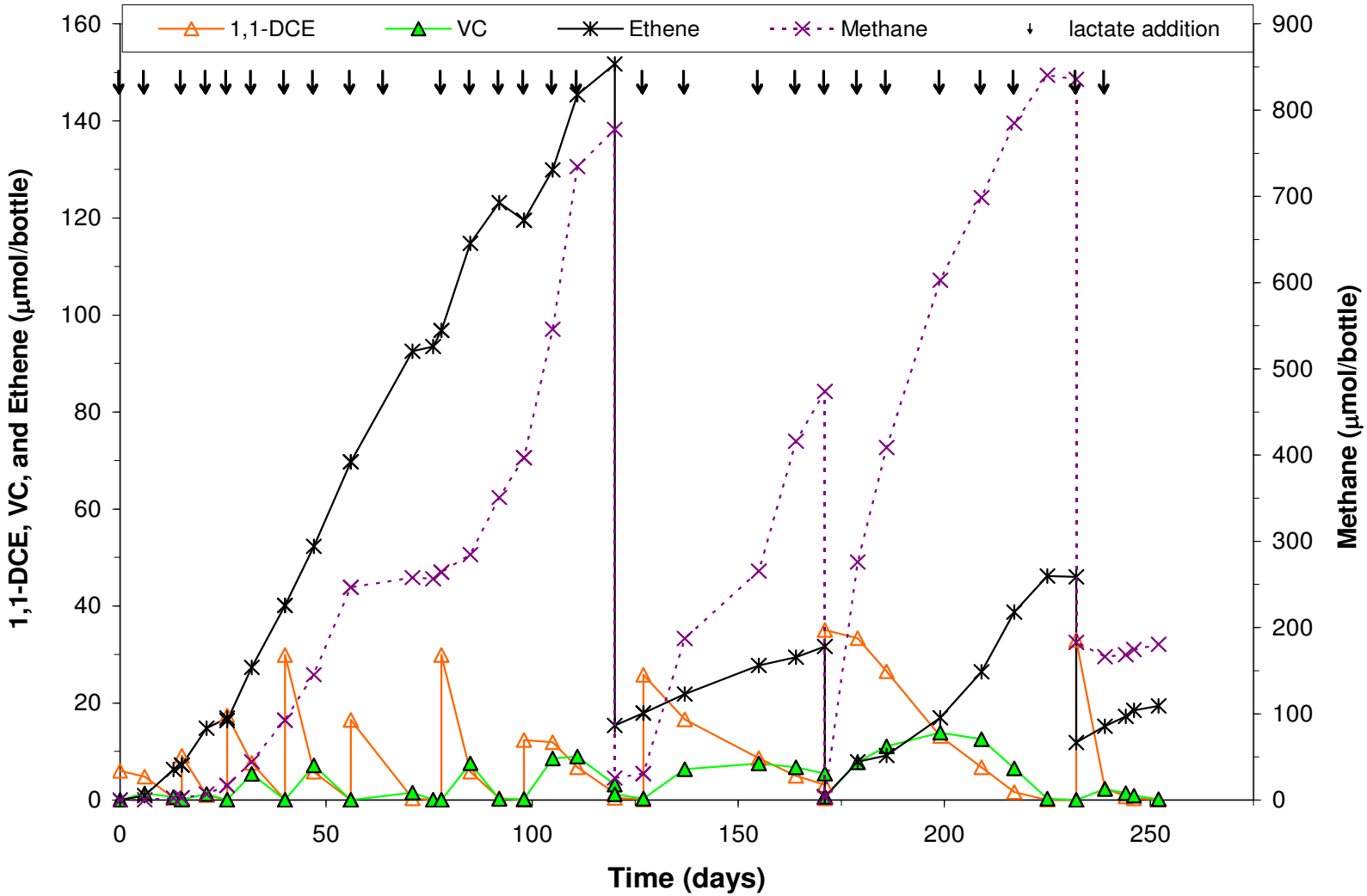

Figure 4.19 Results for one of the Yield Experiment Phase 1 1,1-DCE bottles (1,1-DCE-3).

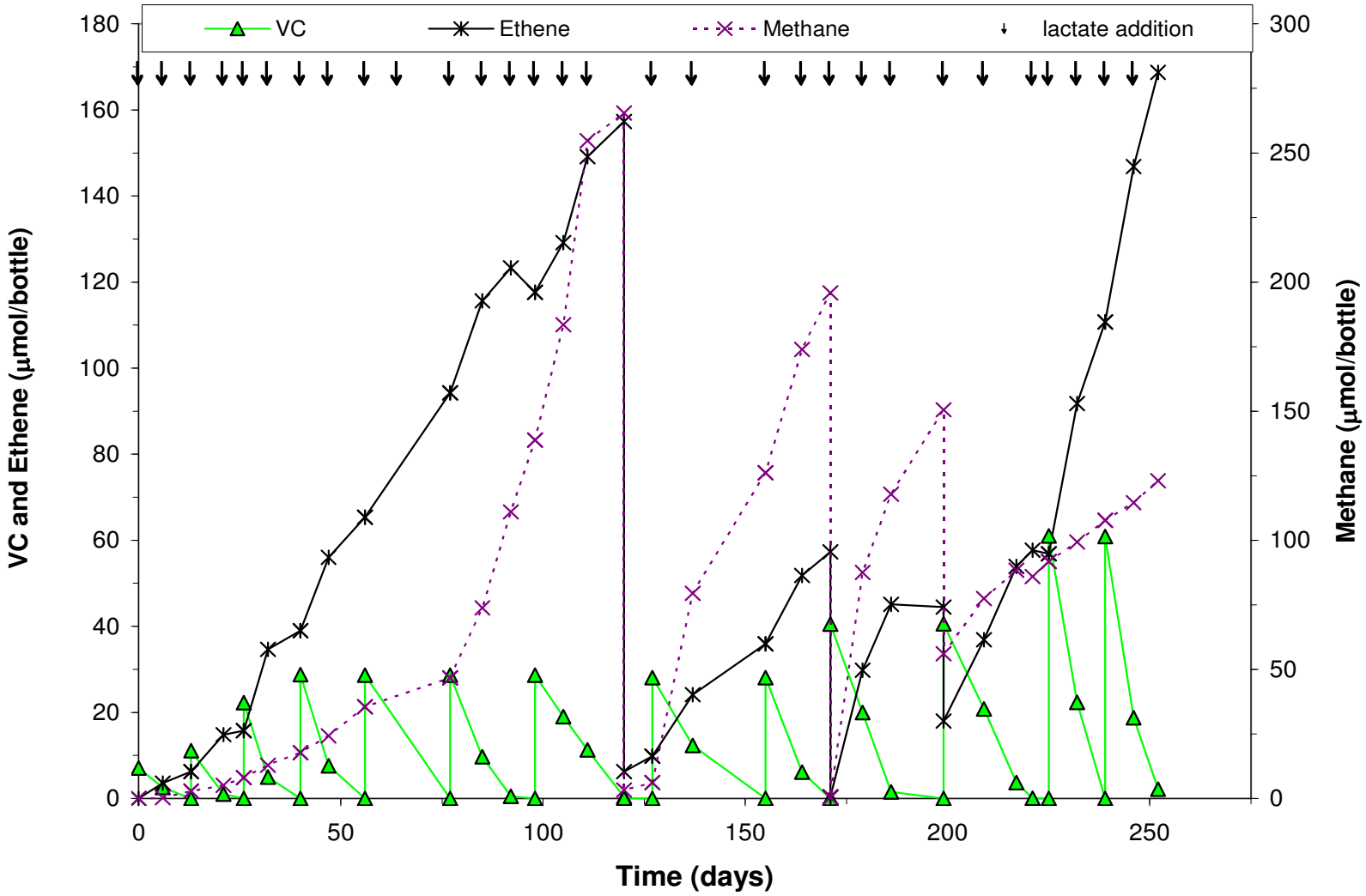

Figure 4.20 Results for one of the Yield Experiment Phase 1 VC bottles (VC-1).

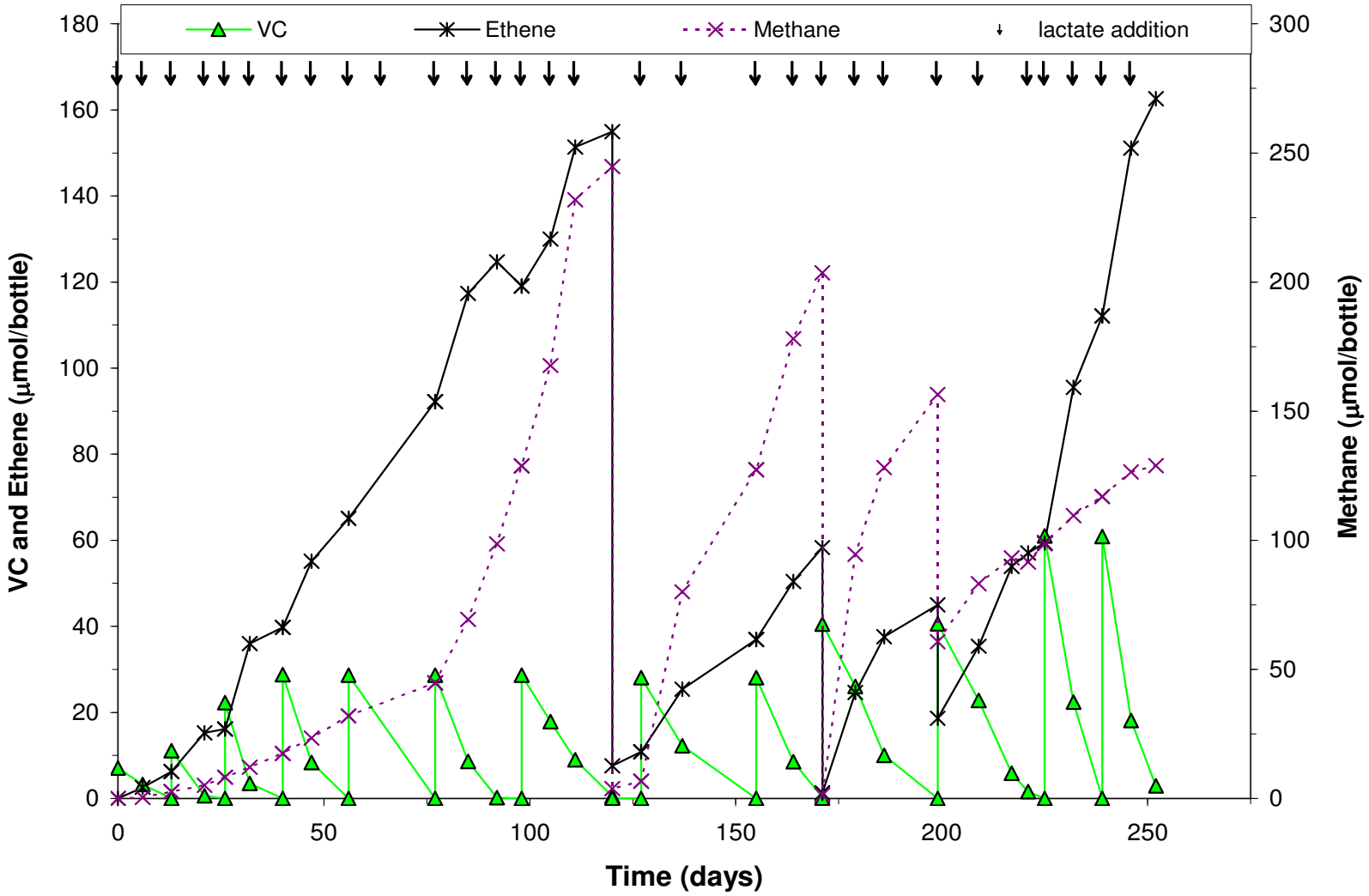

Figure 4.21 Results for one of the Yield Experiment Phase 1 VC bottles (VC-2).

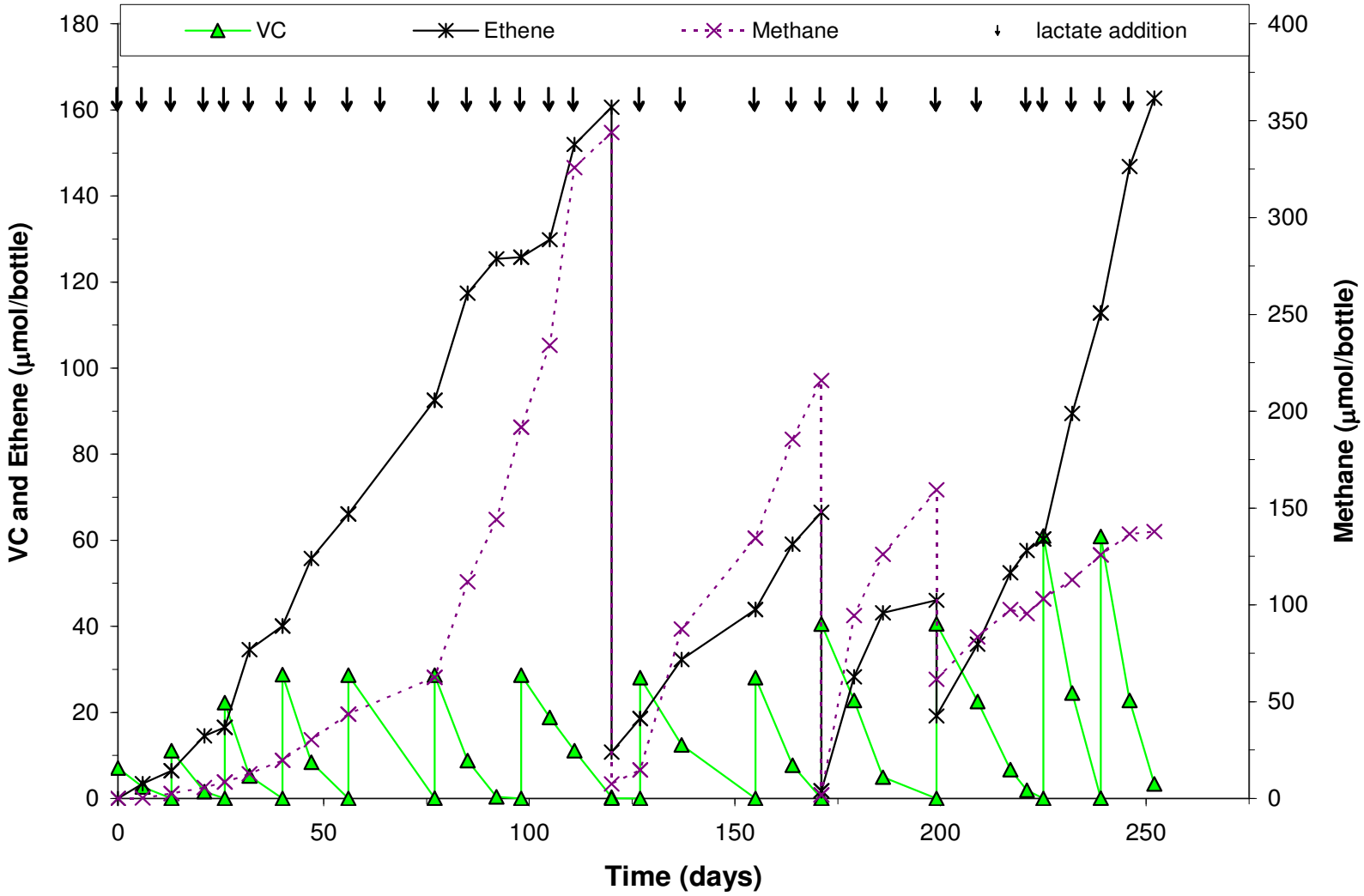

Figure 4.22 Results for one of the Yield Experiment Phase 1 VC bottles (VC-3).

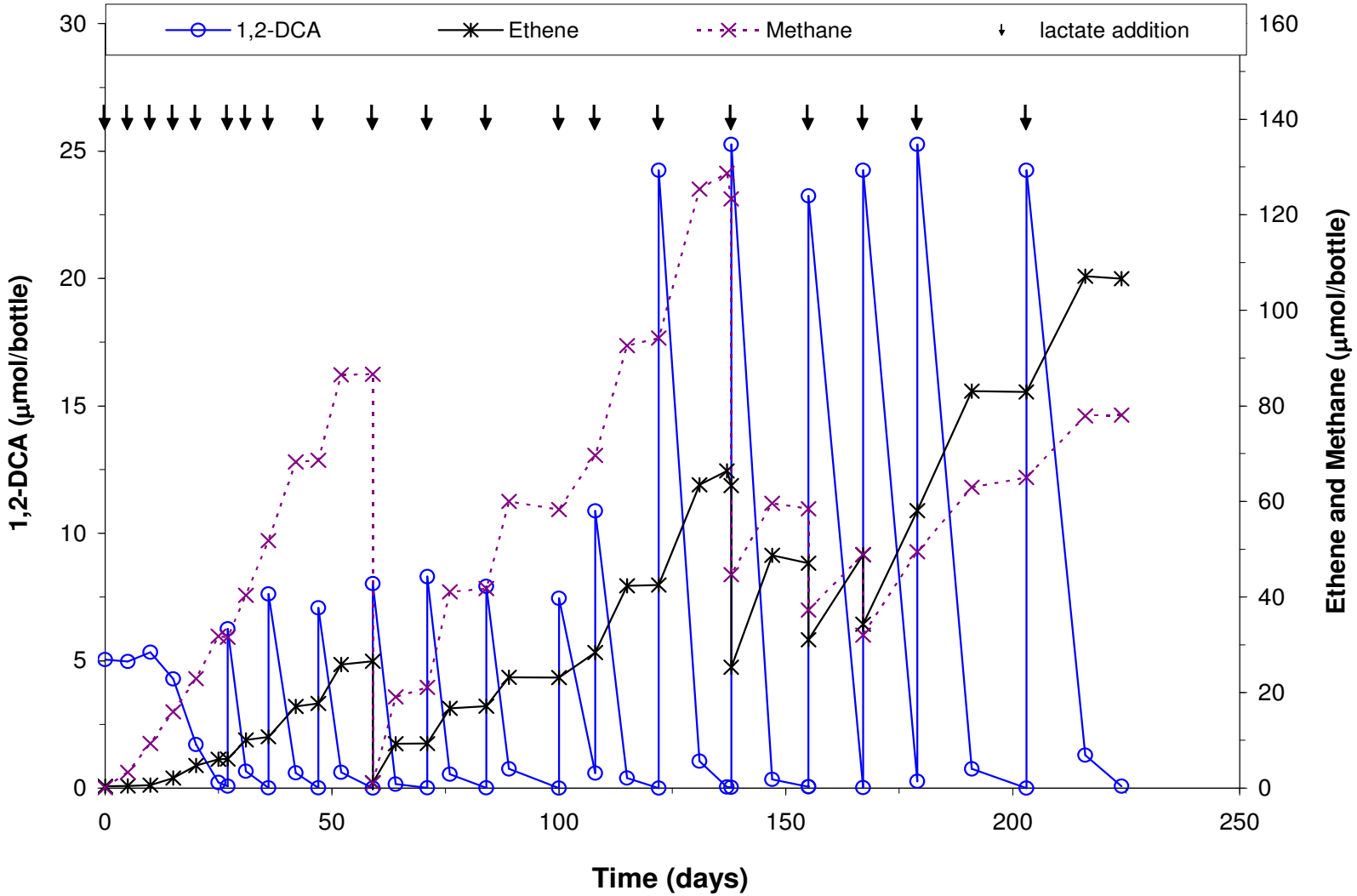

Figure 4.23 Results for one of the Yield Experiment Phase 1 1,2-DCA bottles (1,2-DCA-1).

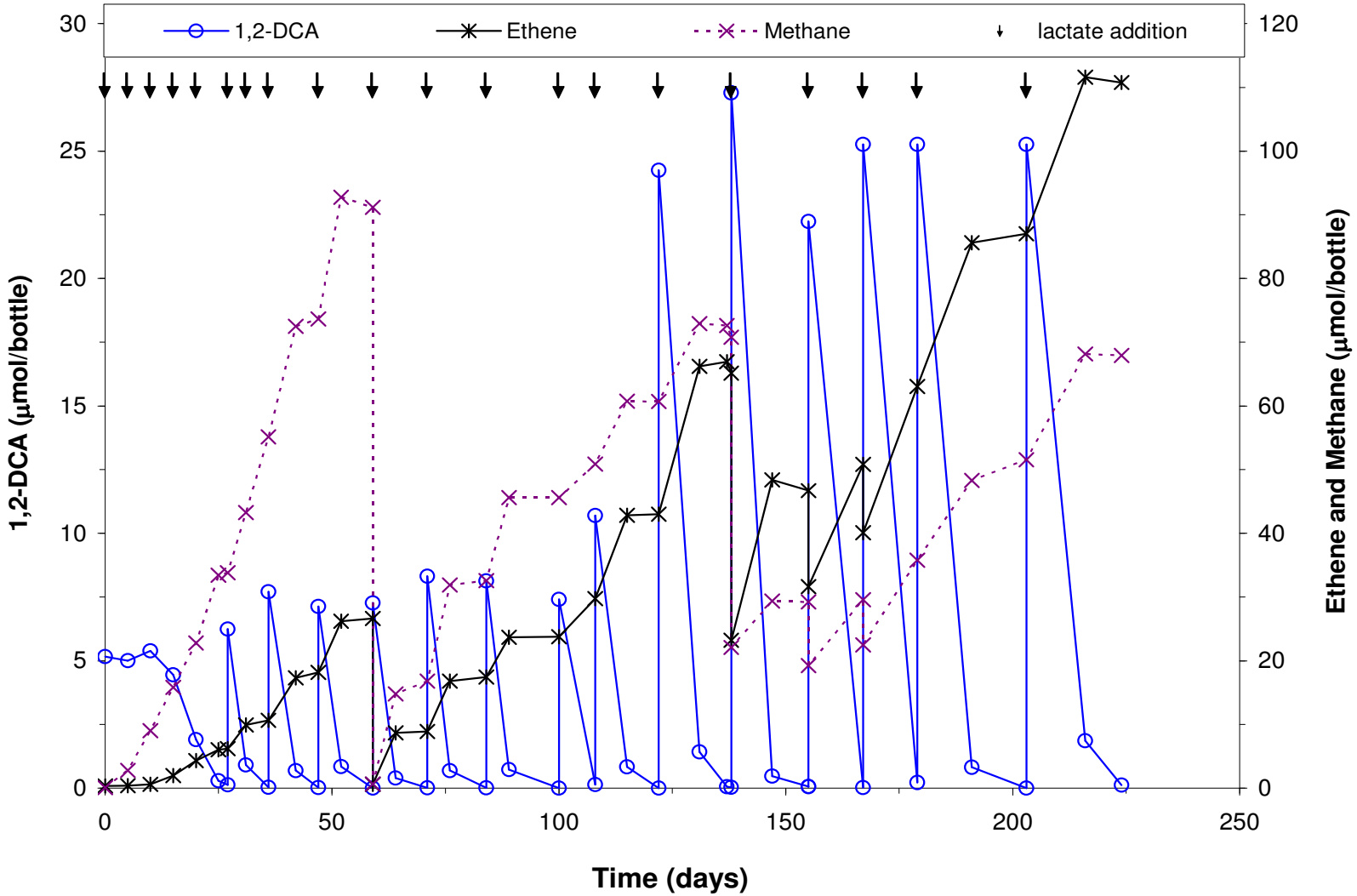

Figure 4.24 Results for one of the Yield Experiment Phase 1 1,2-DCA bottles (1,2-DCA-2).

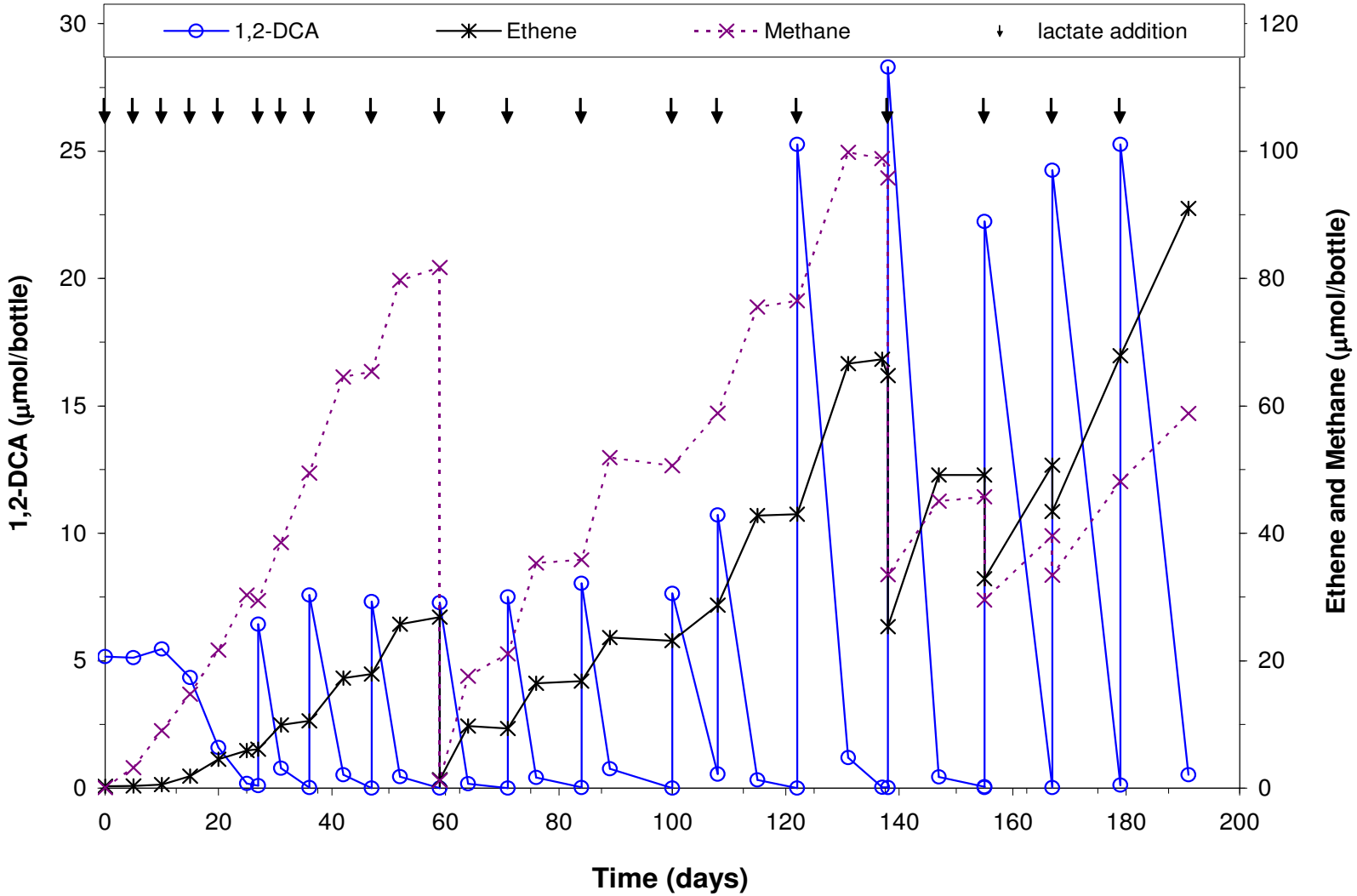

Figure 4.25 Results for one of the Yield Experiment Phase 1 1,2-DCA bottles (1,2-DCA-3).

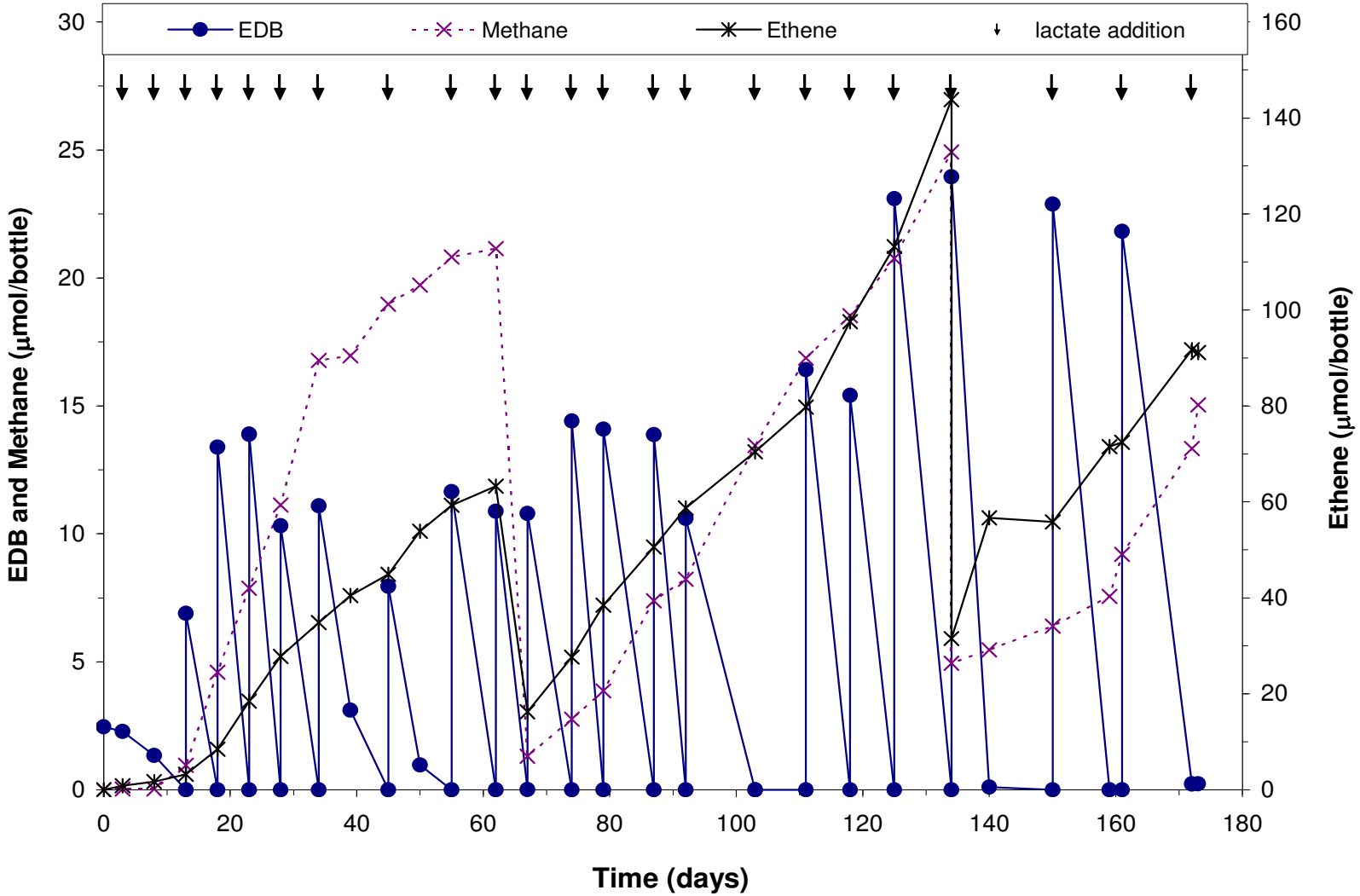

Figure 4.26 Results for one of the Yield Experiment Phase 1 EDB bottles (EDB-1).

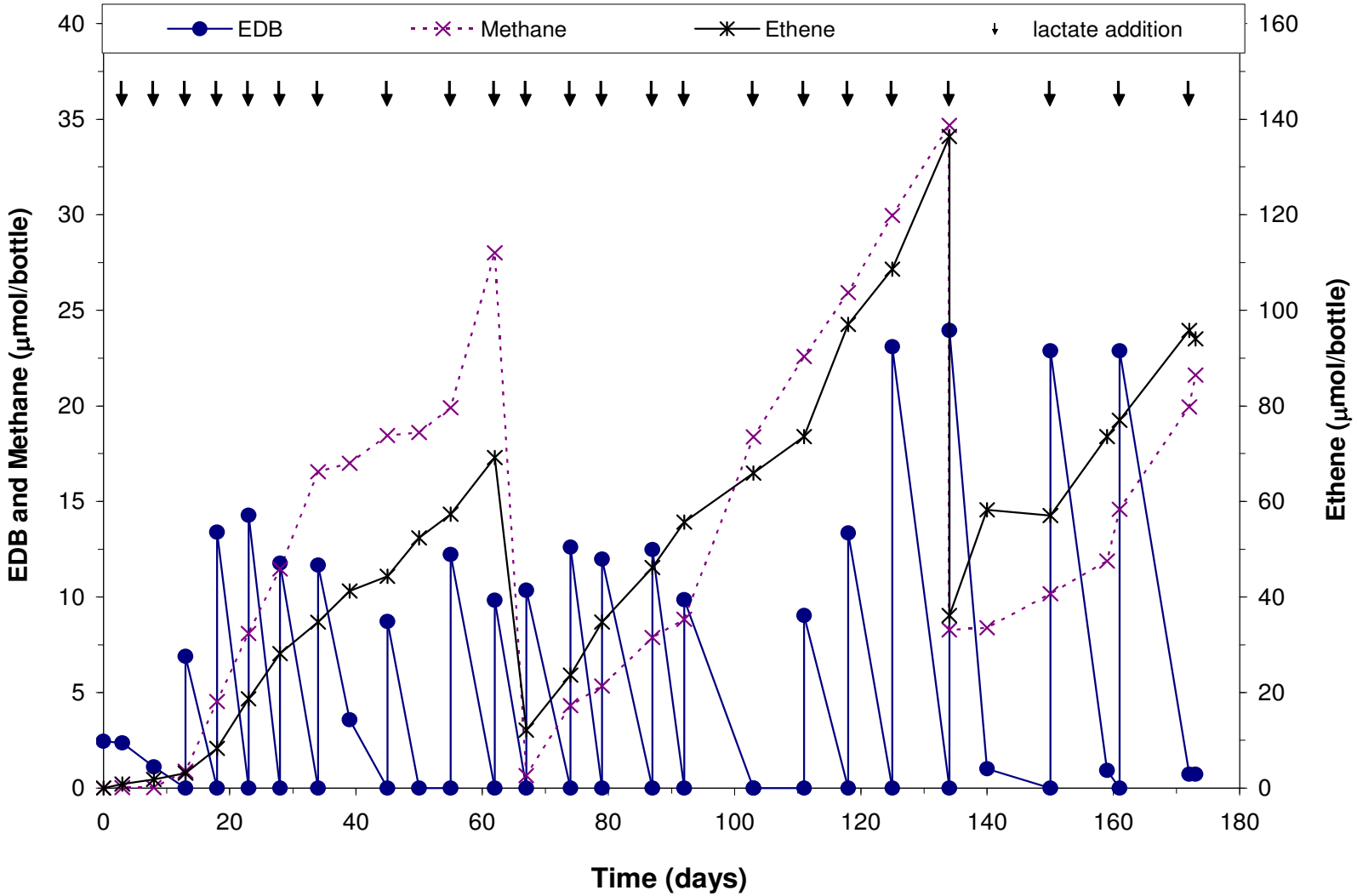

Figure 4.27 Results for one of the Yield Experiment Phase 1 EDB bottles (EDB-2).

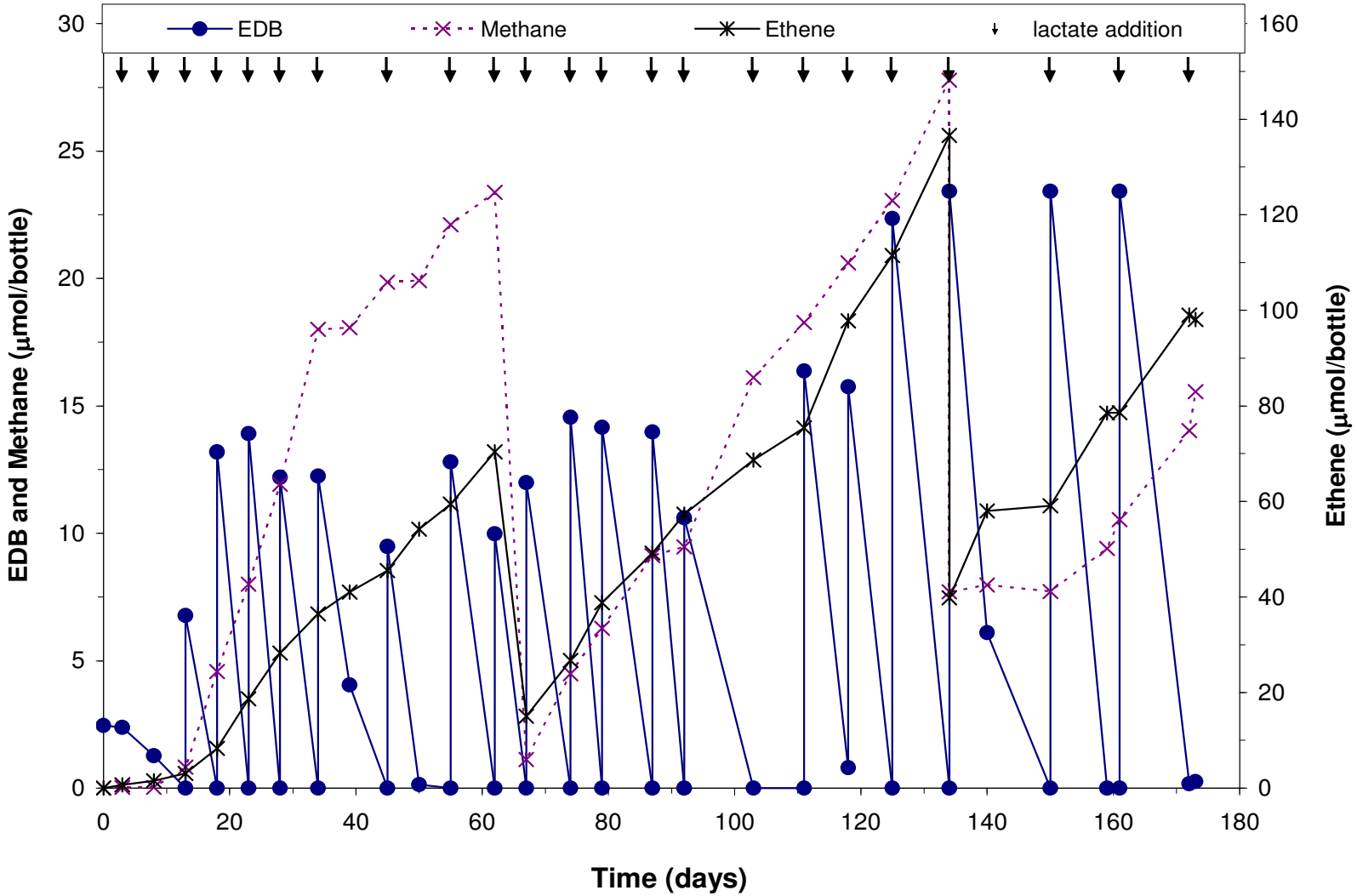

Figure 4.28 Results for one of the Yield Experiment Phase 1 EDB bottles (EDB-3).

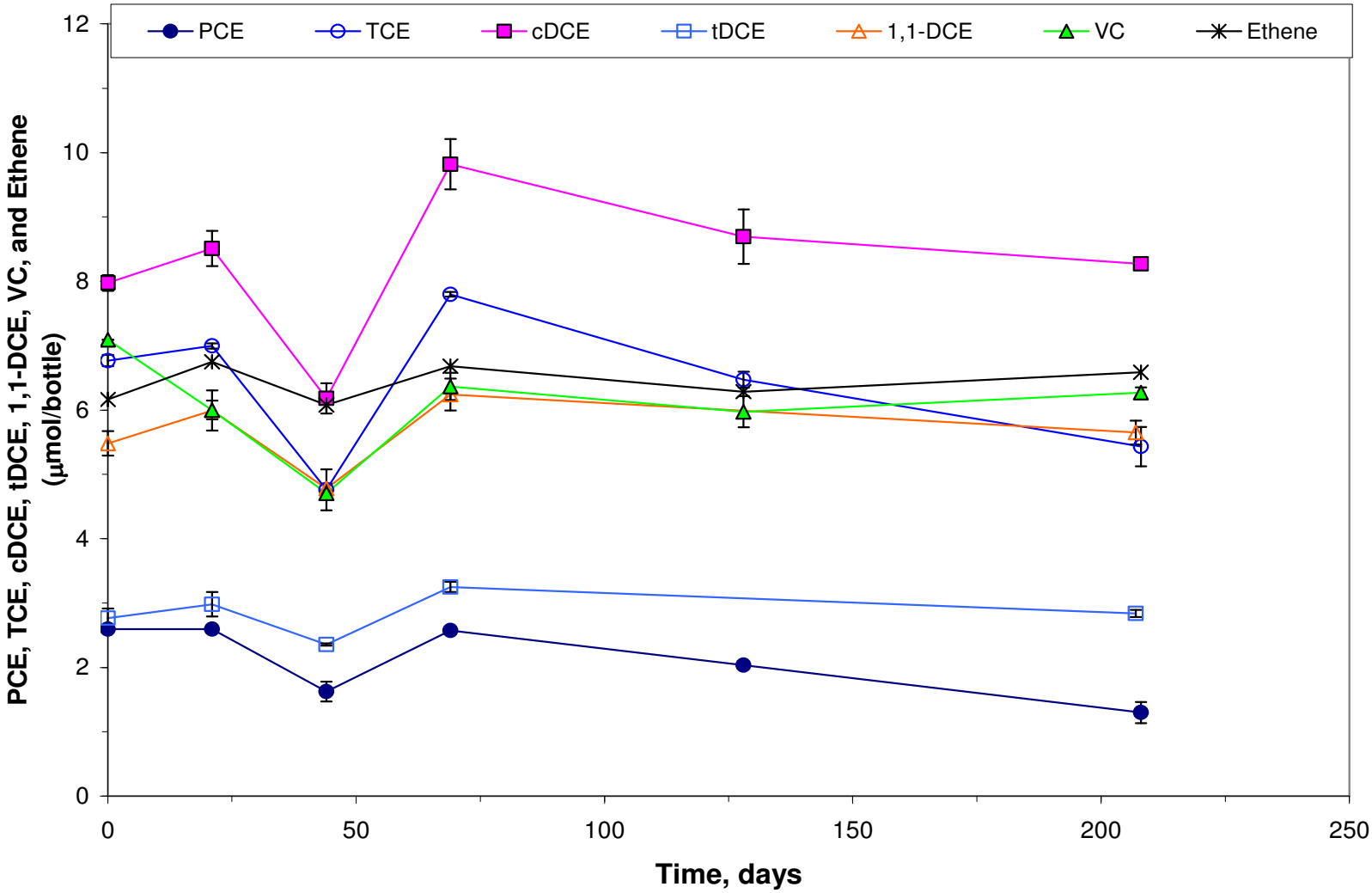

Figure 4.29 Results for the first set of Yield Experiment Phase I WCs (average of triplicates). Error bars represent ± one standard deviation.

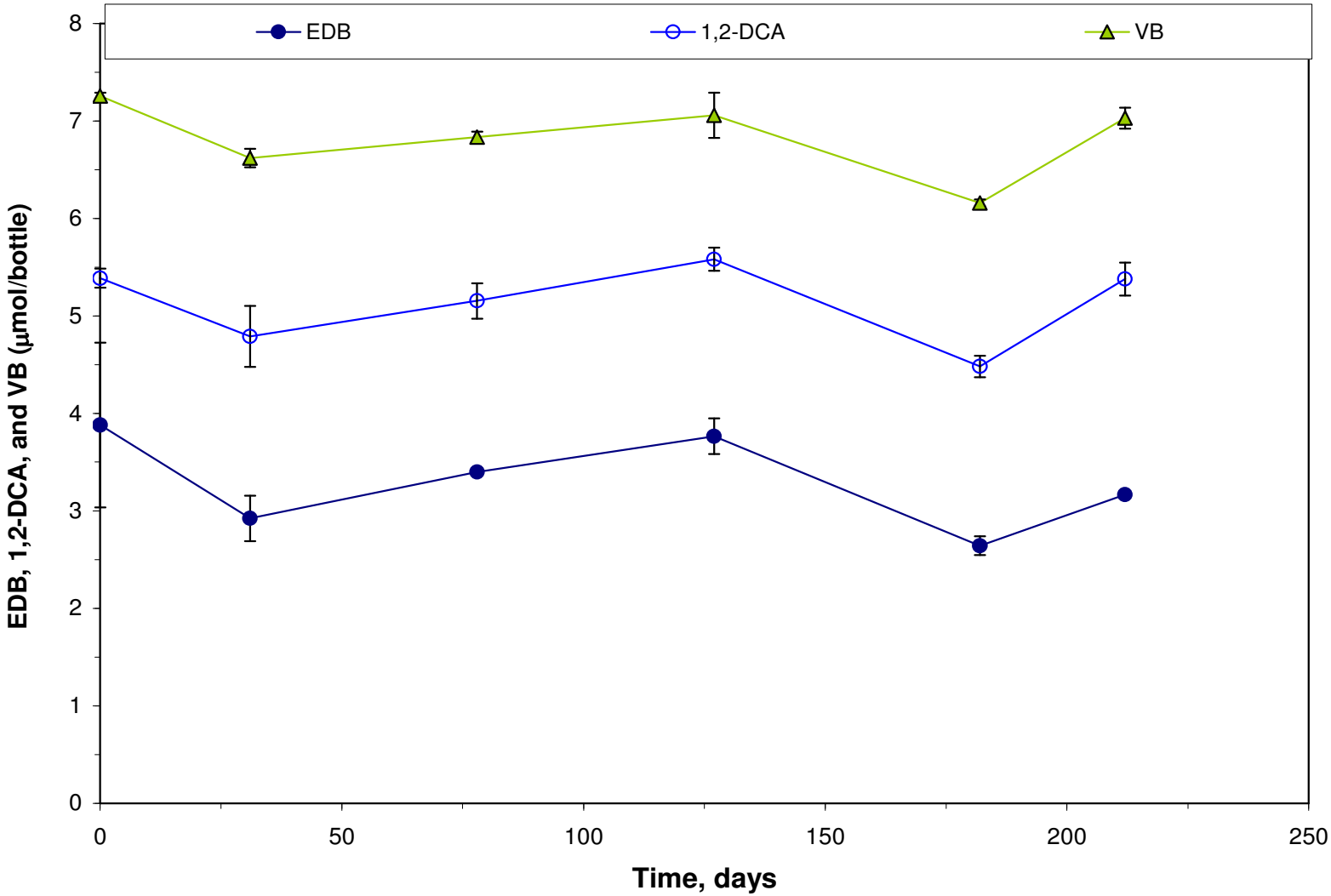

Figure 4.30 Results for the second set of Yield Experiment Phase I WCs (average of triplicates). Error bars represent ± one standard deviation.

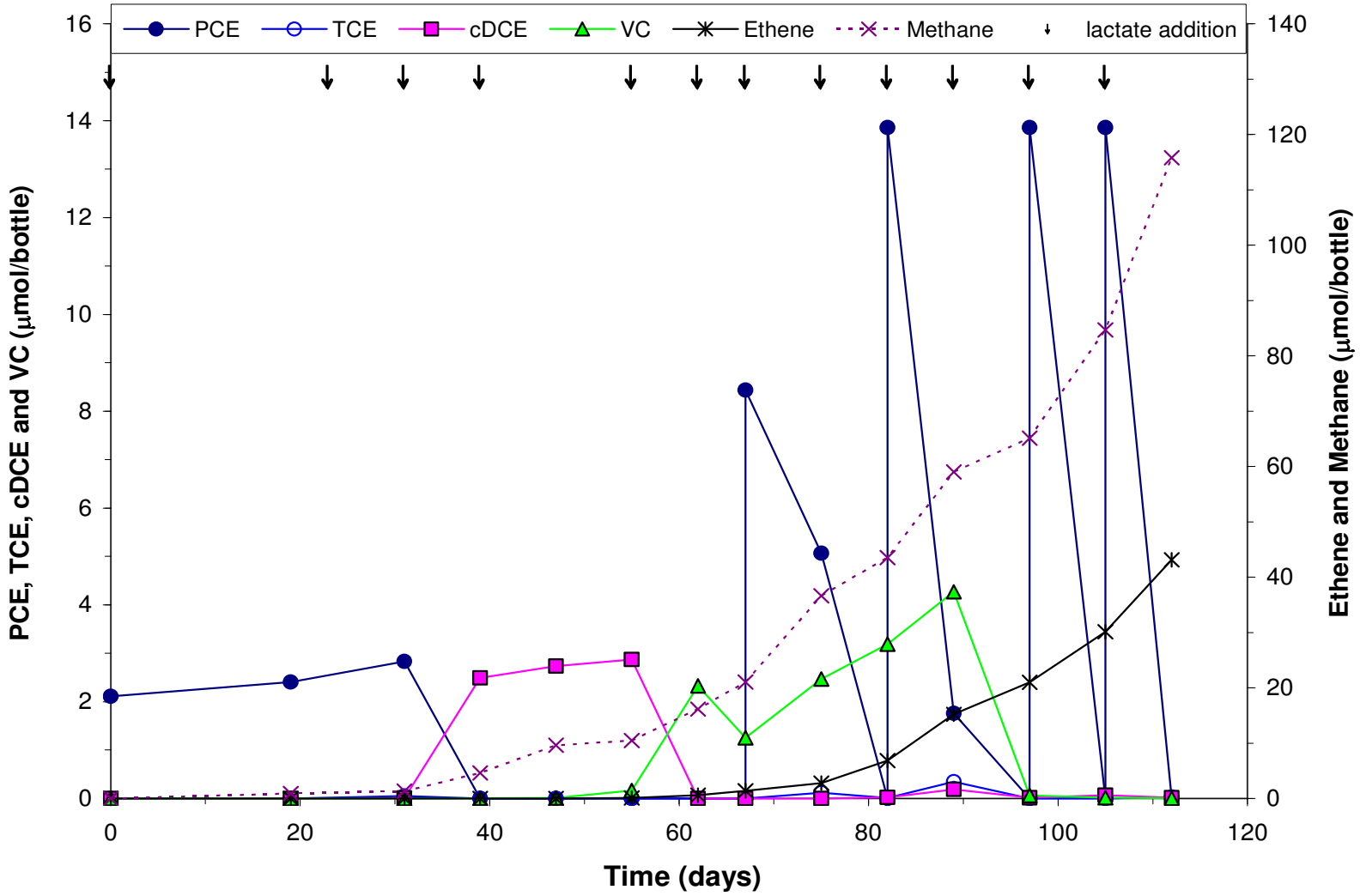

Figure 4.31 Results for one of the Yield Experiment Phase 2 PCE bottles (PCE-1).

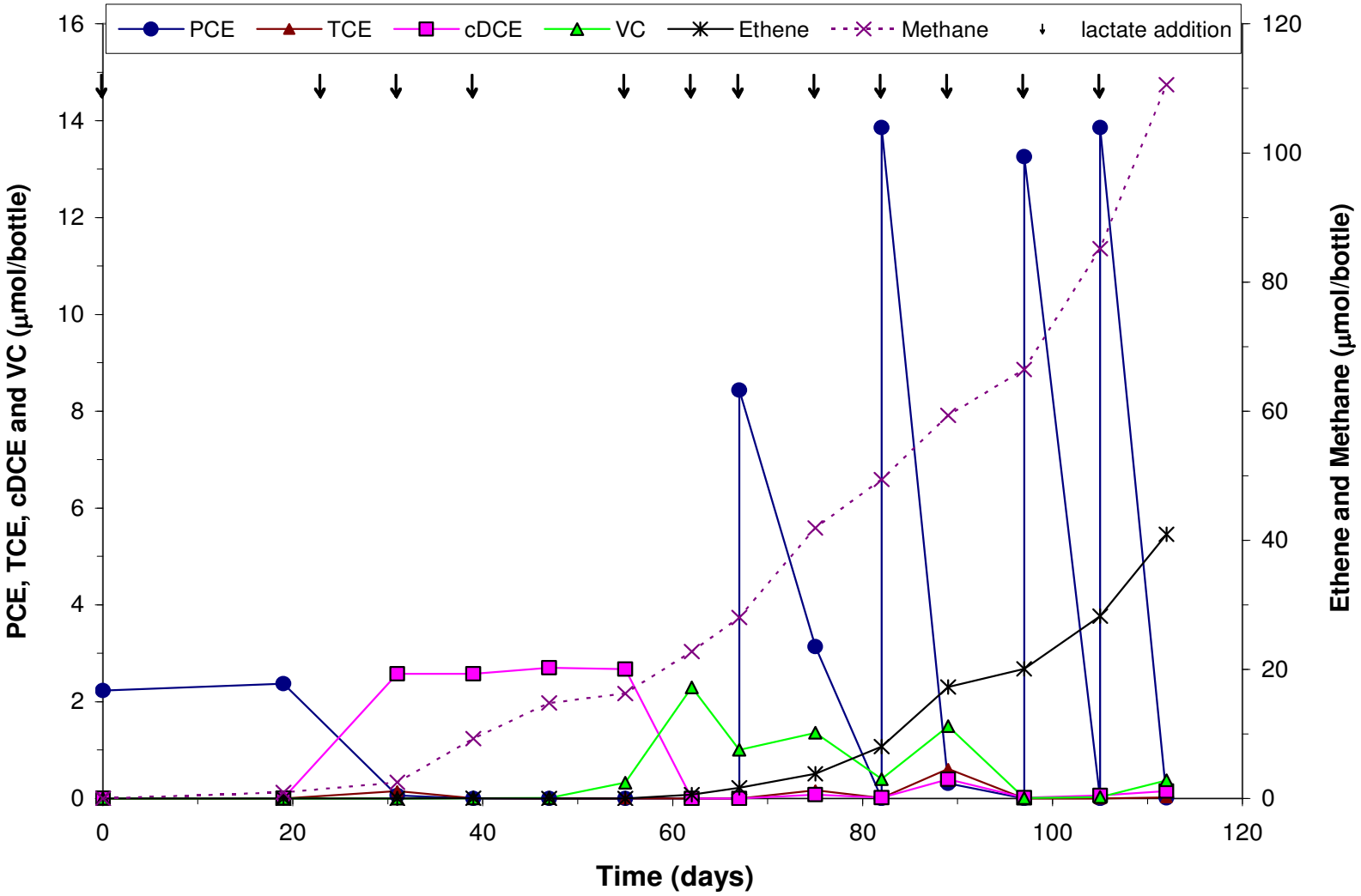

Figure 4.32 Results for one of the Yield Experiment Phase 2 PCE bottles (PCE-2).

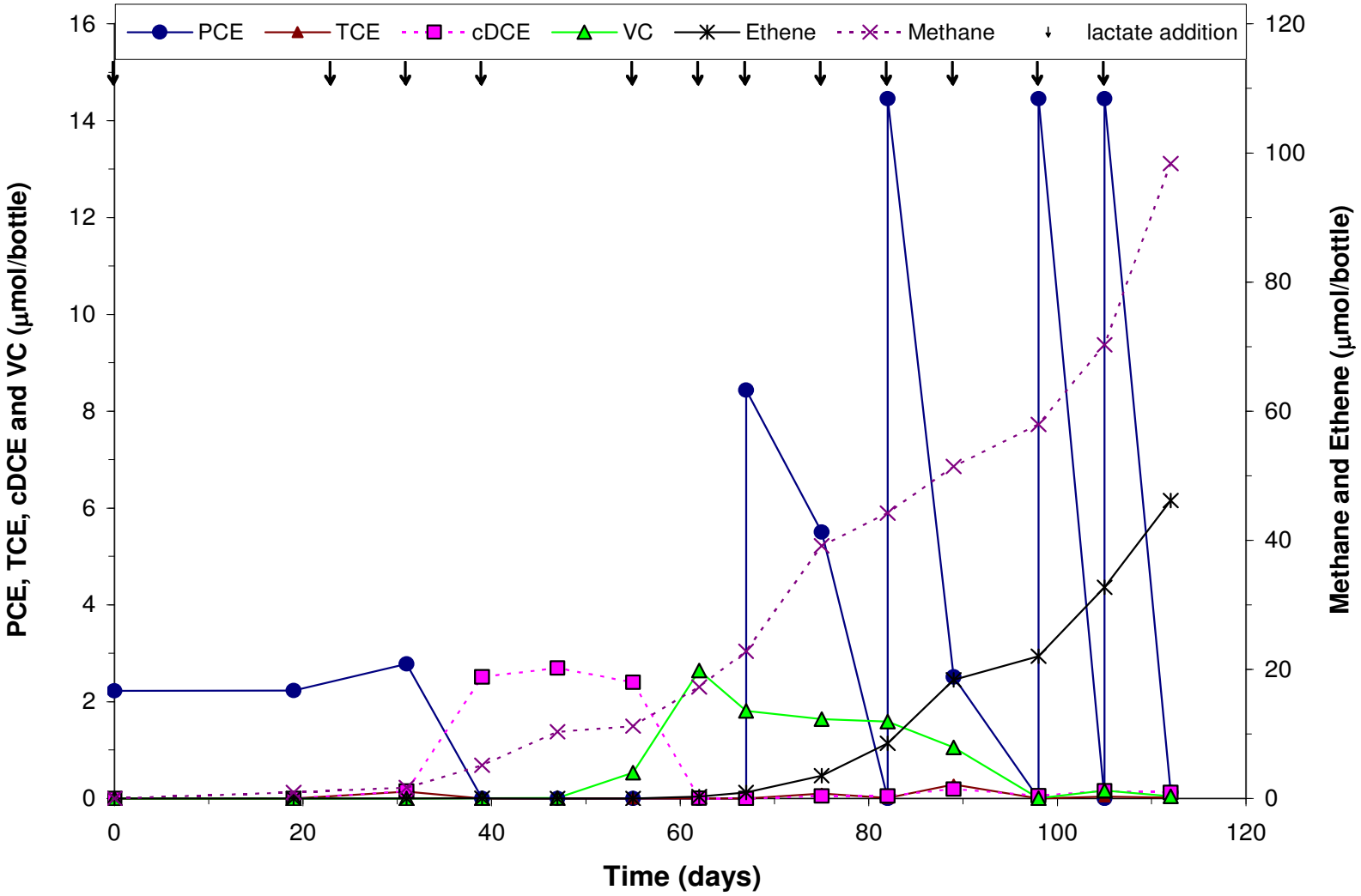

Figure 4.33 Results for one of the Yield Experiment Phase 2 PCE bottles (PCE-3).

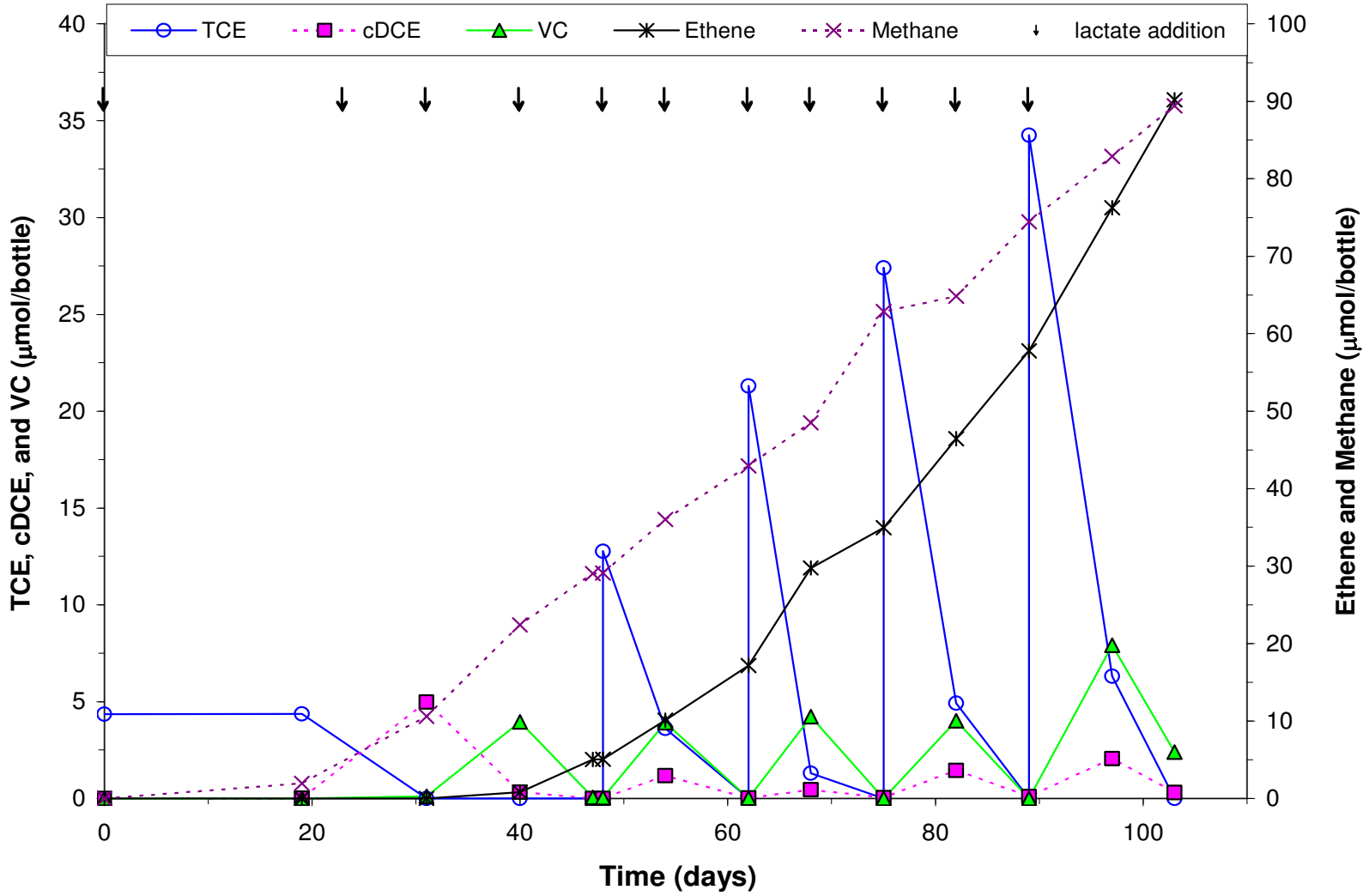

Figure 4.34 Results for one of the Yield Experiment Phase 2 TCE bottles (TCE-1).

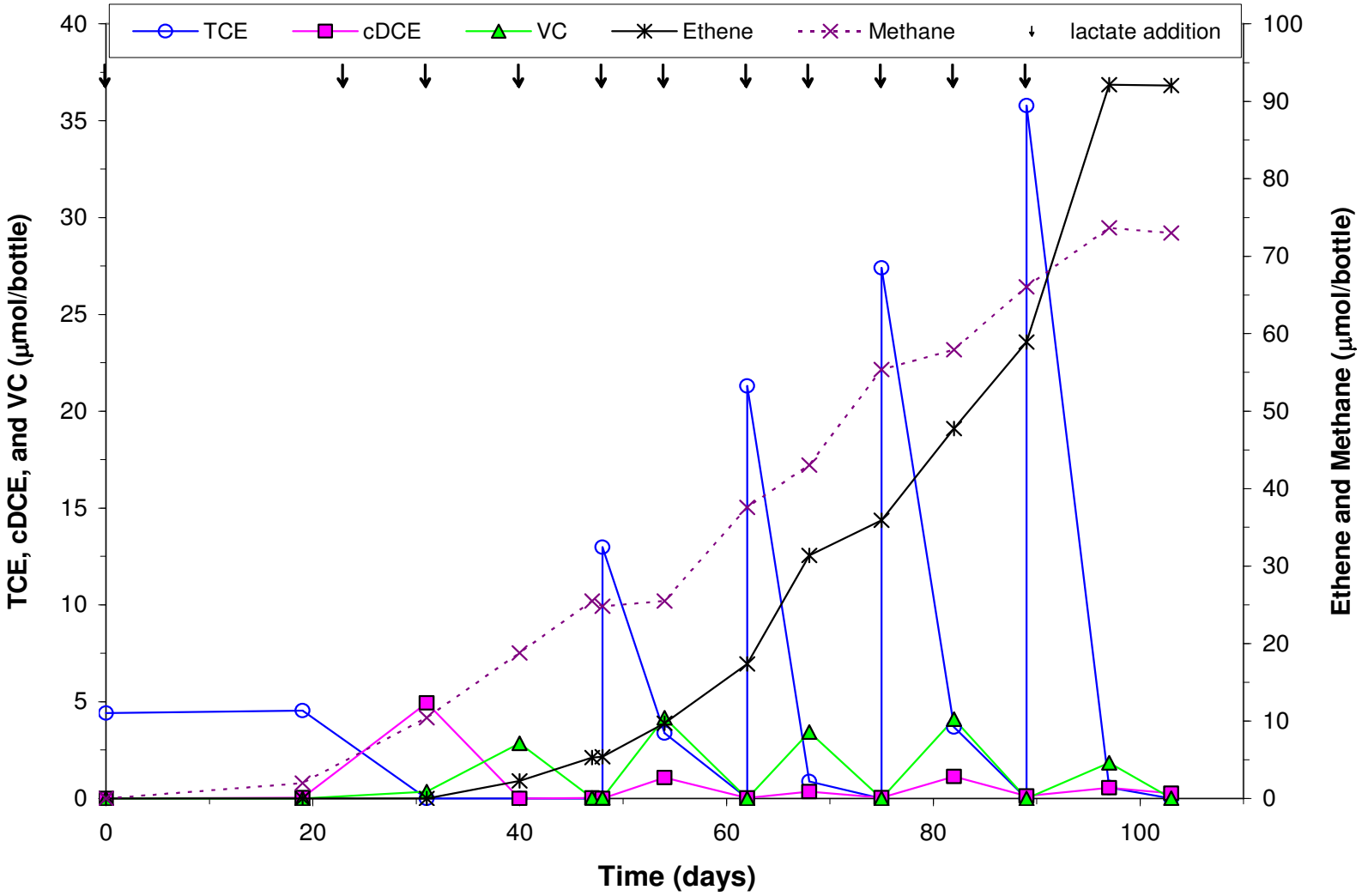

Figure 4.35 Results for one of the Yield Experiment Phase 2 TCE bottles (TCE-2).

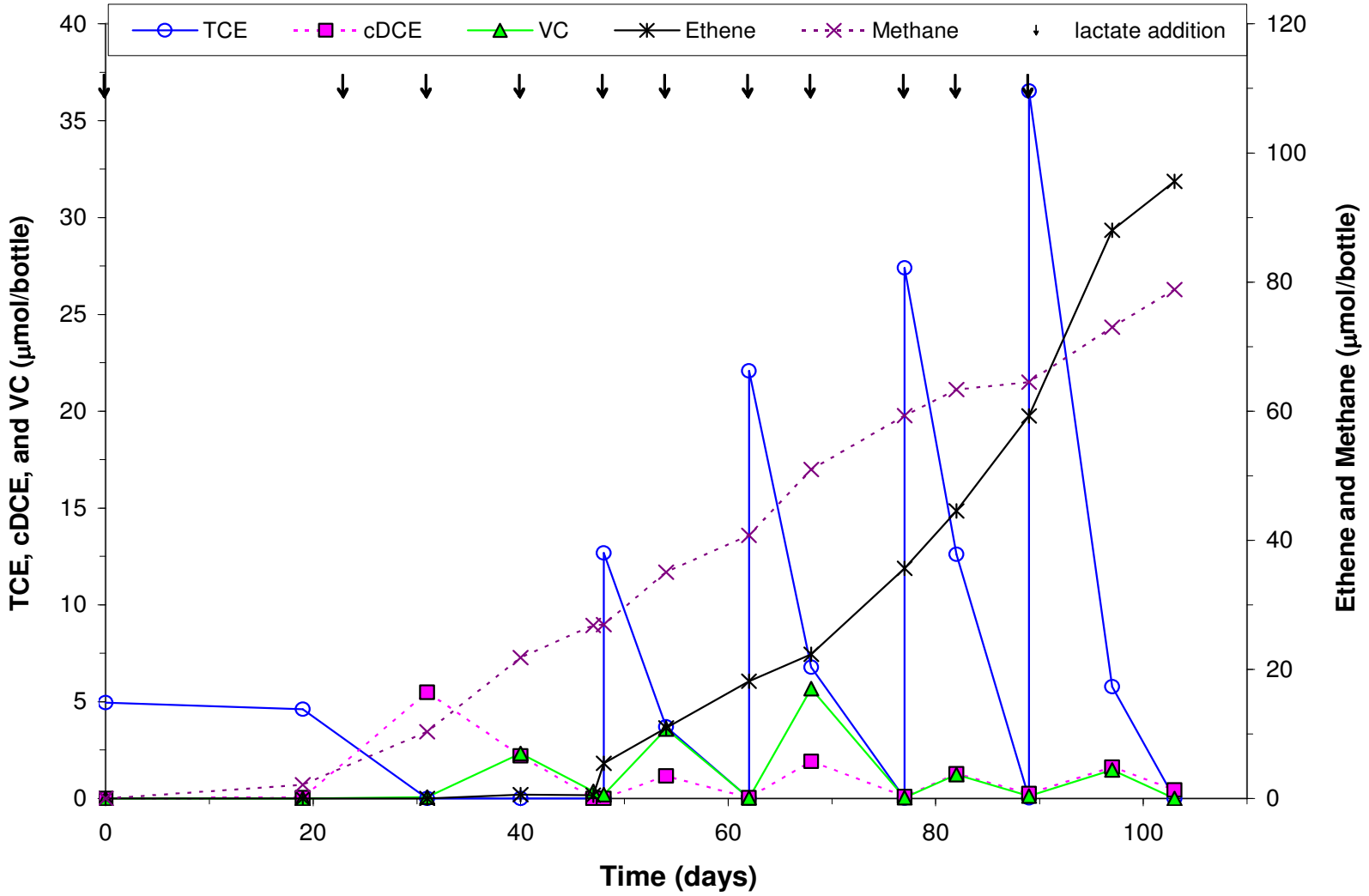

Figure 4.36 Results for one of the Yield Experiment Phase 2 TCE bottles (TCE-3).

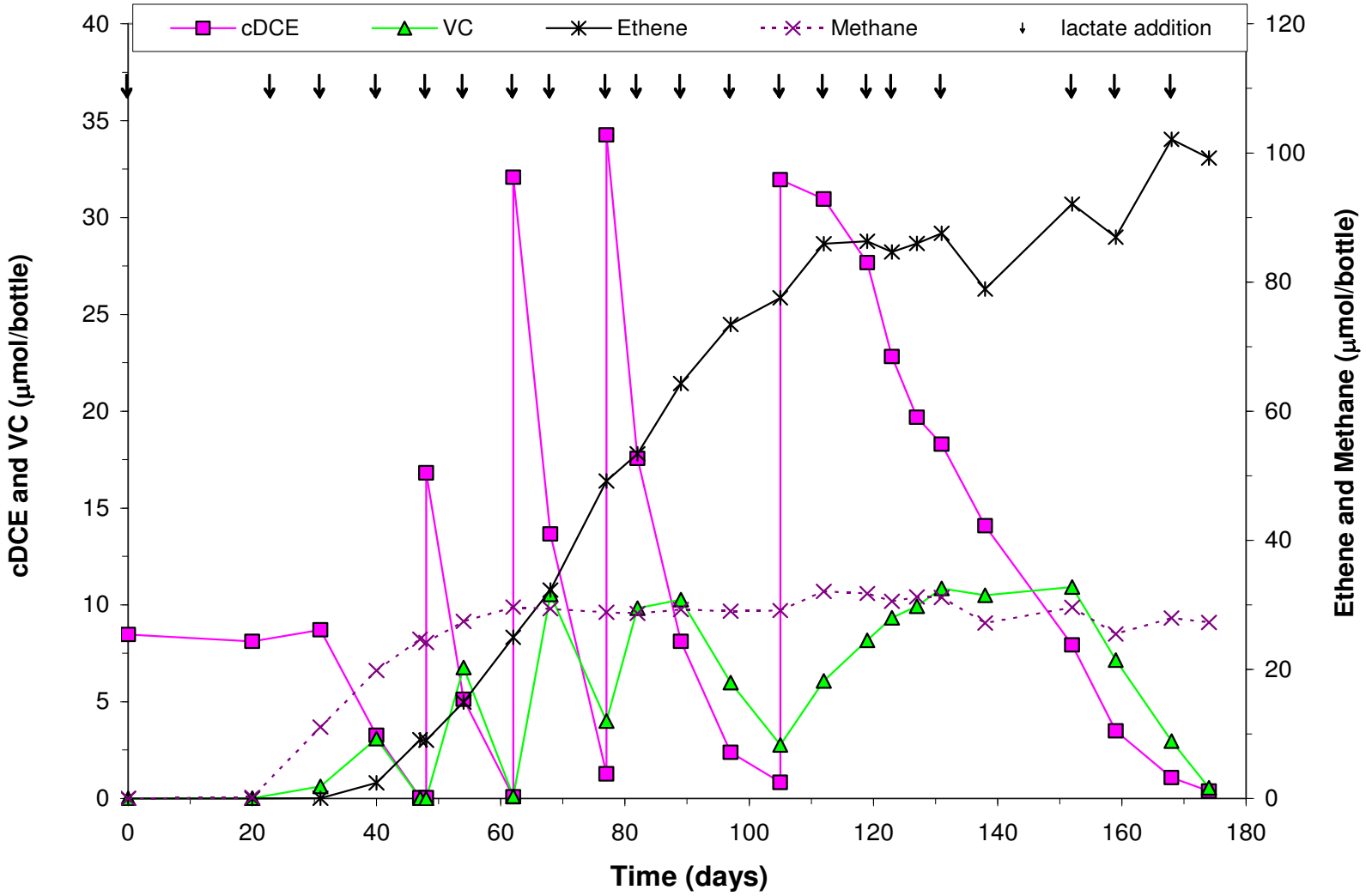

Figure 4.37 Results for one of the Yield Experiment Phase 2 cDCE bottles (cDCE-1).

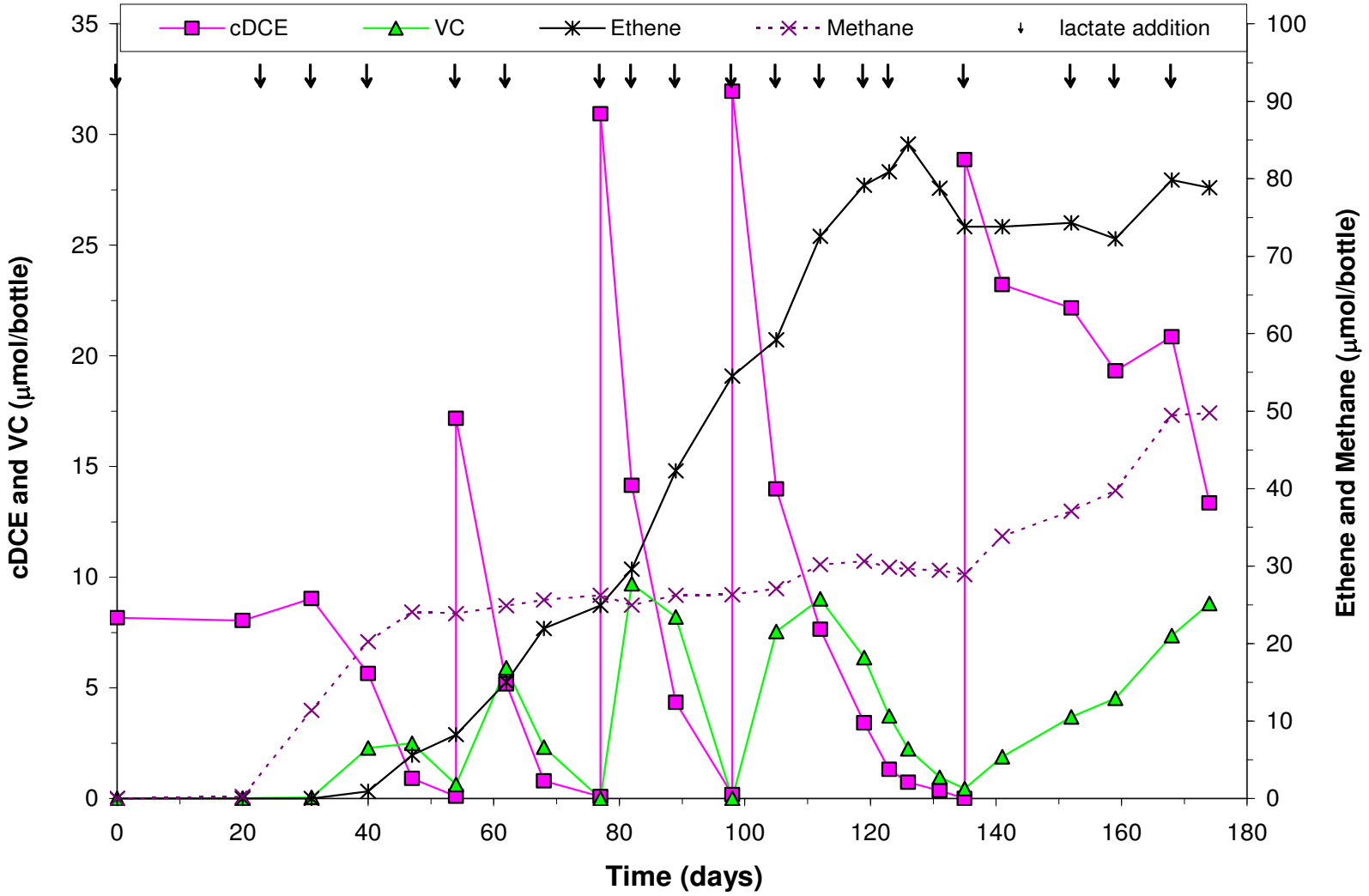

Figure 4.38 Results for one of the Yield Experiment Phase 2 cDCE bottles (cDCE-2).

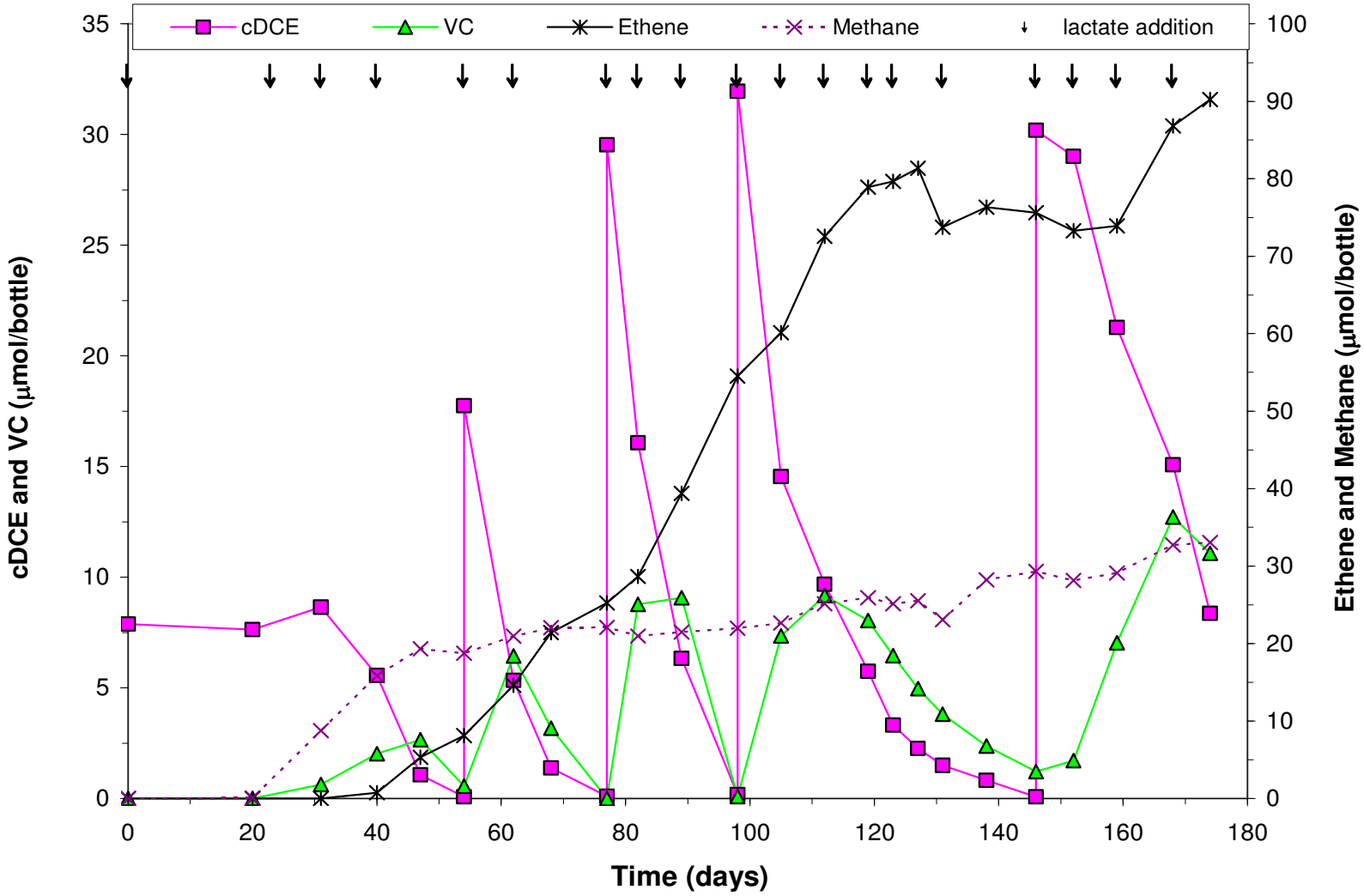

Figure 4.39 Results for one of the Yield Experiment Phase 2 cDCE bottles (cDCE-3).

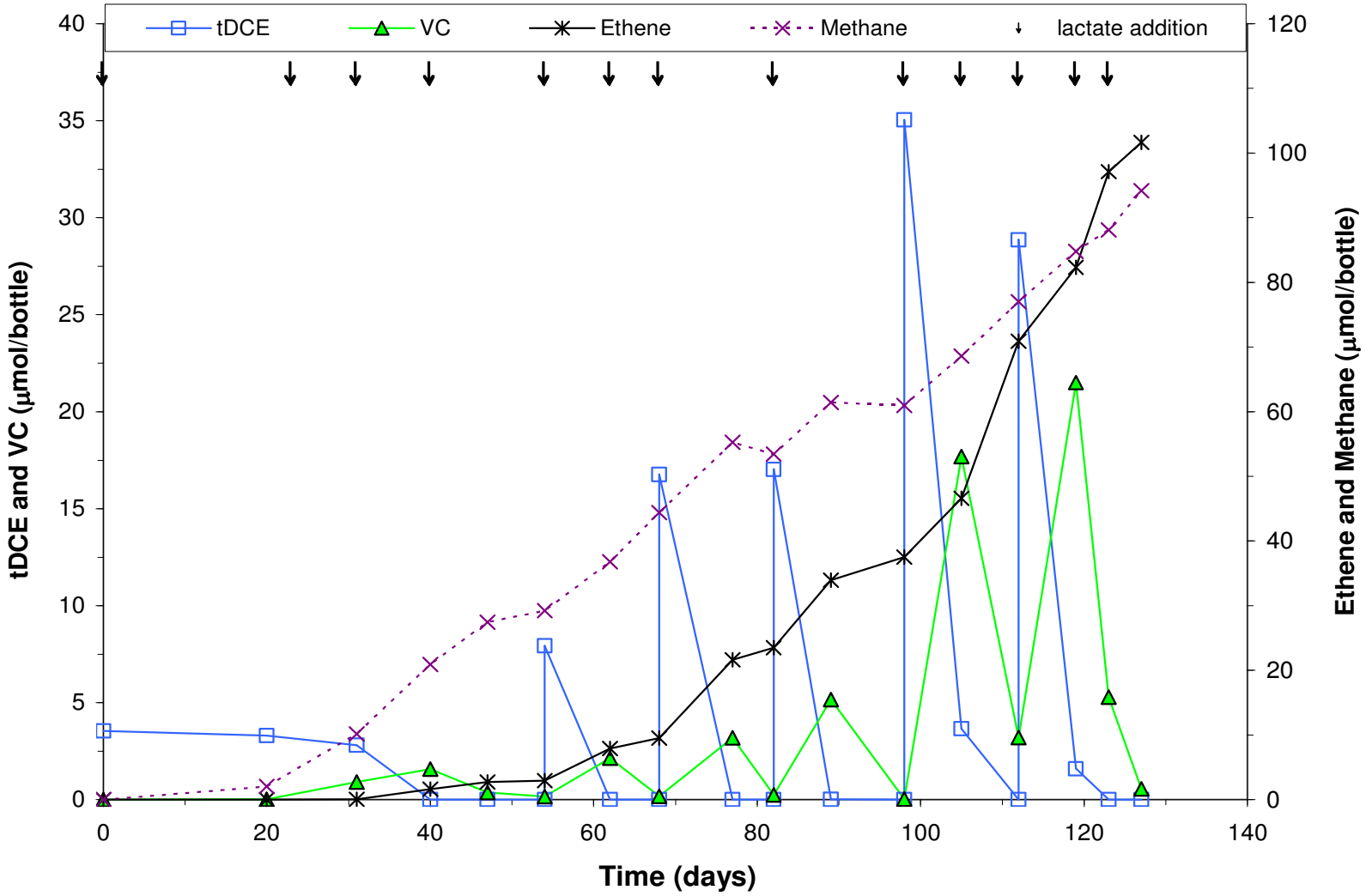

Figure 4.40 Results for one of the Yield Experiment Phase 2 tDCE bottles (tDCE-1).

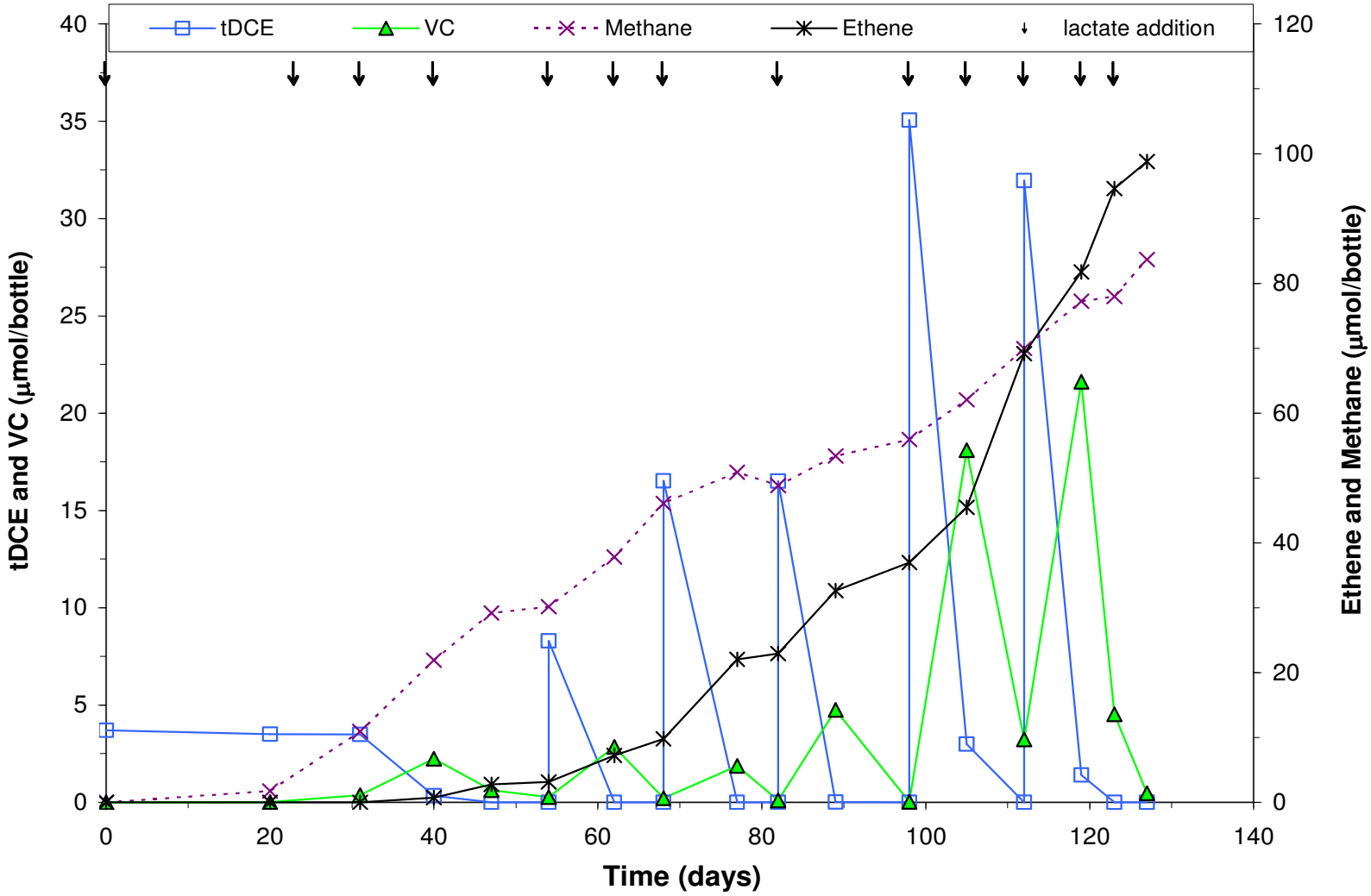

Figure 4.41 Results for one of the Yield Experiment Phase 2 tDCE bottles (tDCE-2).

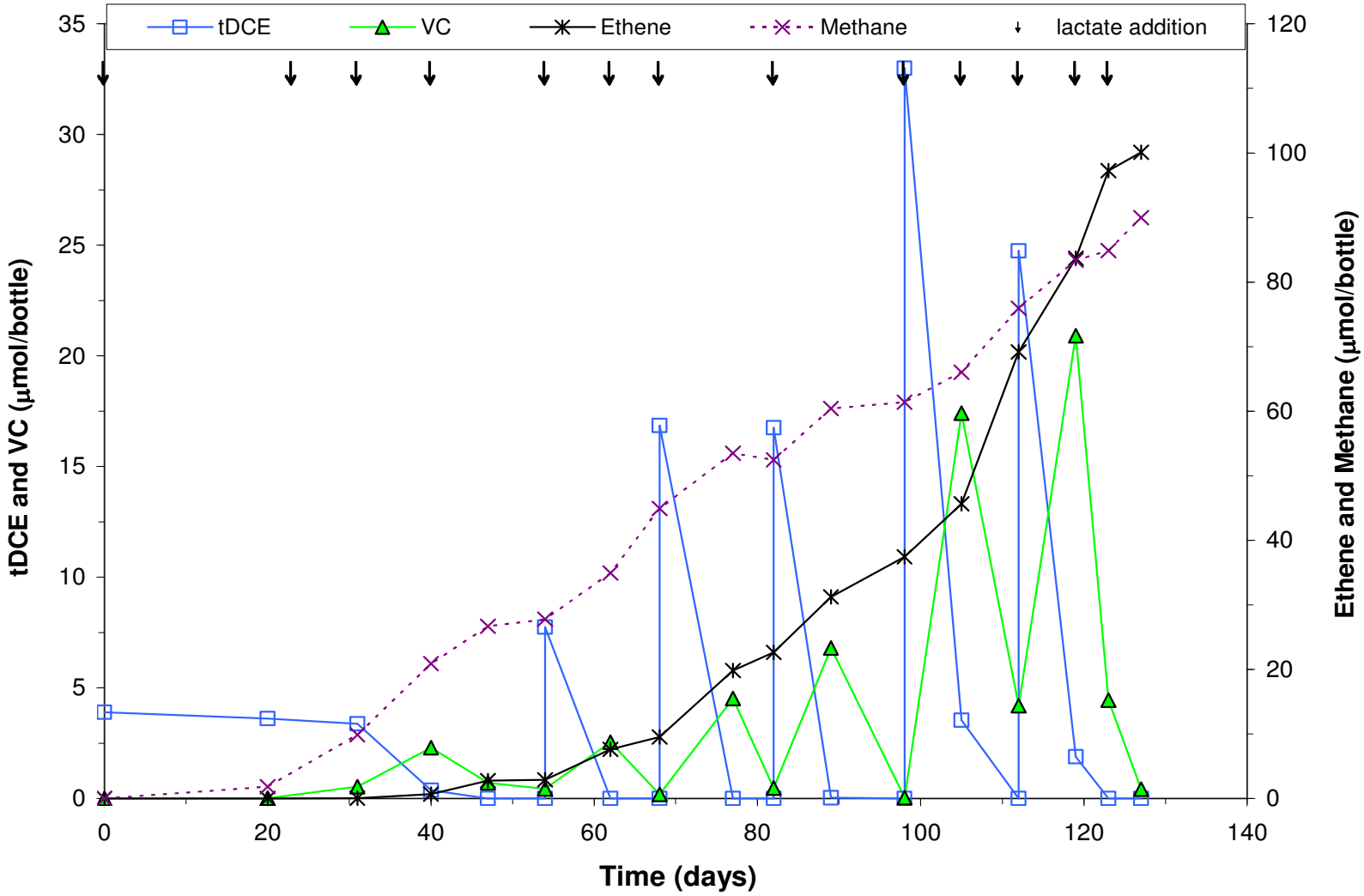

Figure 4.42 Results for one of the Yield Experiment Phase 2 tDCE bottles (tDCE-3).

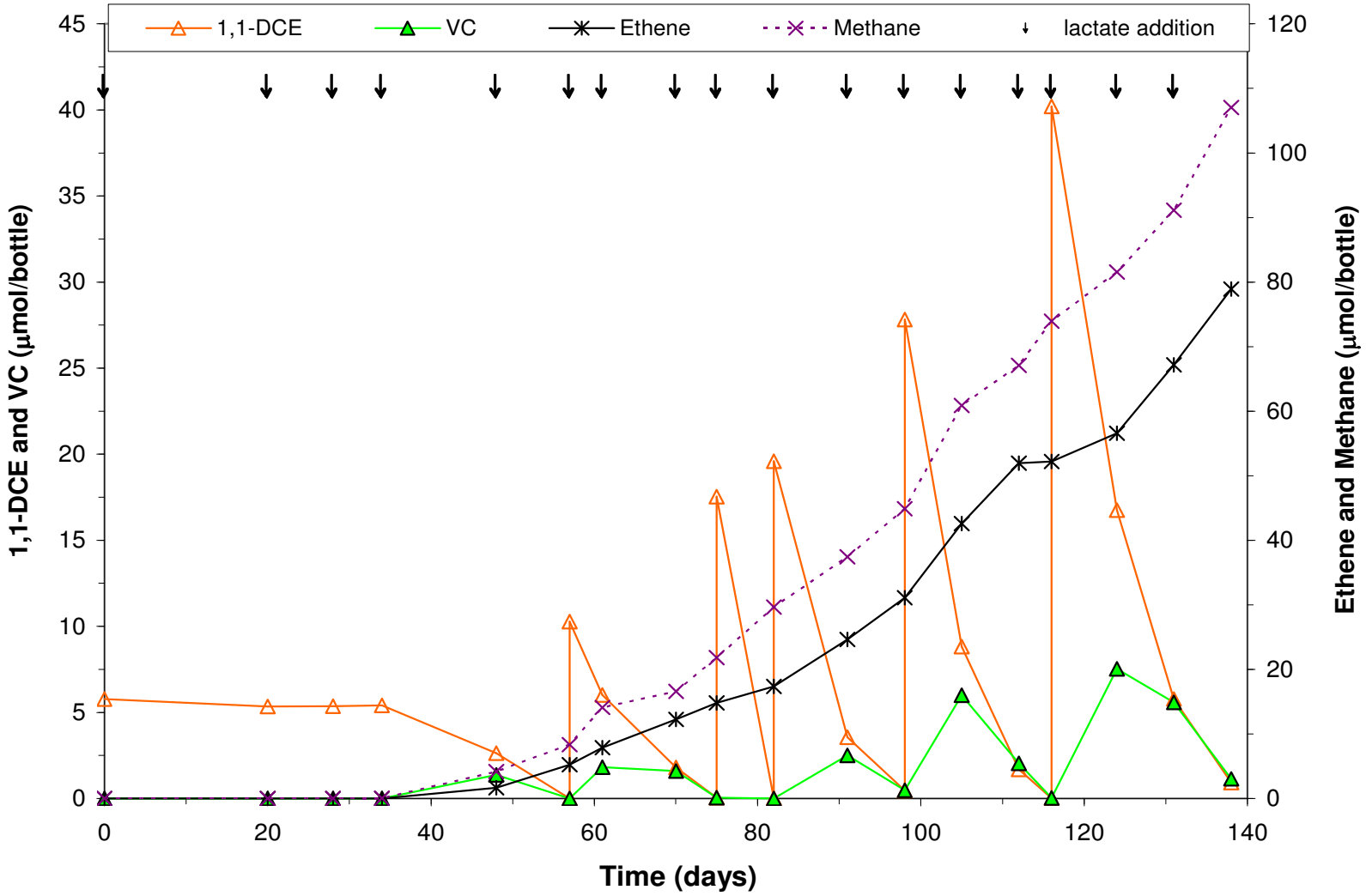

Figure 4.43 Results for one of the Yield Experiment Phase 2 1,1-DCE bottles (1,1-DCE-1).

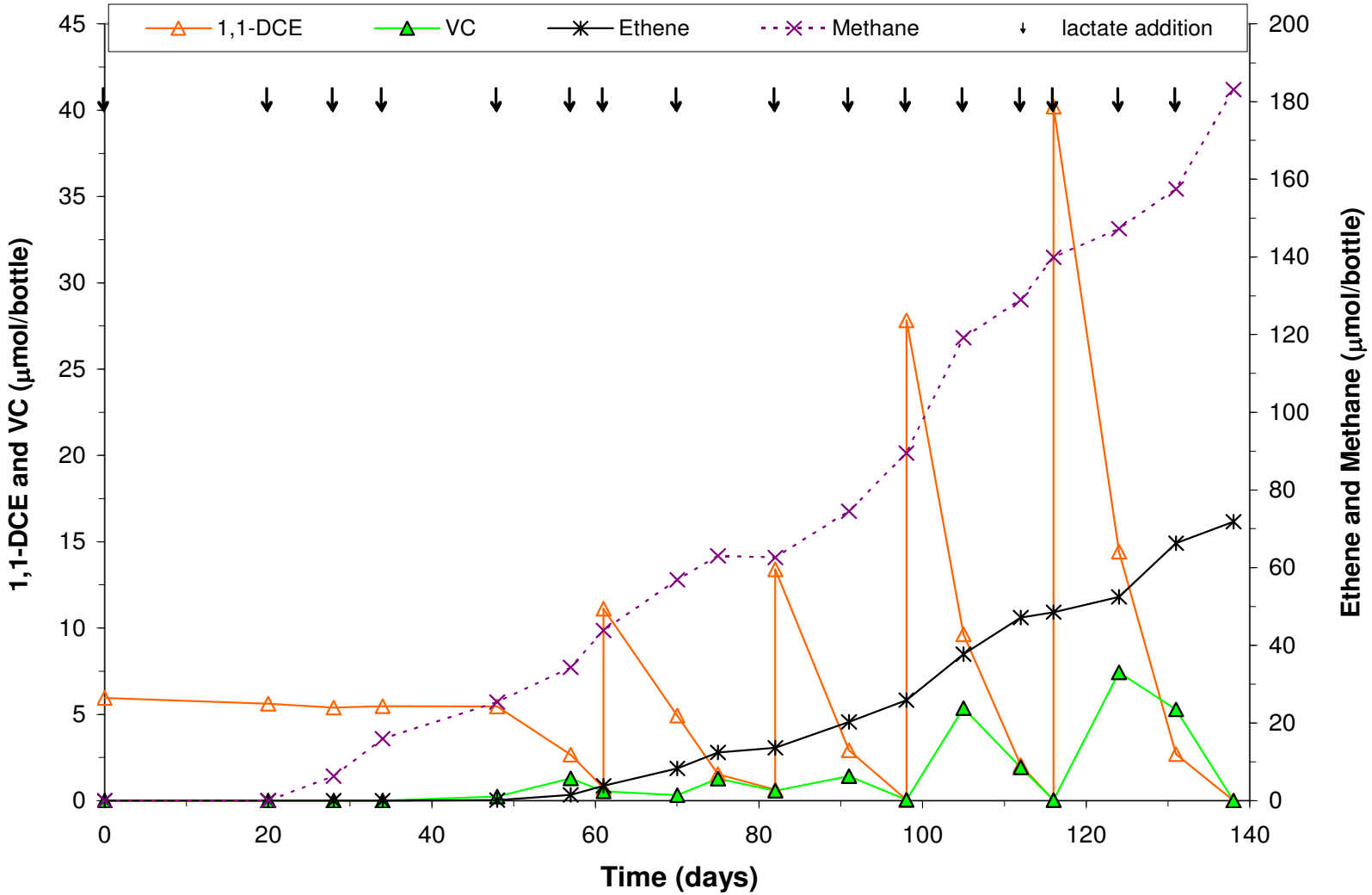

Figure 4.44 Results for one of the Yield Experiment Phase 2 1,1-DCE bottles (1,1-DCE-2).

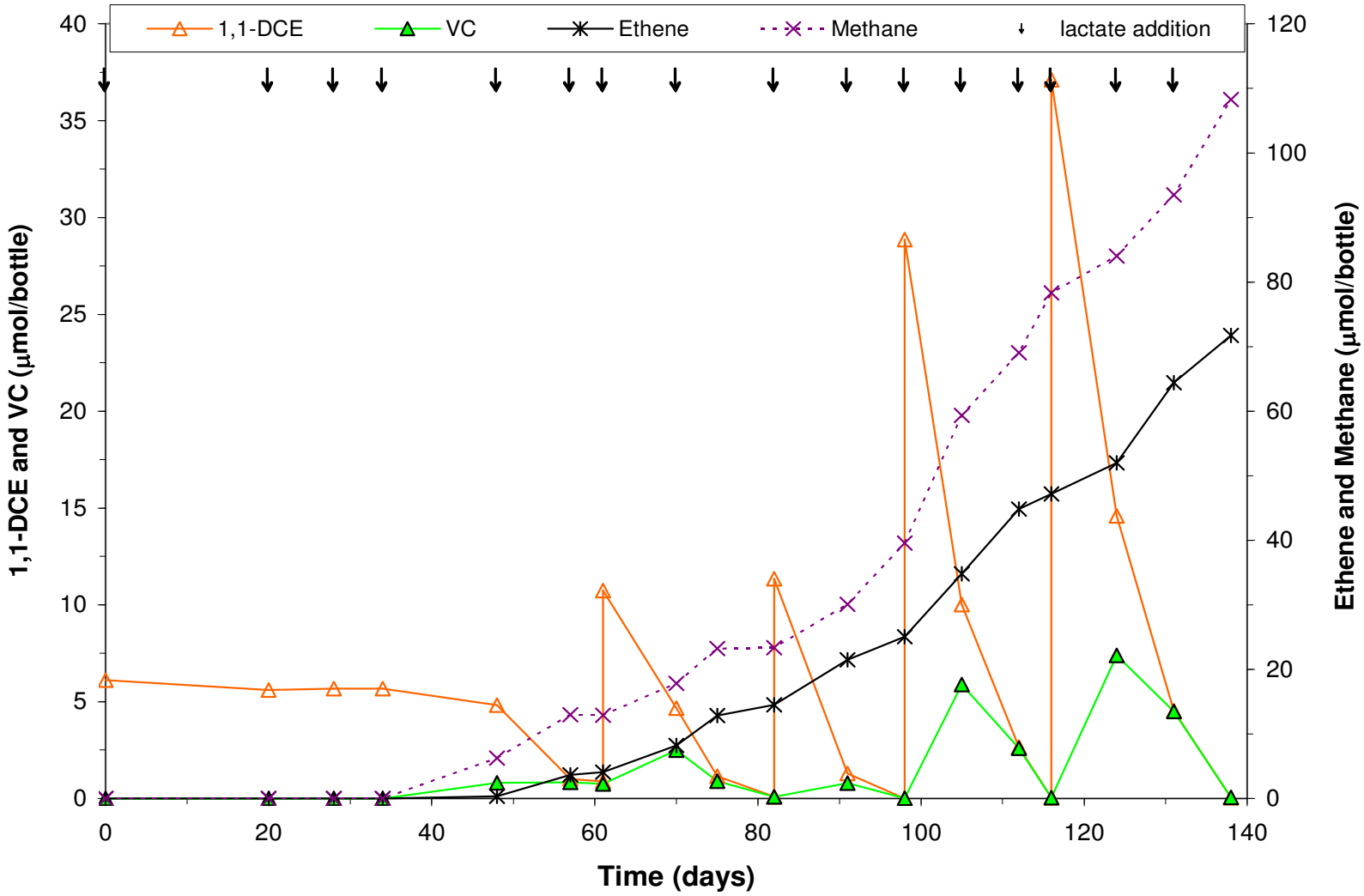

Figure 4.45 Results for one of the Yield Experiment Phase 2 1,1-DCE bottles (1,1-DCE-3).

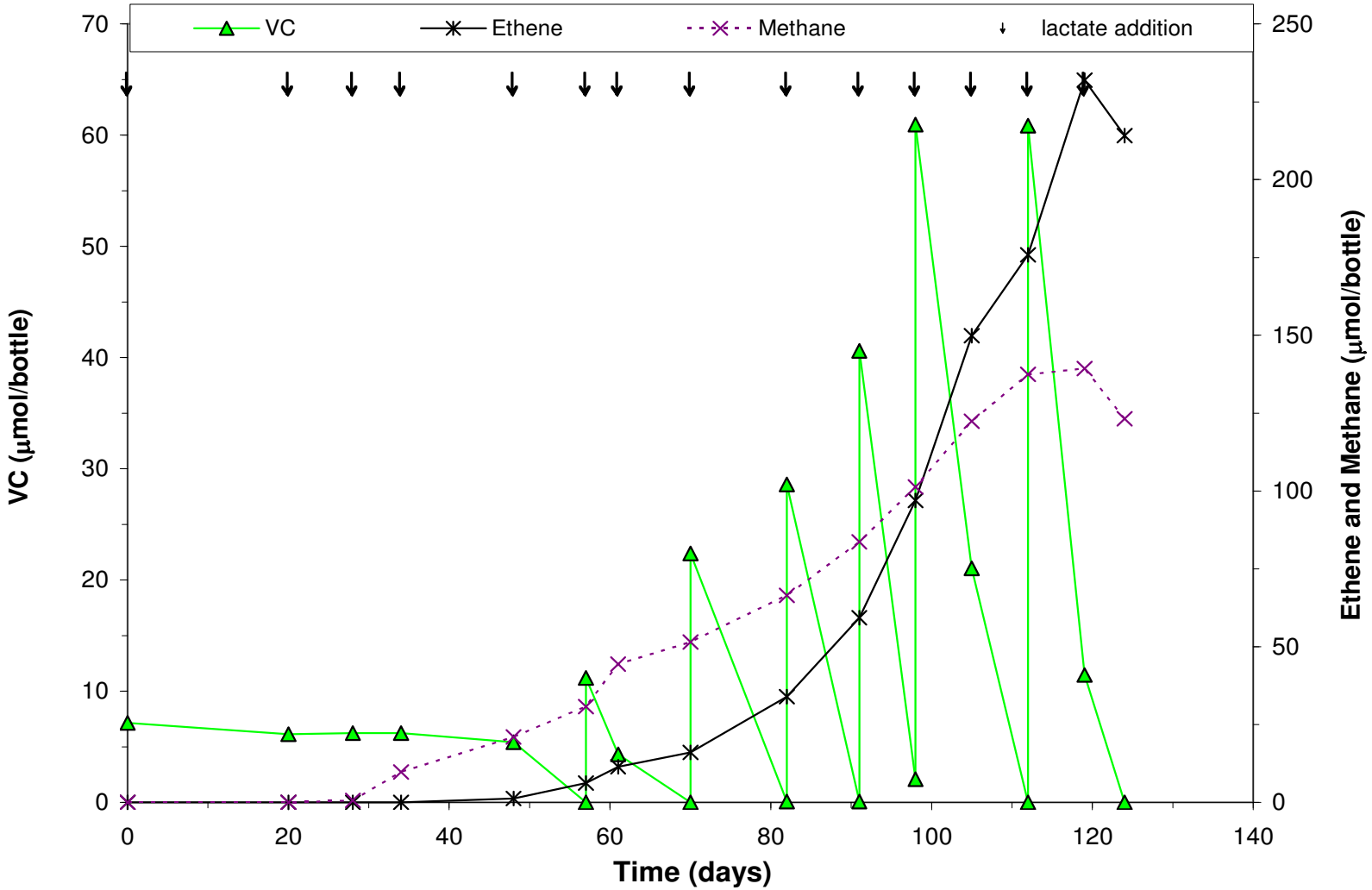

Figure 4.46 Results for one of the Yield Experiment Phase 2 VC bottles (VC-1).

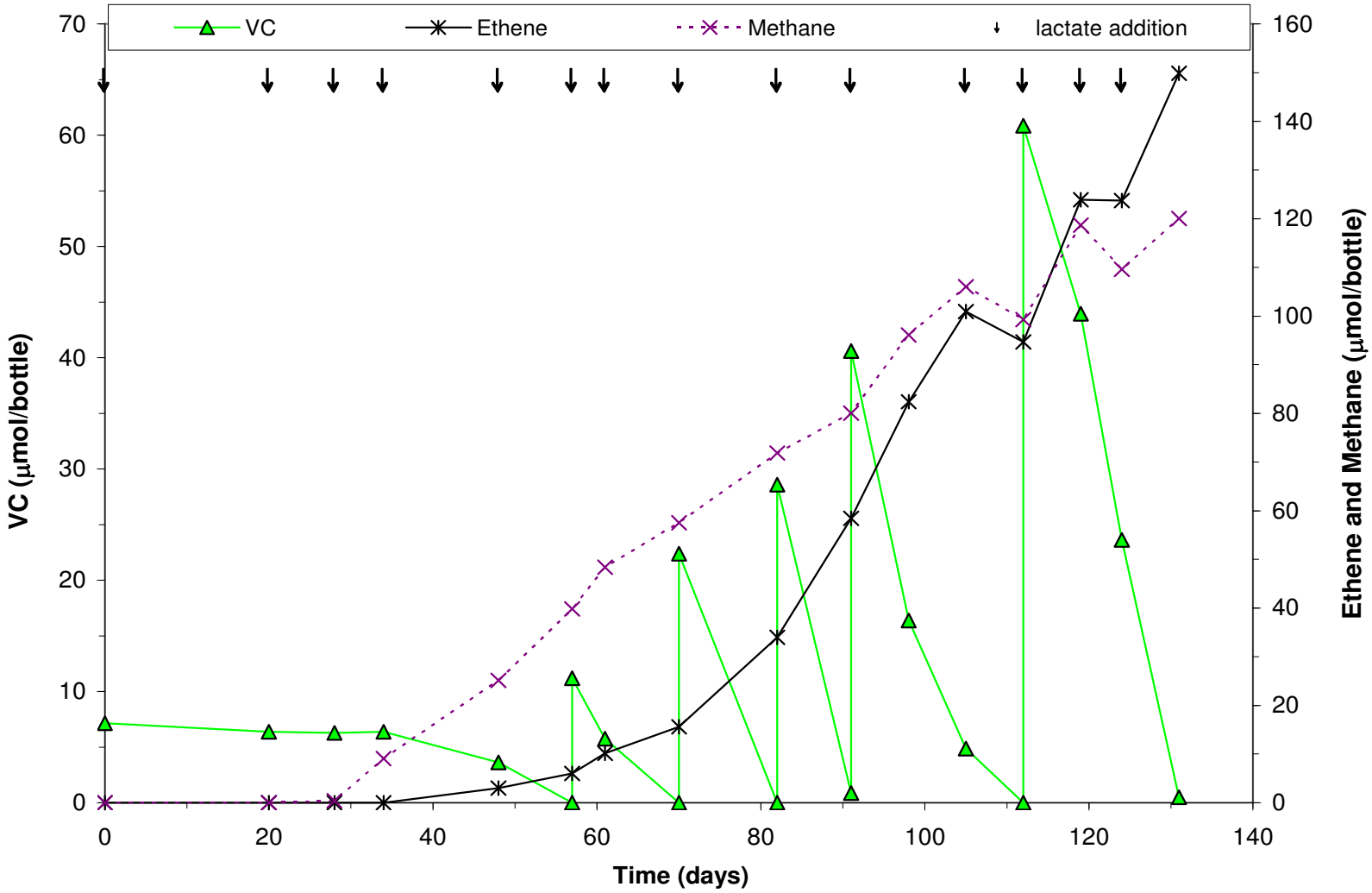

Figure 4.47 Results for one of the Yield Experiment Phase 2 VC bottles (VC-2).

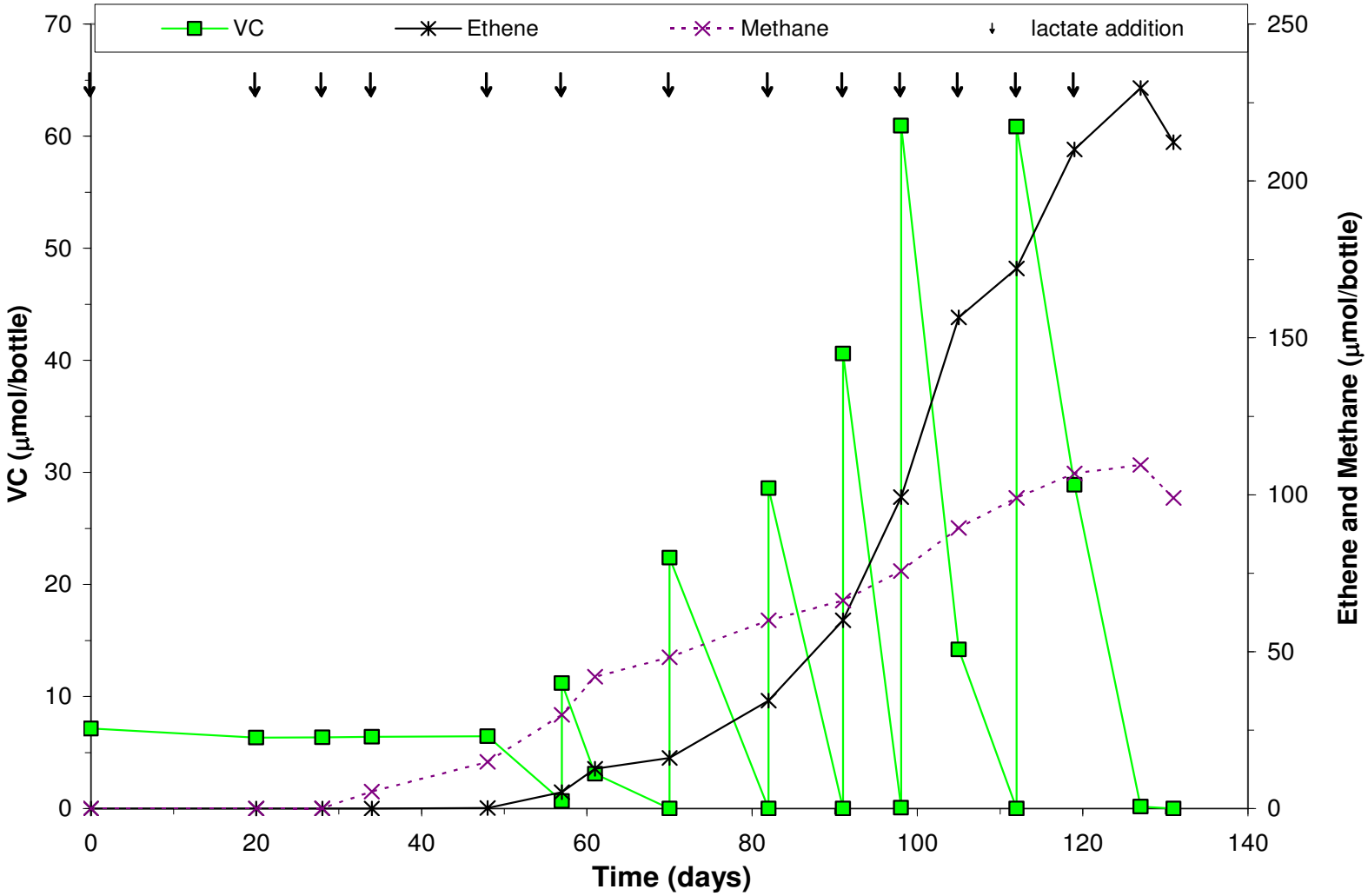

Figure 4.48 Results for one of the Yield Experiment Phase 2 VC bottles (VC-3).

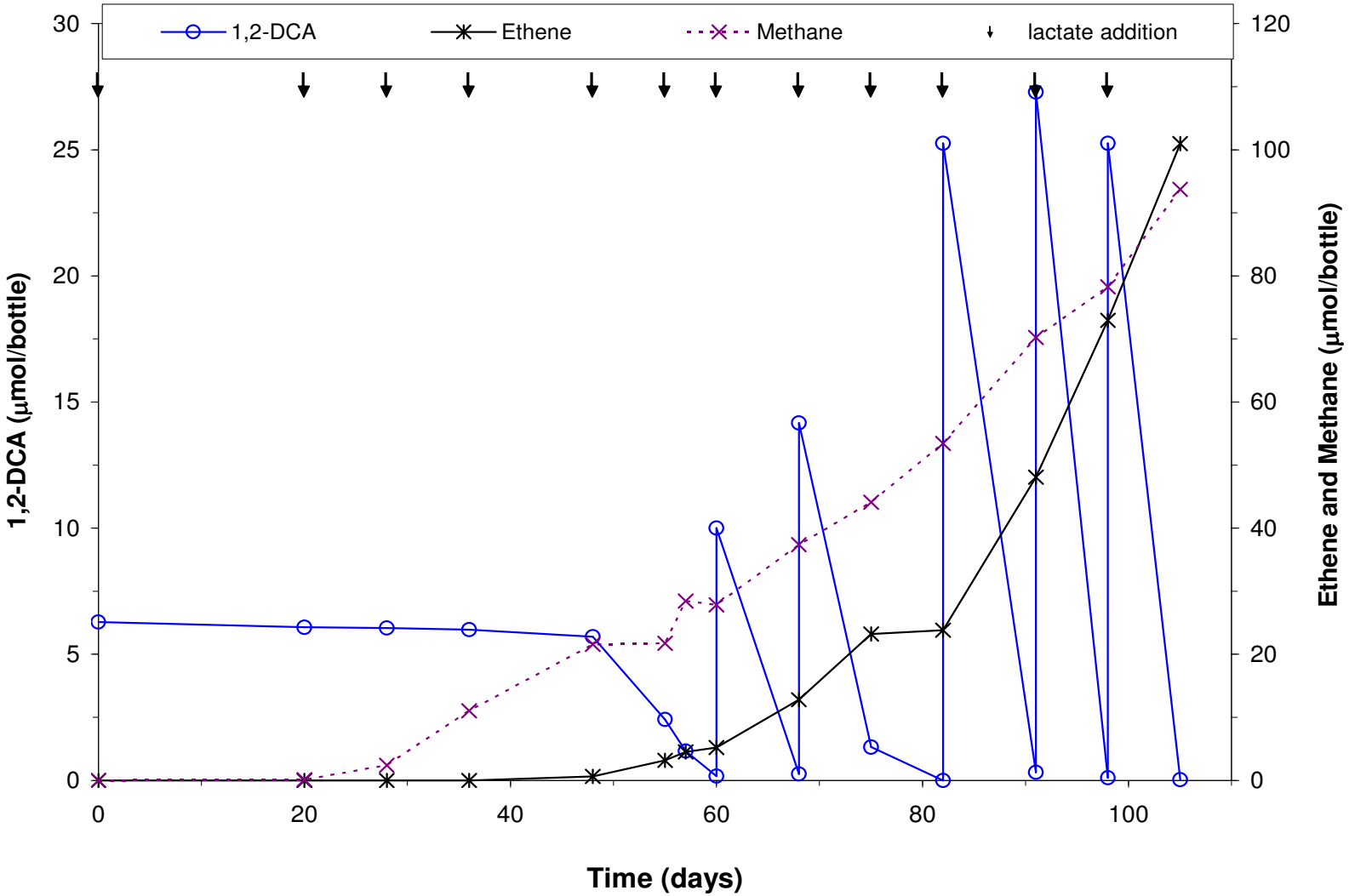

Figure 4.49 Results for one of the Yield Experiment Phase 2 1,2-DCA bottles (1,2-DCA-1).

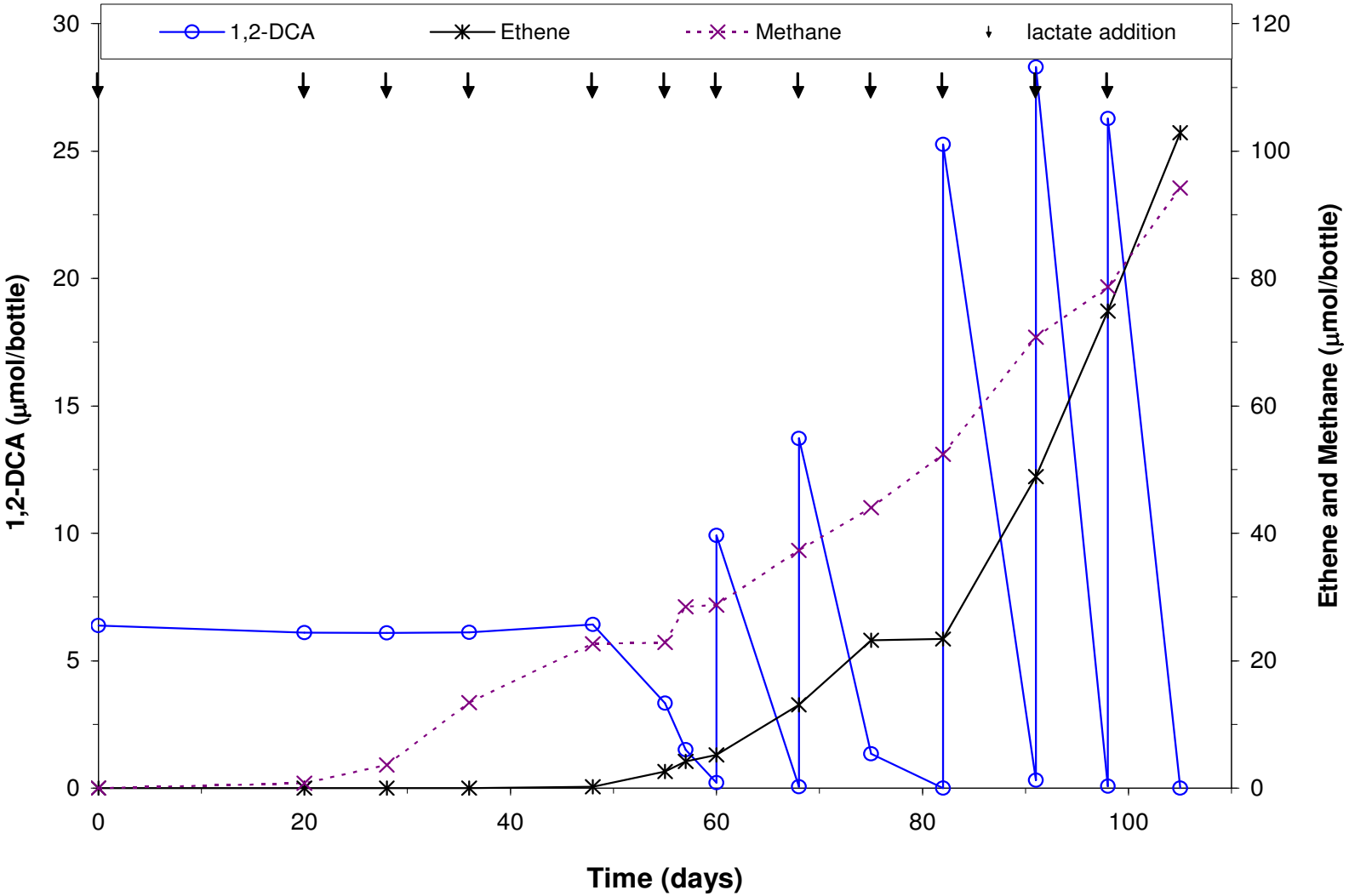

Figure 4.50 Results for one of the Yield Experiment Phase 2 1,2-DCA bottles (1,2-DCA-2).

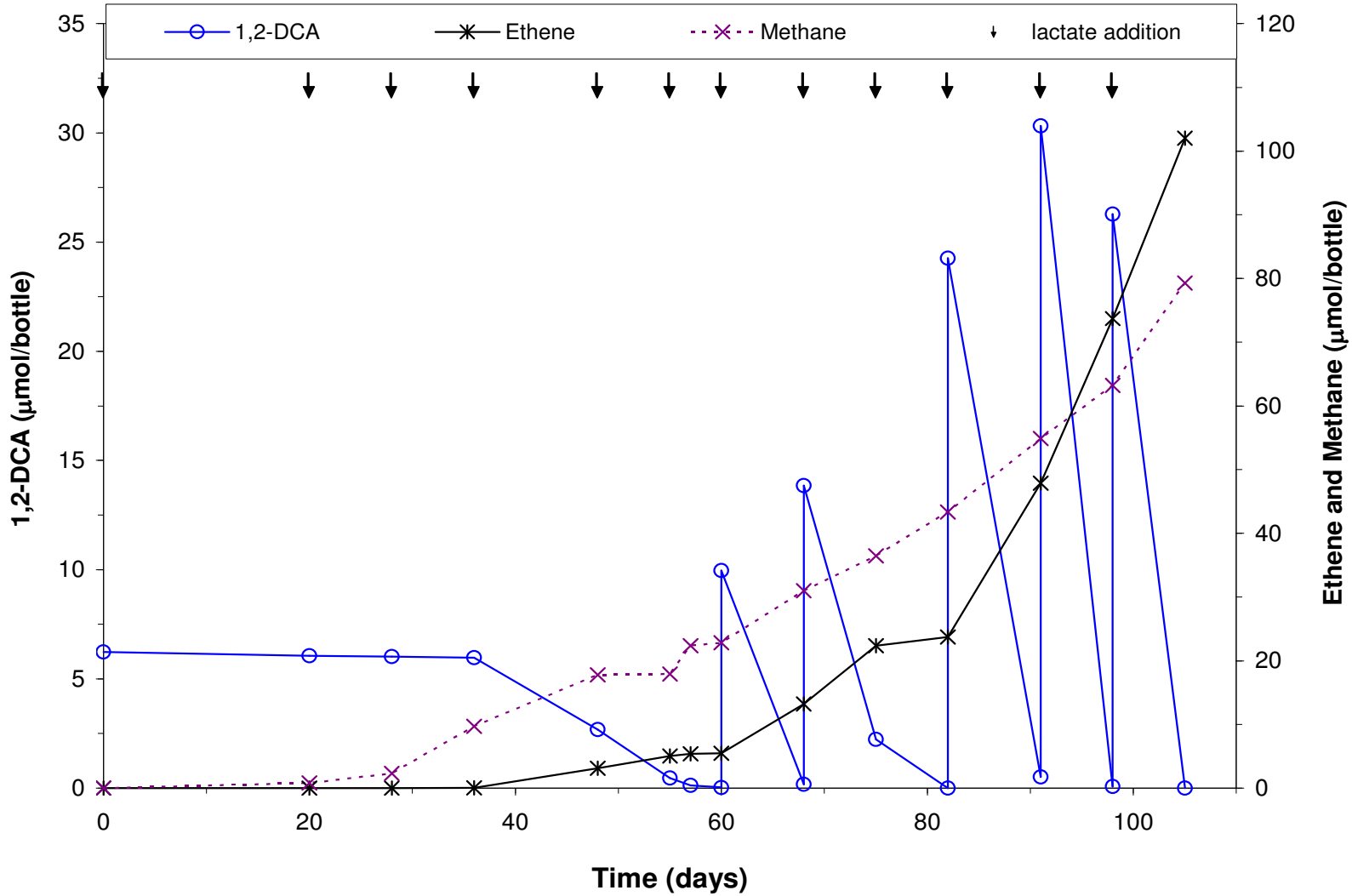

Figure 4.51 Results for one of the Yield Experiment Phase 2 1,2-DCA bottles (1,2-DCA-3).

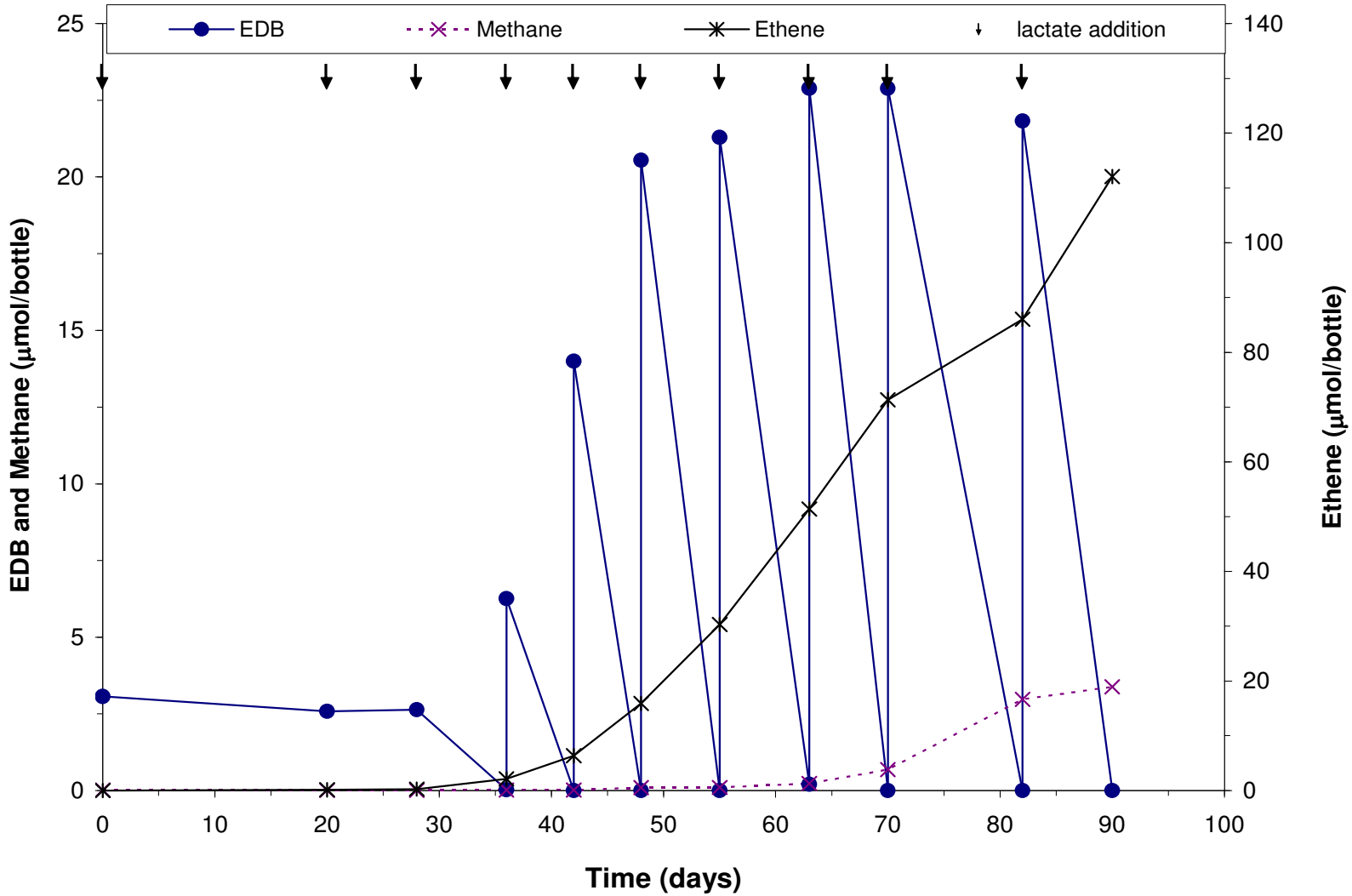

Figure 4.52 Results for one of the Yield Experiment Phase 2 EDB bottles (EDB-1).

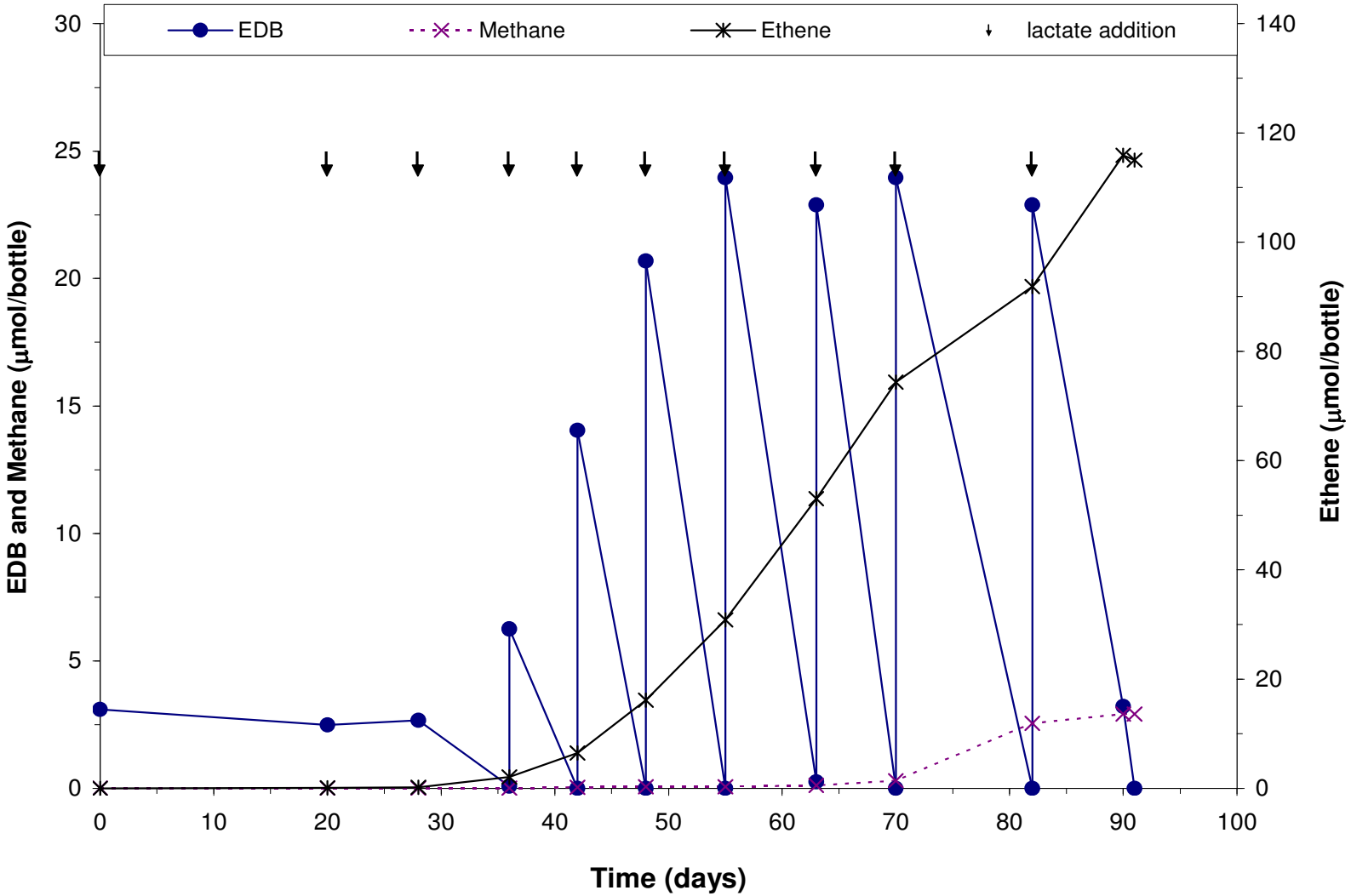

Figure 4.53 Results for one of the Yield Experiment Phase 2 EDB bottles (EDB-2).

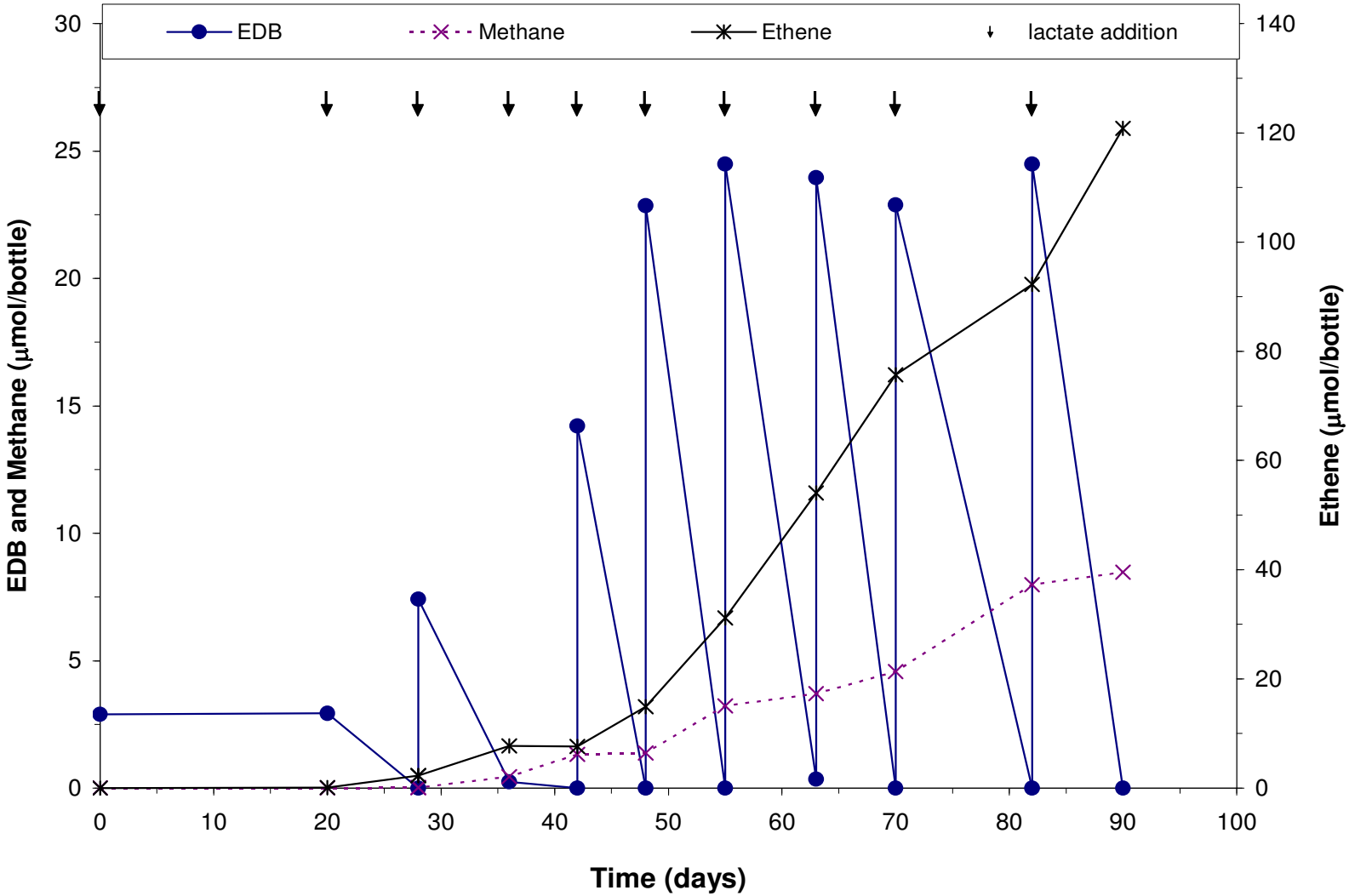

Figure 4.54 Results for one of the Yield Experiment Phase 2 EDB bottles (EDB-3).

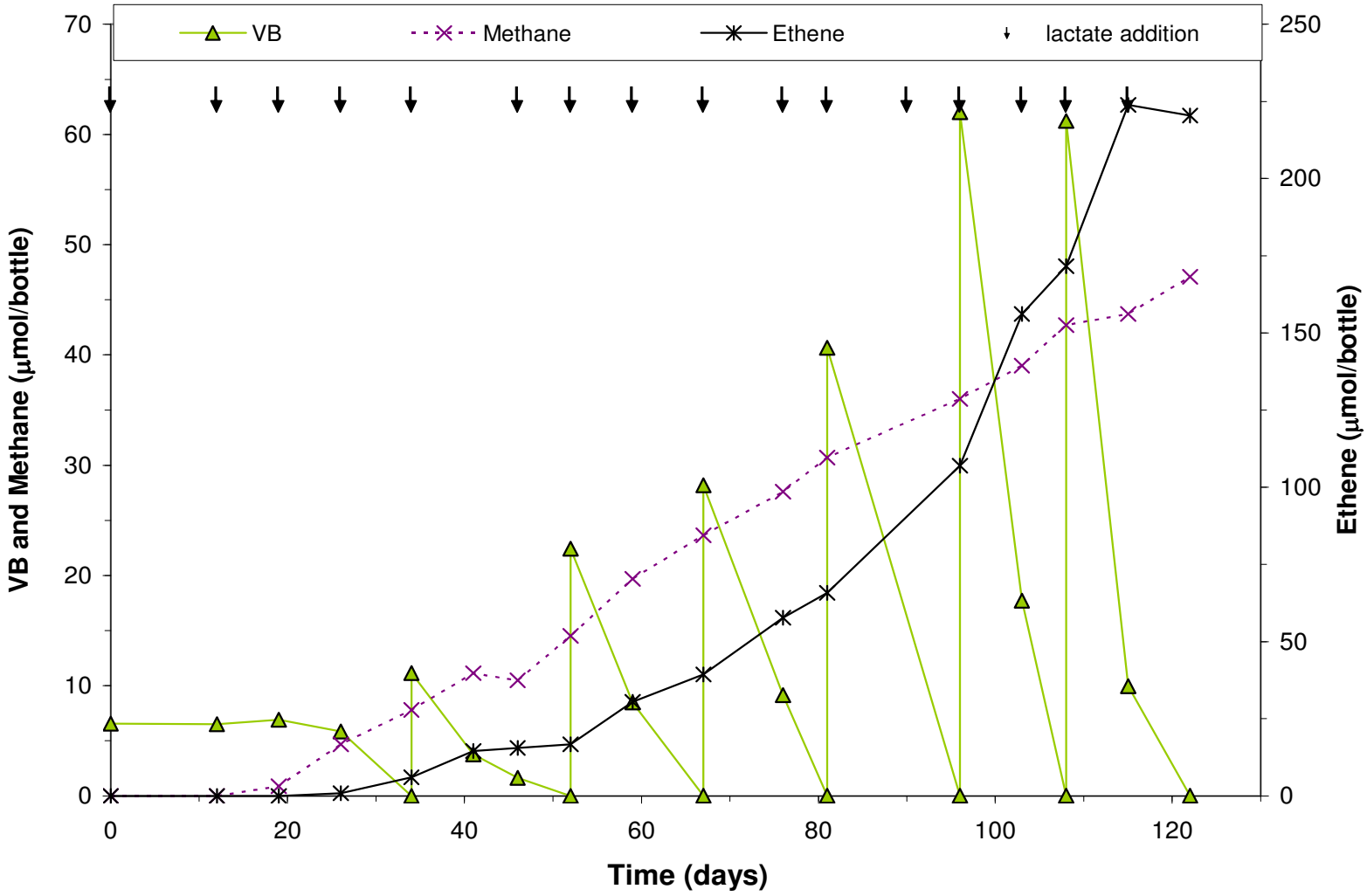

Figure 4.55 Results for one of the Yield Experiment Phase 2 VB bottles (VB-1).

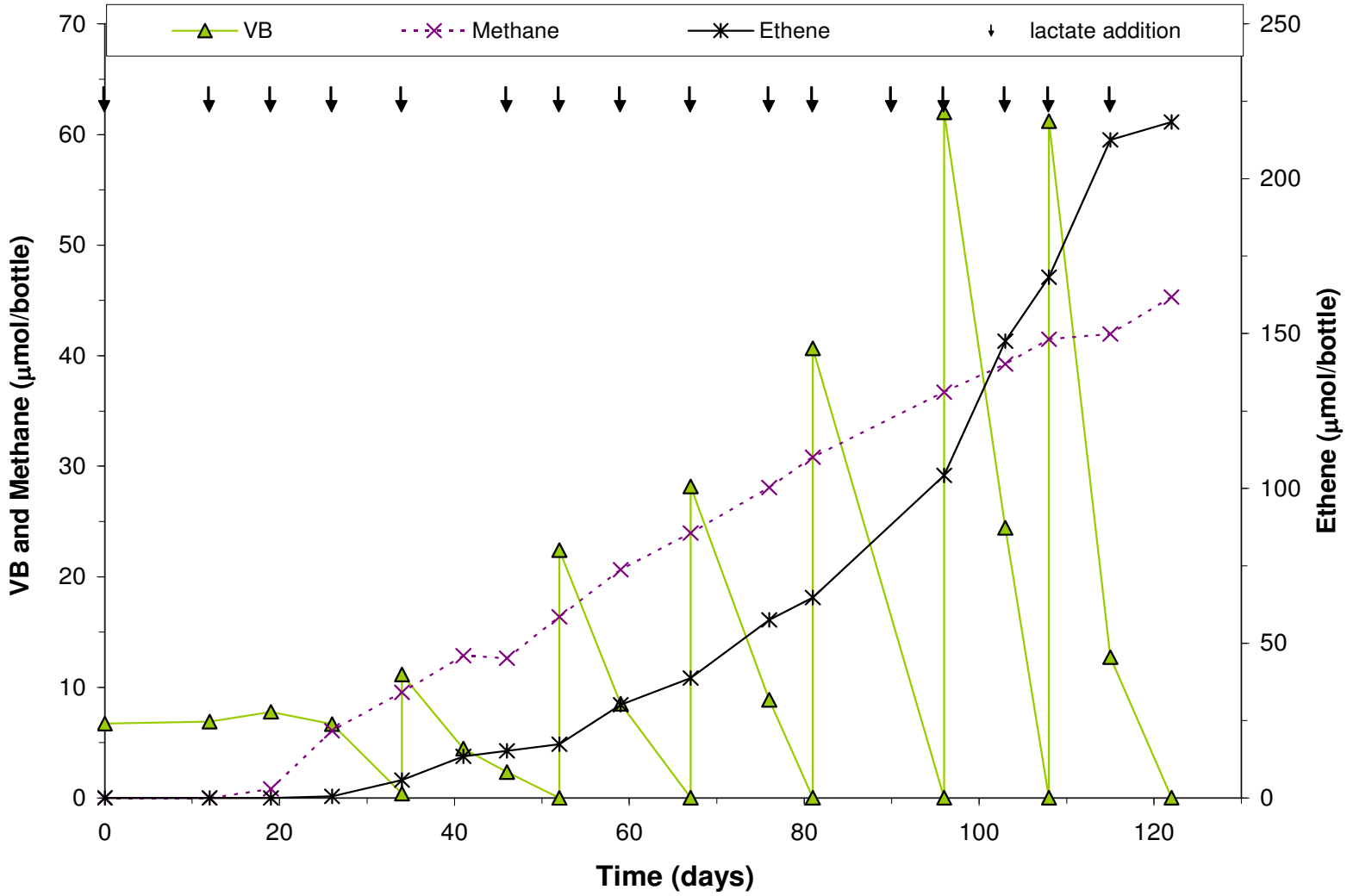

Figure 4.56 Results for one of the Yield Experiment Phase 2 VB bottles (VB-2).

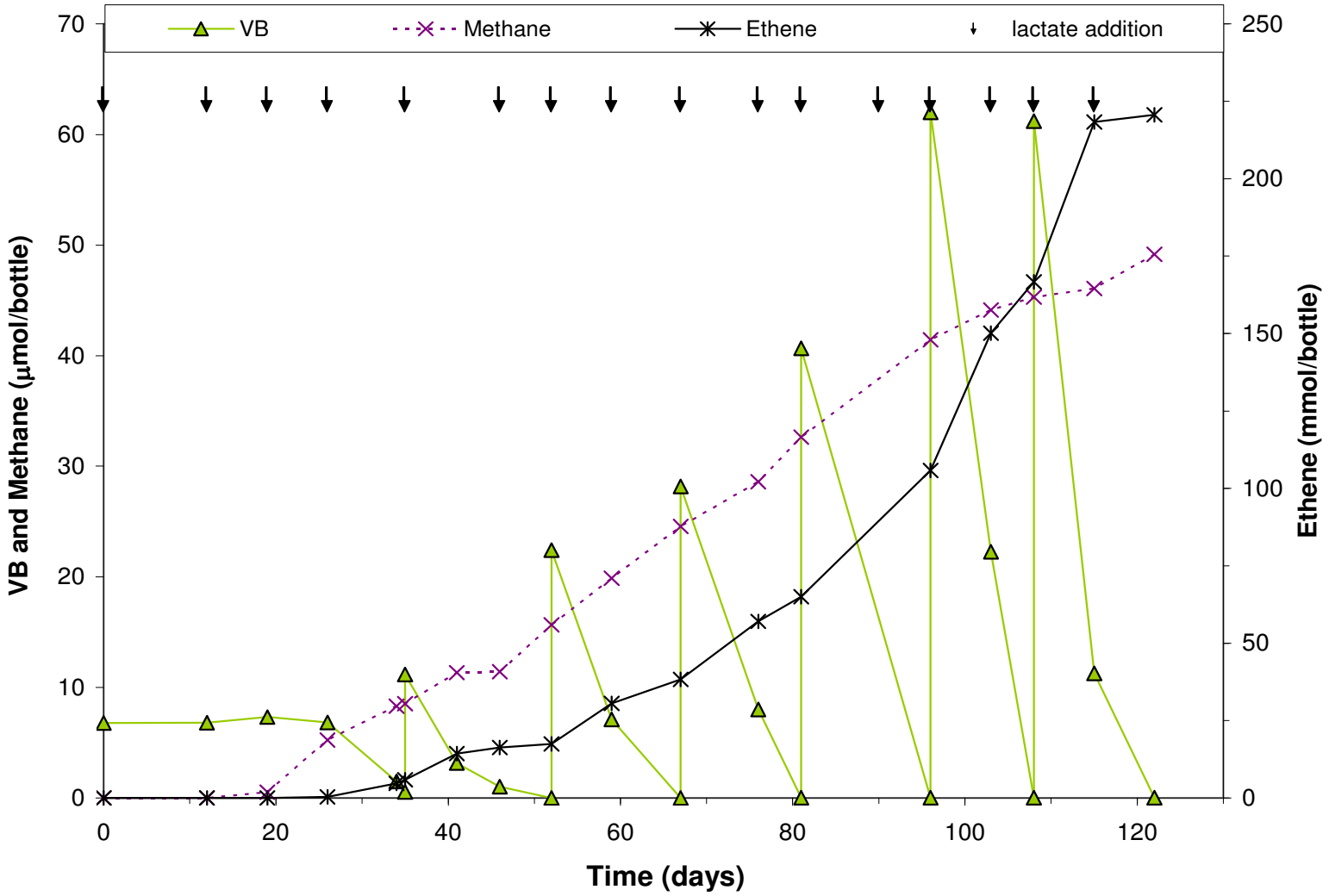

Figure 4.57 Results for one of the Yield Experiment Phase 2 VB bottles (VB-3).

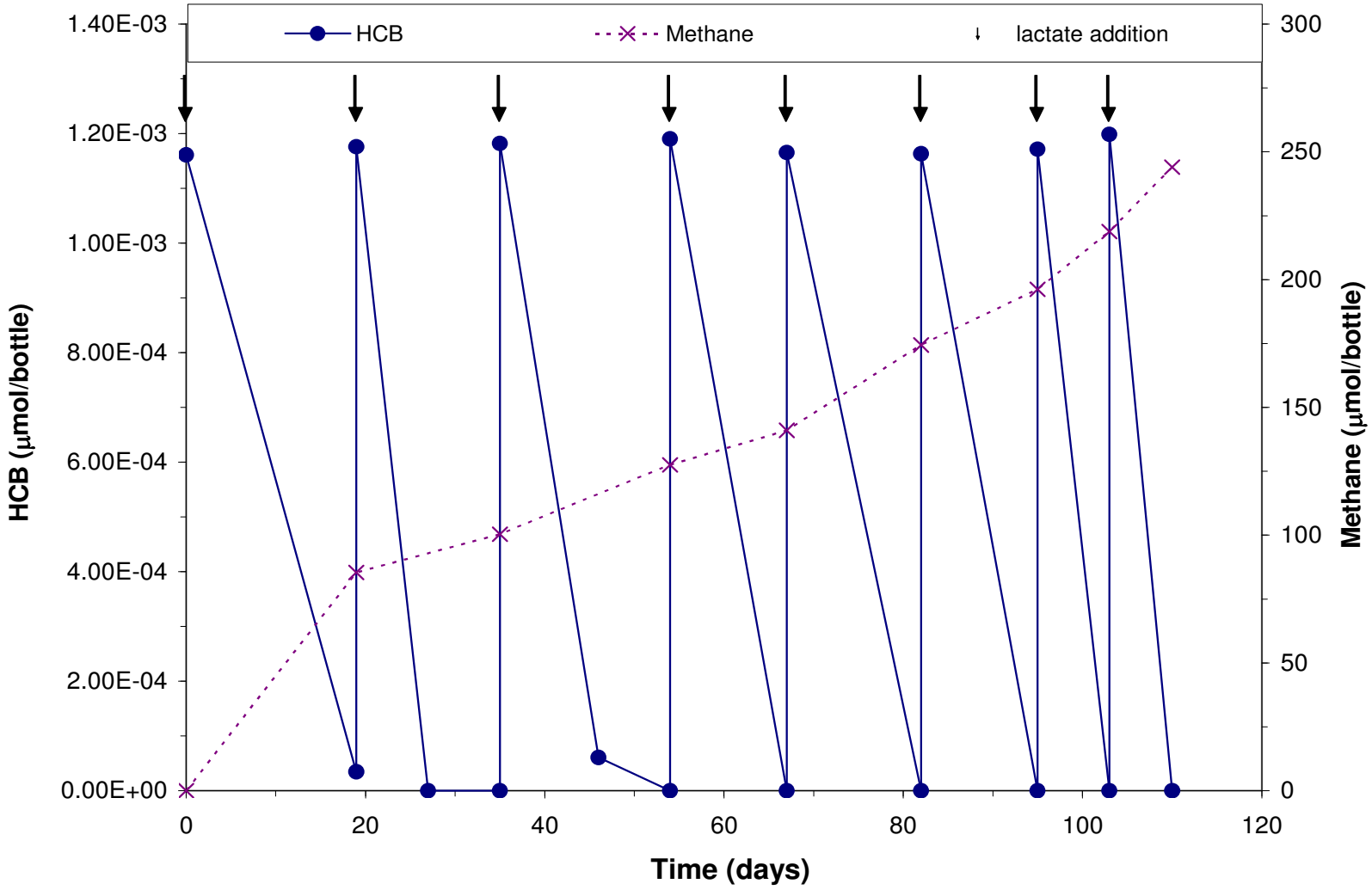

Figure 4.58a Results for one of the TEA Experiment HCB bottles (HCB-1); data for HCB and methane.

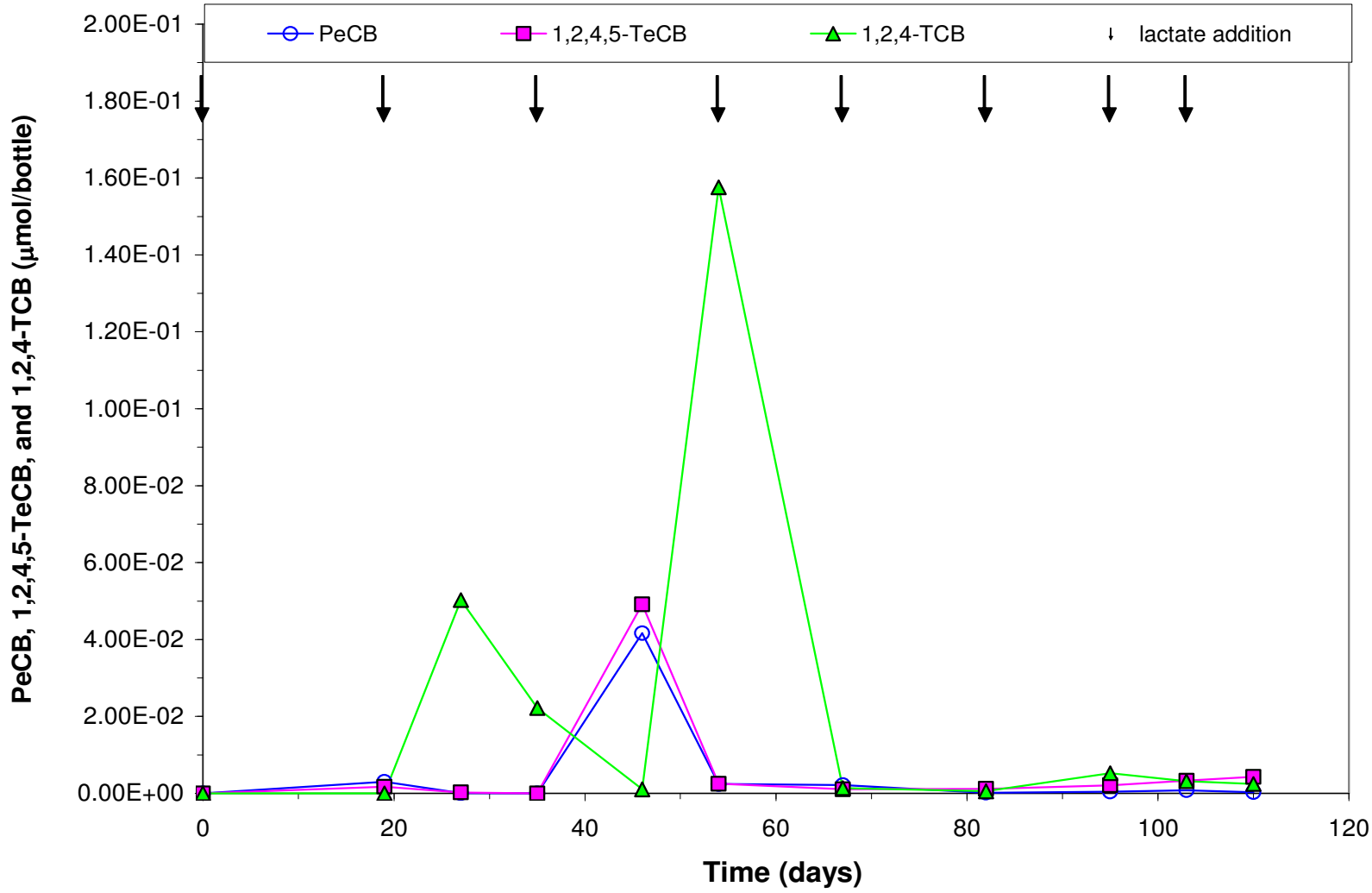

Figure 4.58b Results for one of the TEA Experiment HCB bottles (HCB-1); data for PeCB, 1,2,4,5-TeCB, and 1,2,4-TCB.

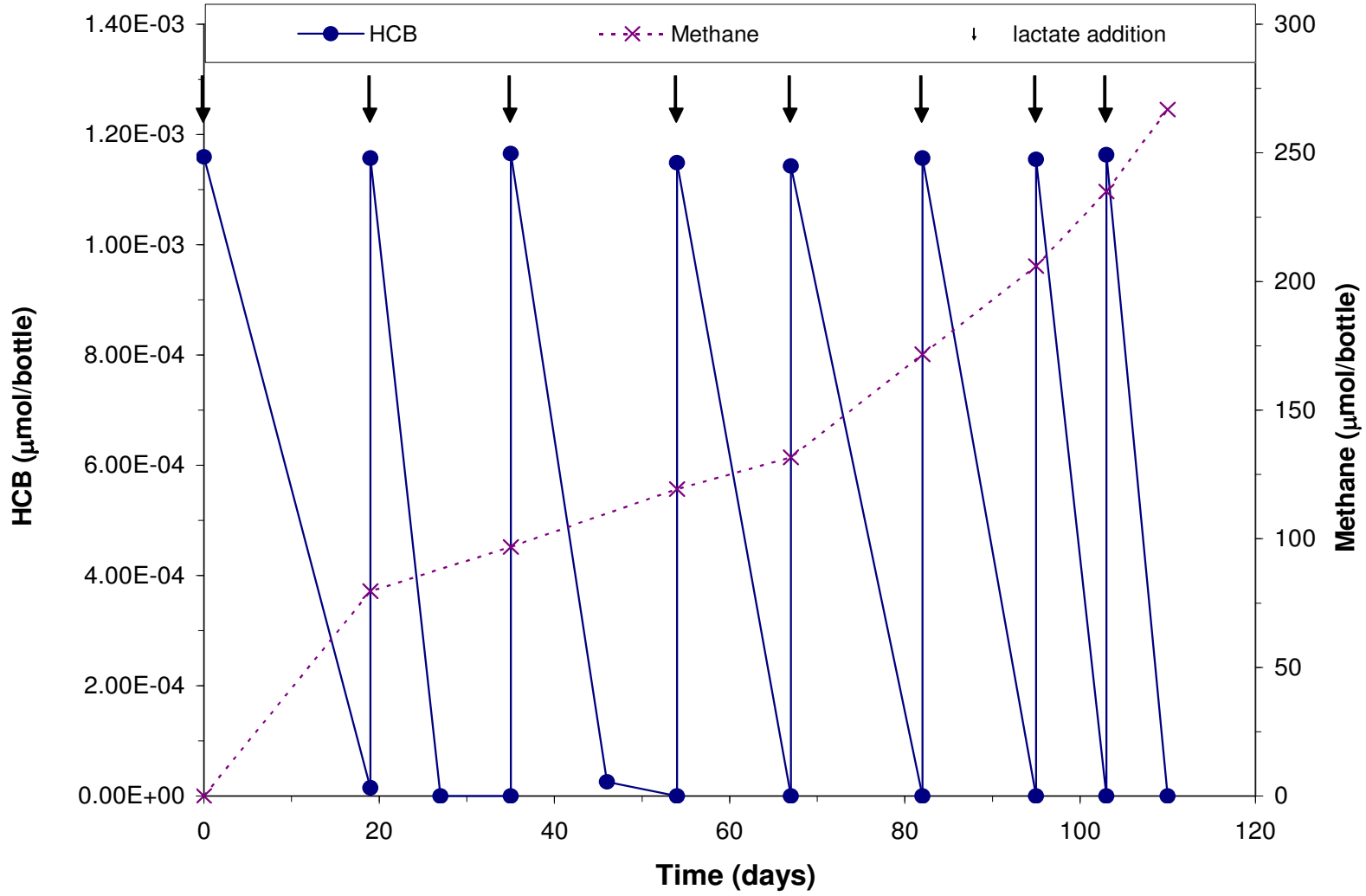

Figure 4.59a Results for one of the TEA Experiment HCB bottles (HCB-2); data for HCB and methane.

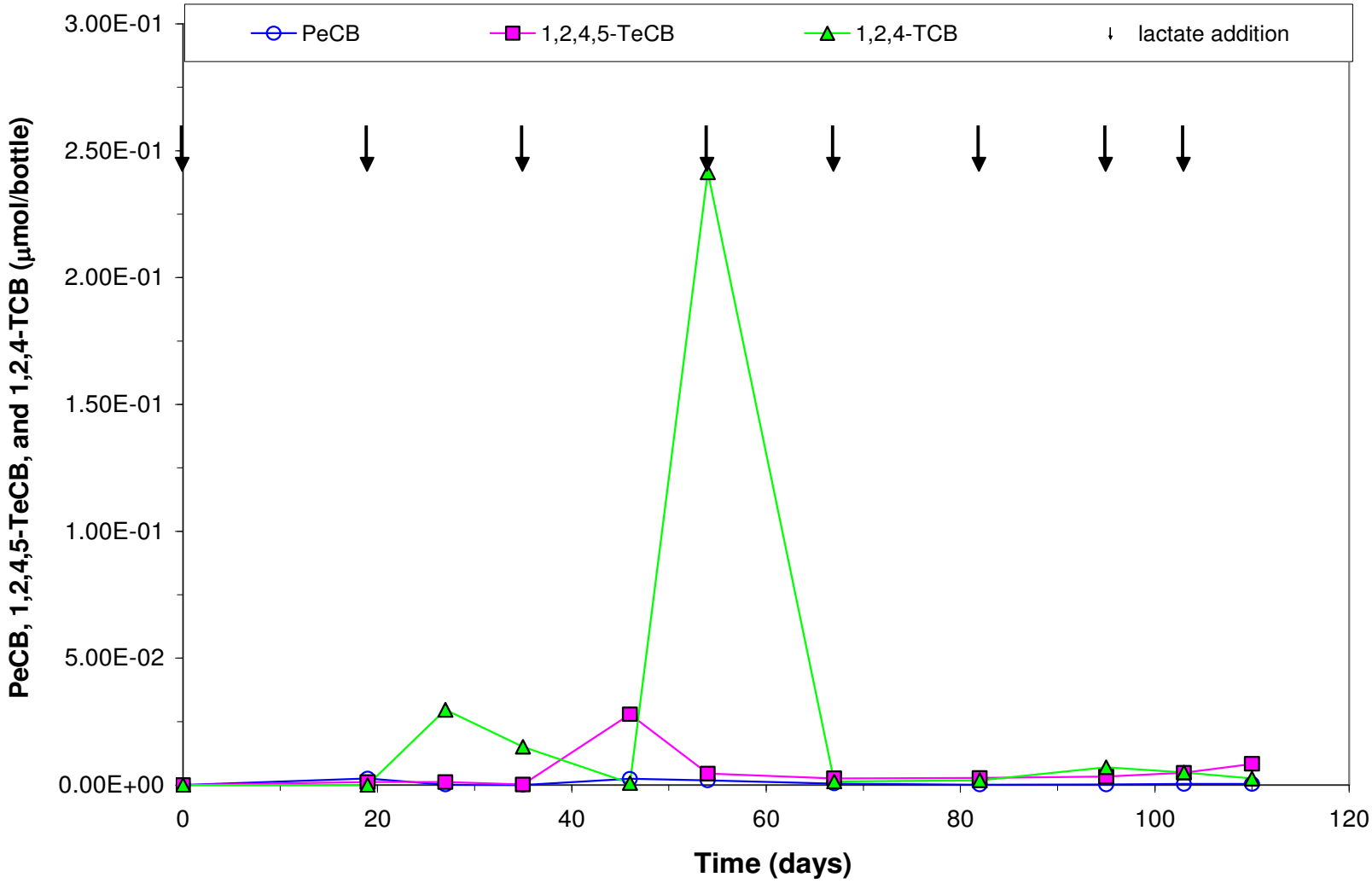

Figure 4.59b Results for one of the TEA Experiment HCB bottles (HCB-2); data for PeCB, 1,2,4,5-TeCB, and 1,2,4-TCB.

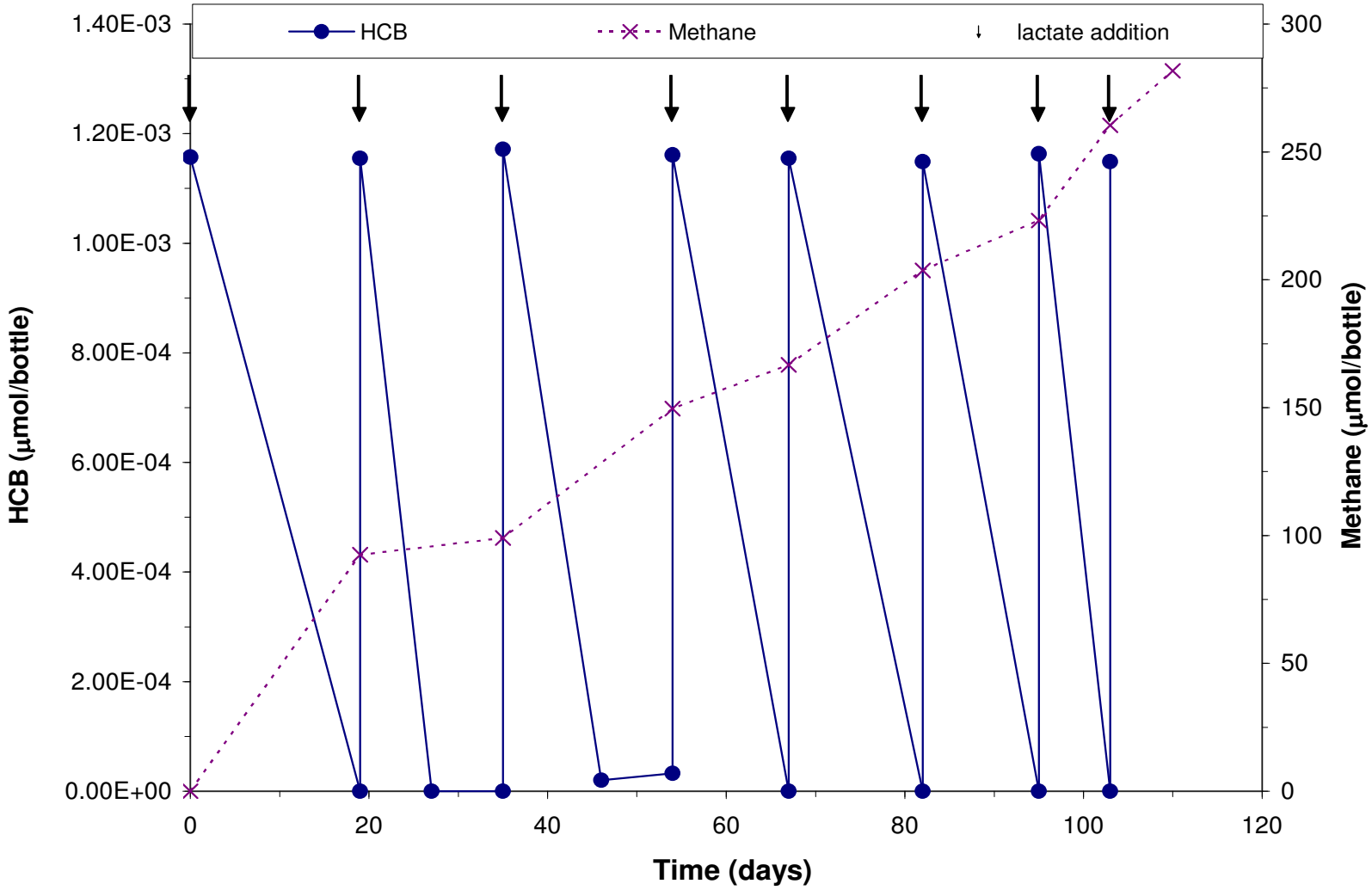

Figure 4.60a Results for one of the TEA Experiment HCB bottles (HCB-3); data for HCB and methane.

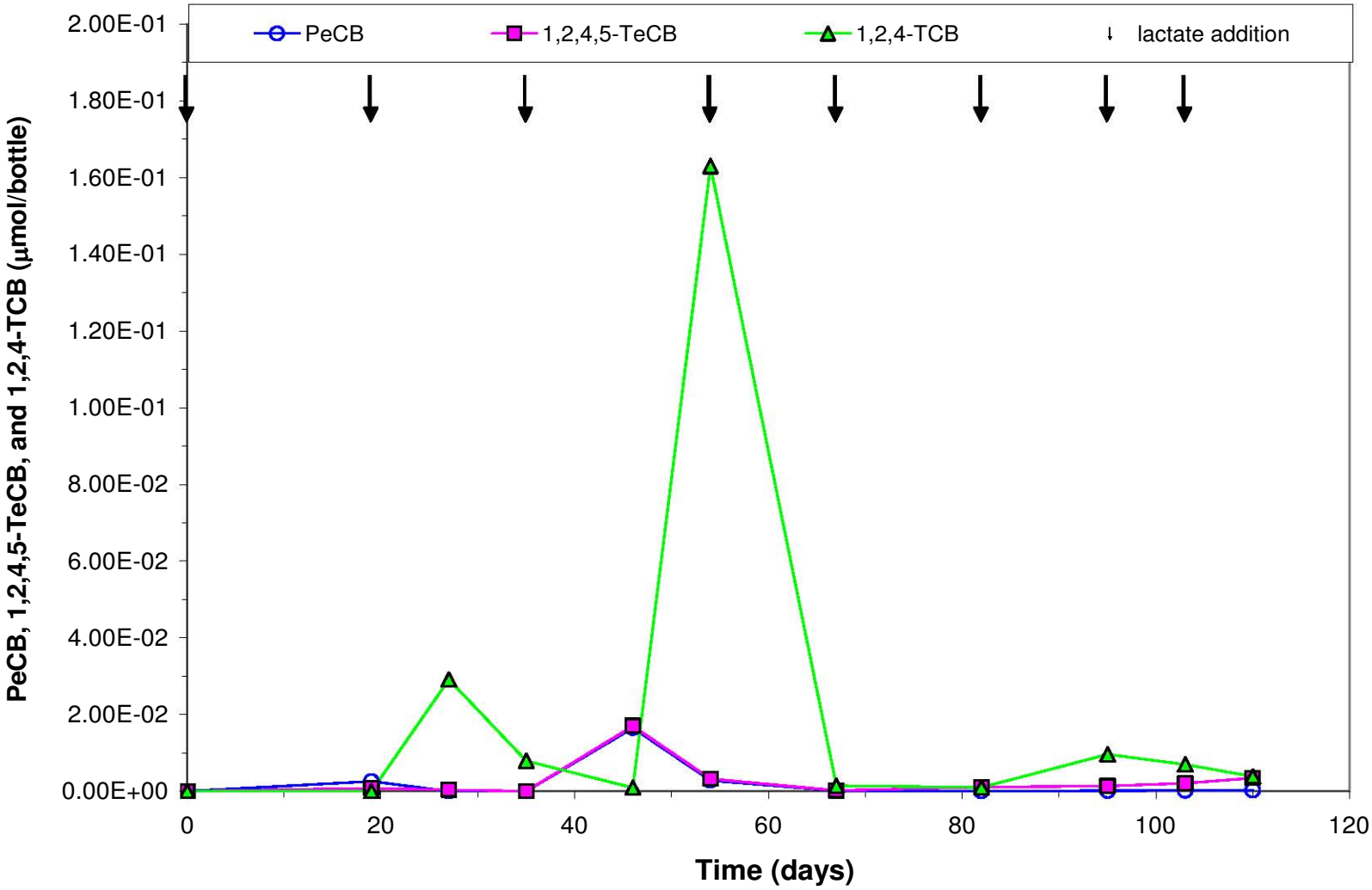

Figure 4.60b Results for one of the TEA Experiment HCB bottles (HCB-3); data for PeCB, 1,2,4,5-TeCB, and 1,2,4-TCB.

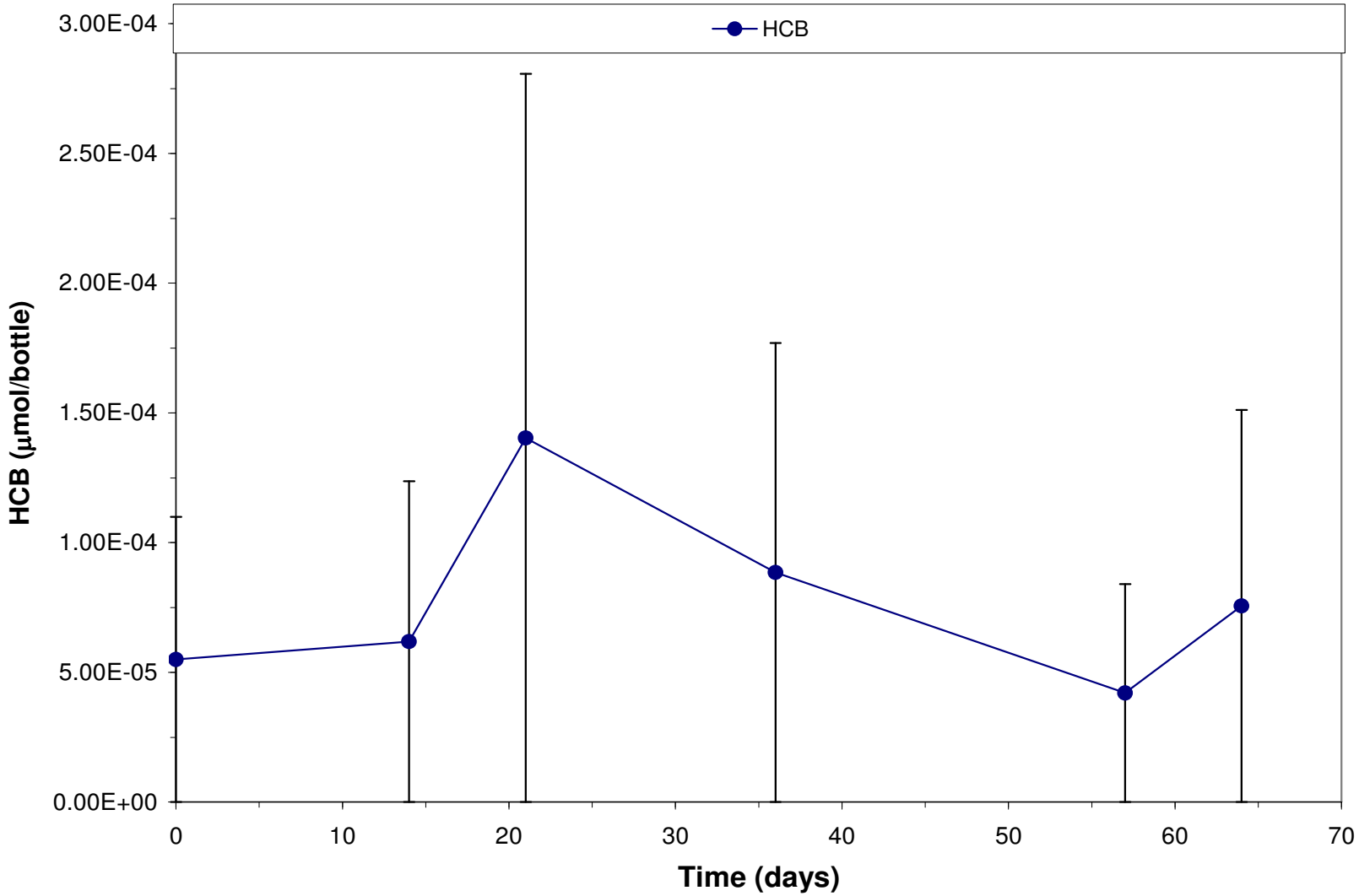

Figure 4.61a Results for the chlorinated benzenes autoclaved controls (average of triplicates); HCB only. Error bars represent ± one standard deviation.

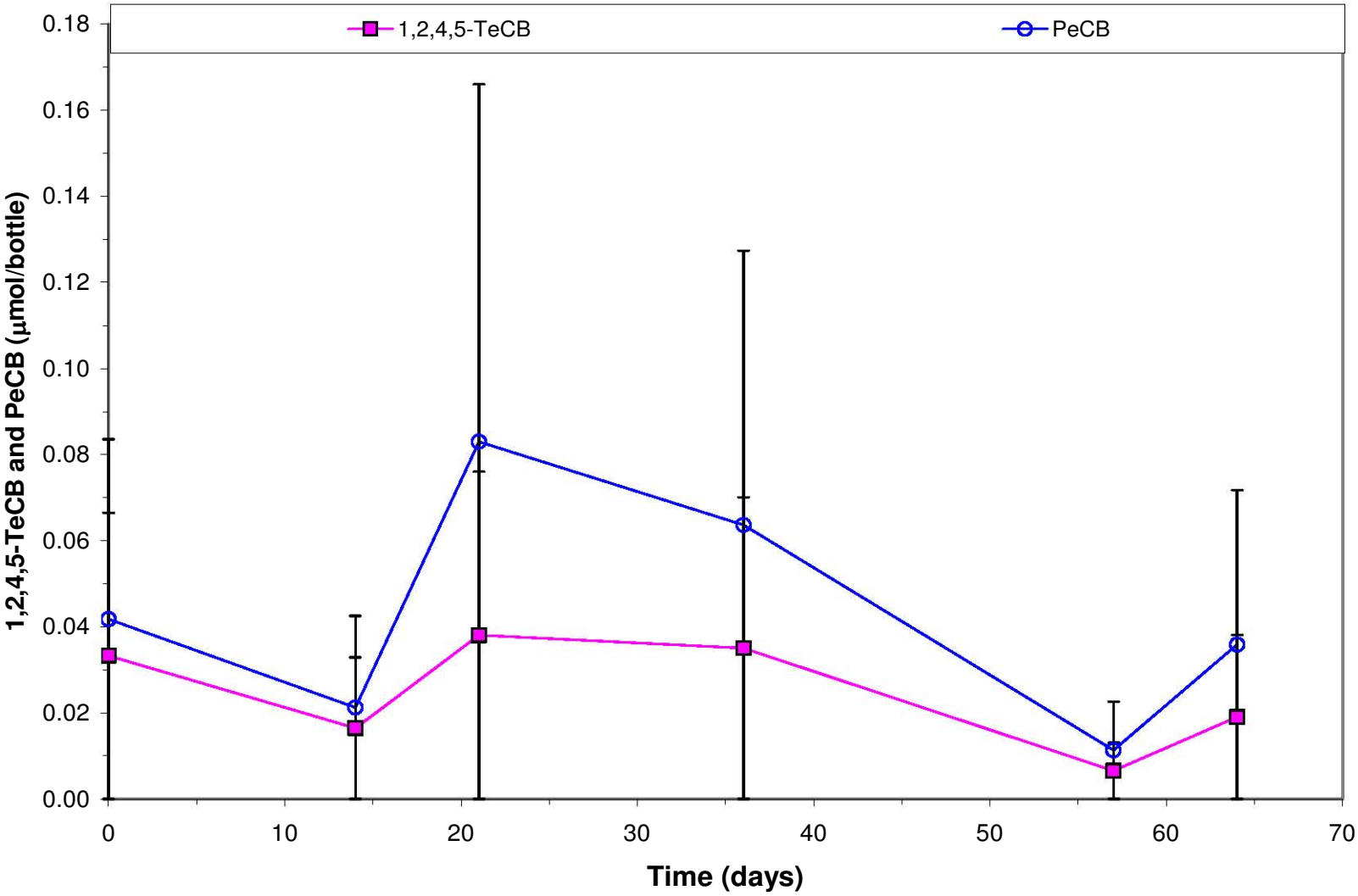

Figure 4.61b Results for the chlorinated benzenes autoclaved controls (average of triplicates); PeCB and 1,2,4,5-TeCB. Error bars represent ± one standard deviation.

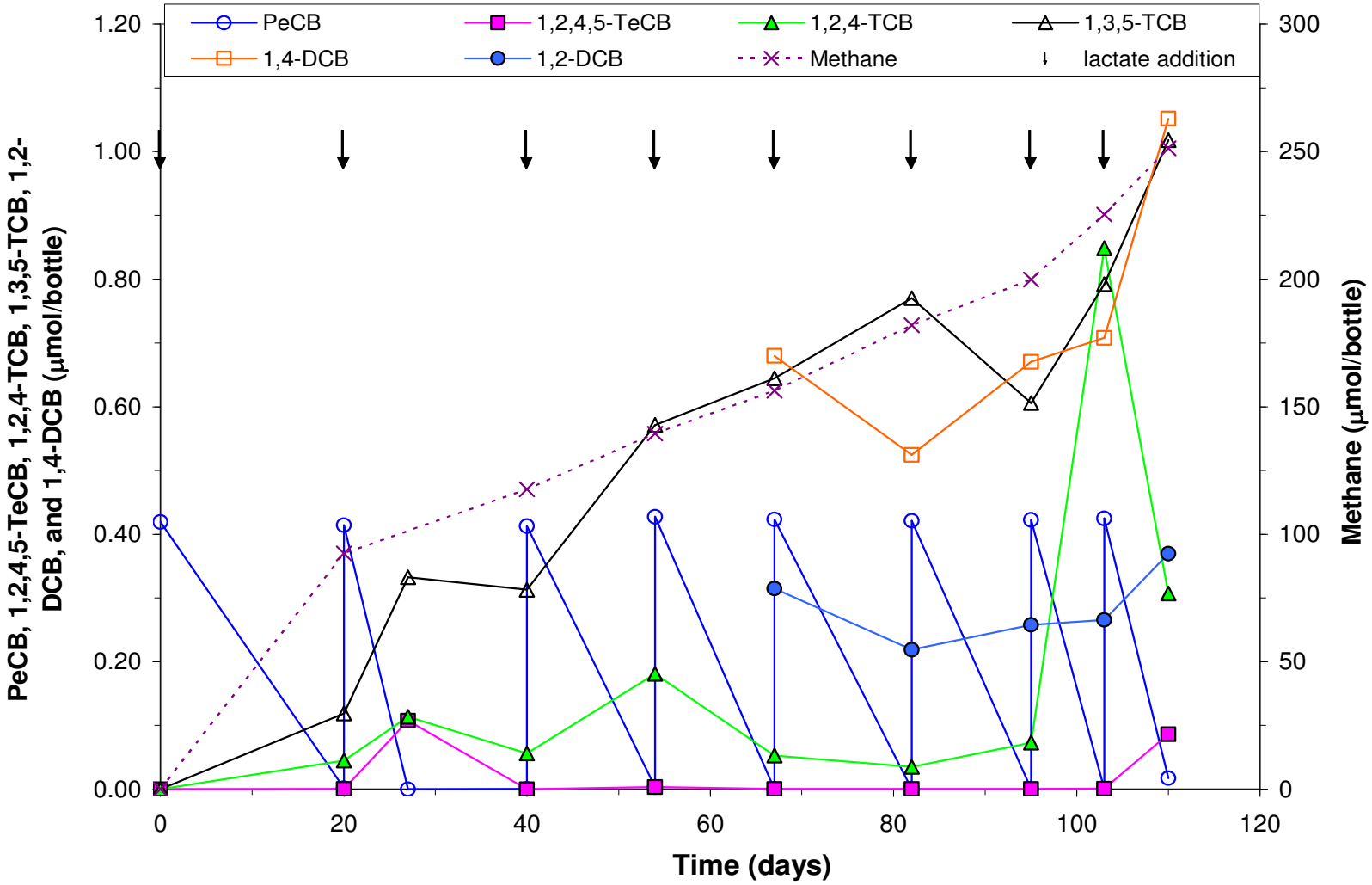

Figure 4.62 Results for one of the TEA Experiment PeCB bottles (PeCB-1).

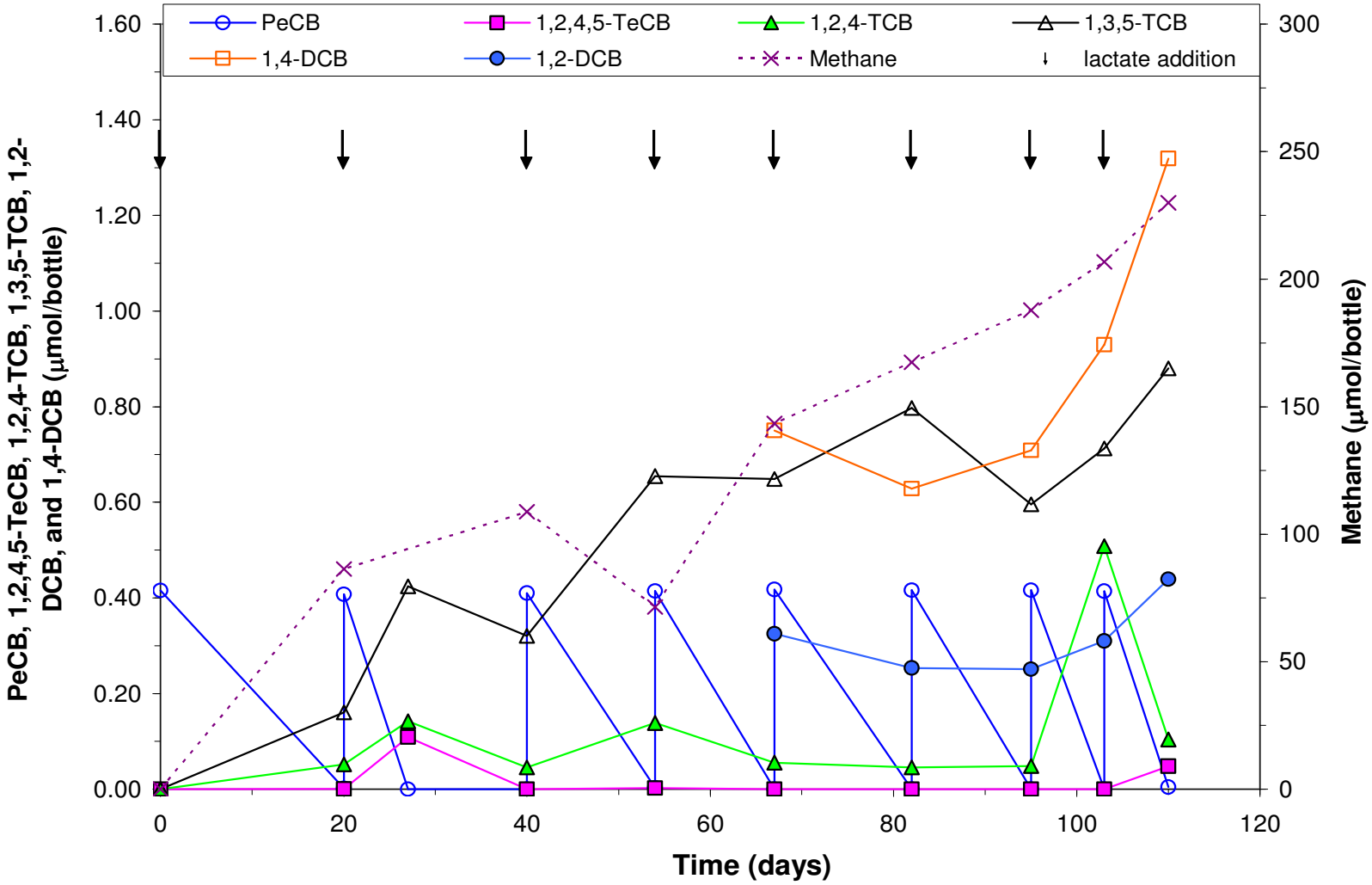

Figure 4.63 Results for one of the TEA Experiment PeCB bottles (PeCB-2).

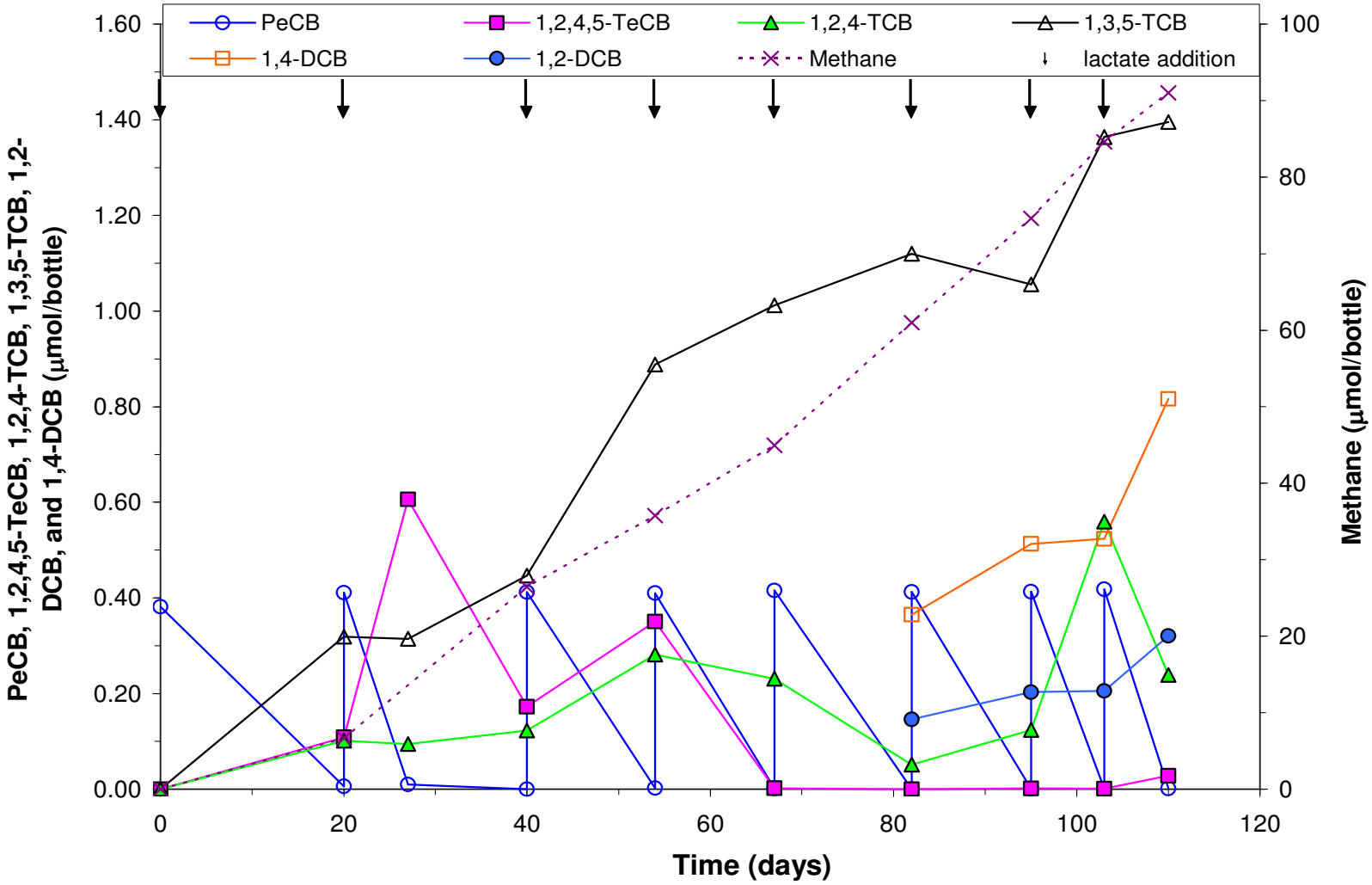

Figure 4.64 Results for one of the TEA Experiment PeCB bottles (PeCB-3).

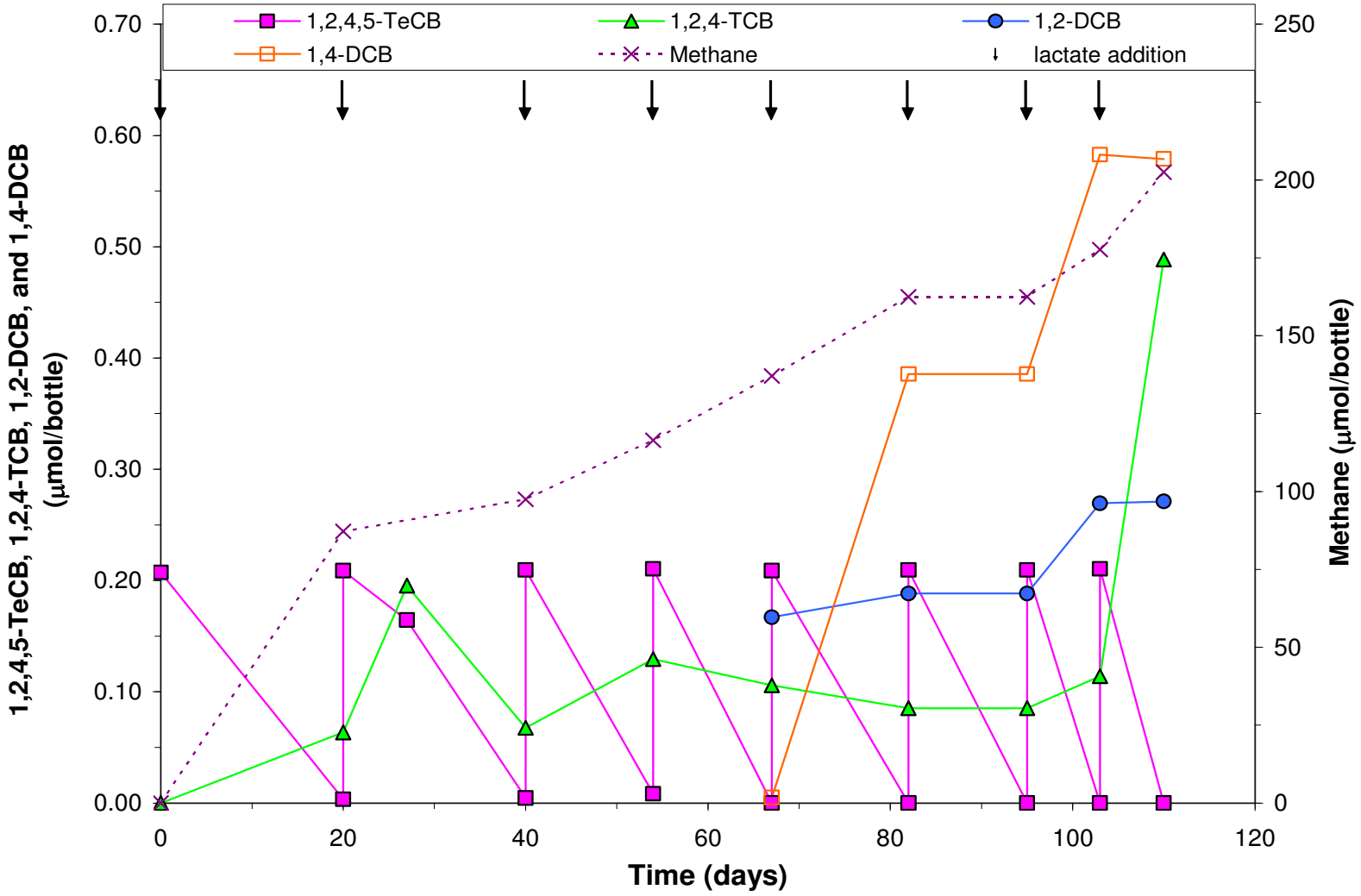

Figure 4.65 Results for one of the TEA Experiment 1,2,4,5-TeCB bottles (1,2,4,5-TeCB-1).

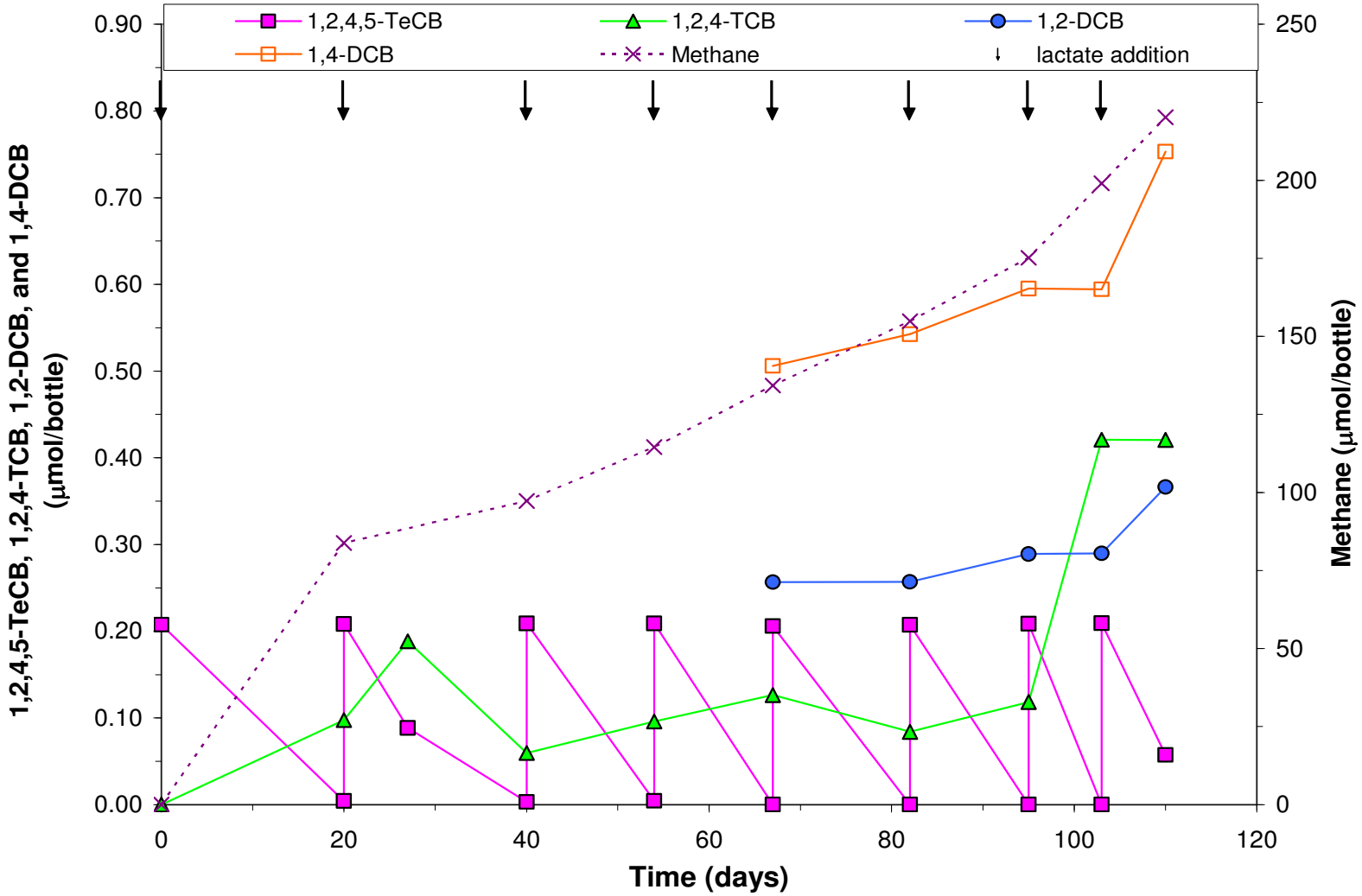

Figure 4.66 Results for one of the TEA Experiment 1,2,4,5-TeCB bottles (1,2,4,5-TeCB-2).

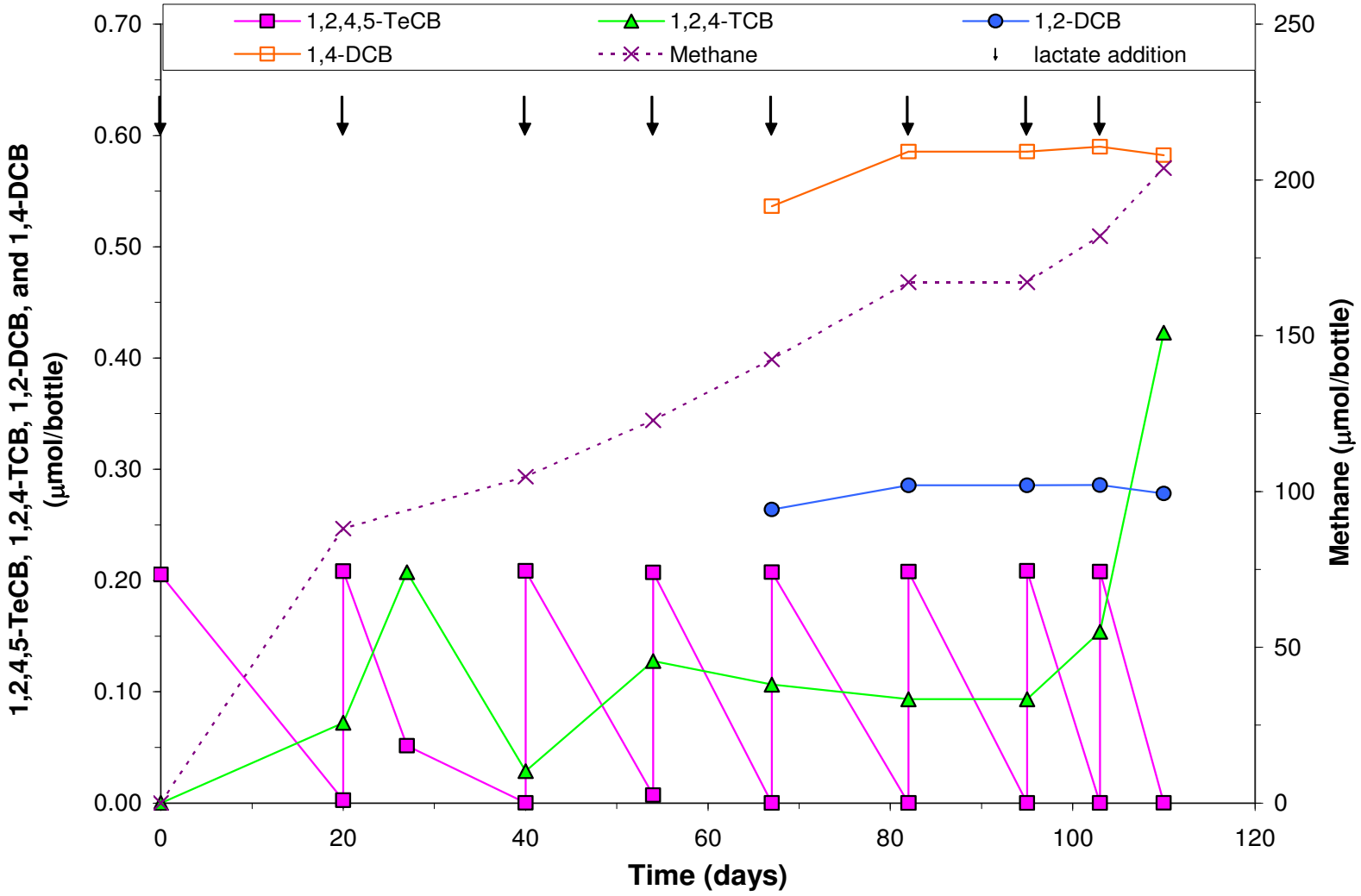

Figure 4.67 Results for one of the TEA Experiment 1,2,4,5-TeCB bottles (1,2,4,5-TeCB-3).

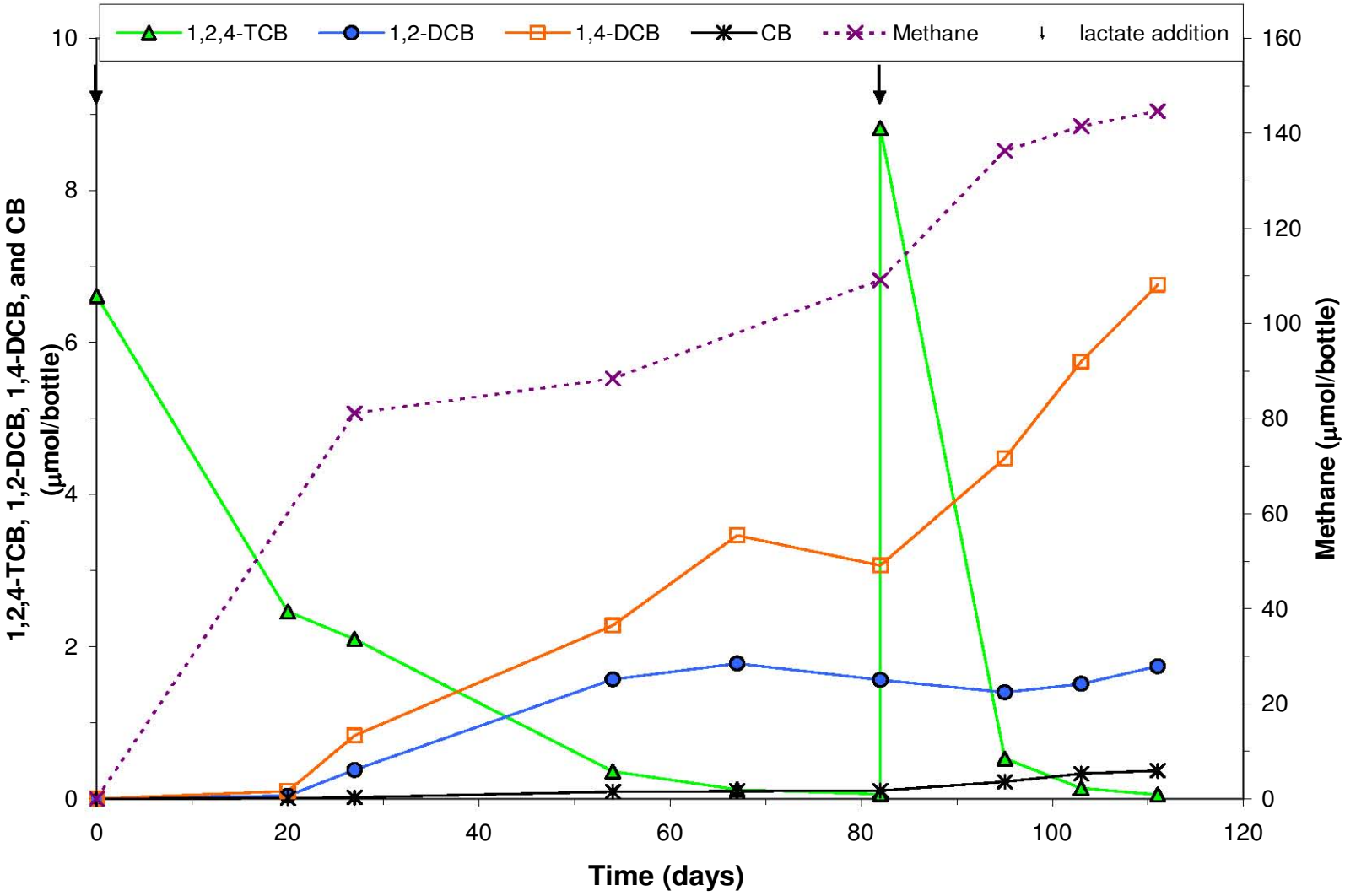

Figure 4.68 Results for one of the TEA Experiment 1,2,4-TCB bottles (1,2,4-TCB-1).

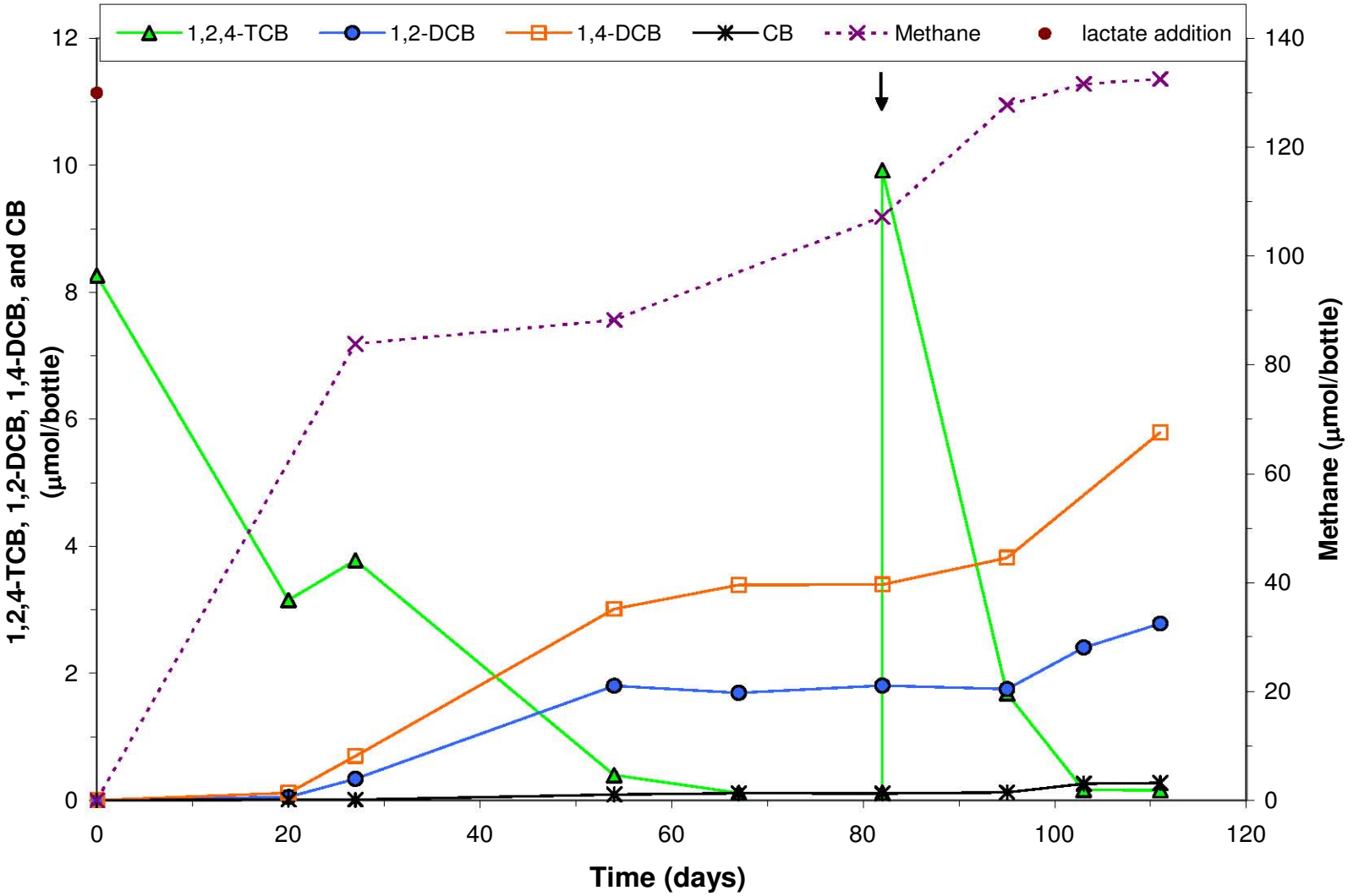

Figure 4.69 Results for one of the TEA Experiment 1,2,4-TCB bottles (1,2,4-TCB-2).

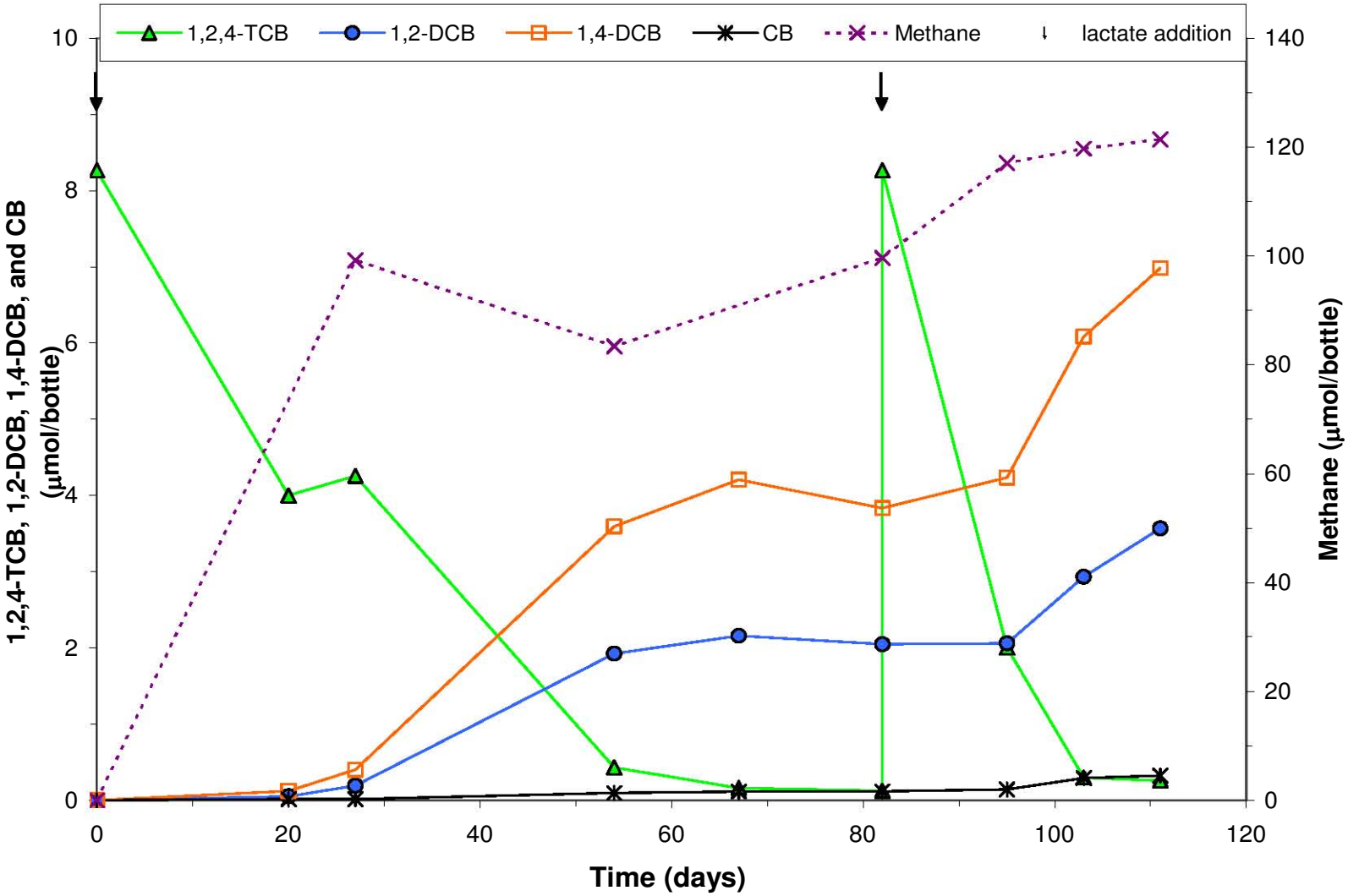

Figure 4.70 Results for one of the TEA Experiment 1,2,4-TCB bottles (1,2,4-TCB-3).

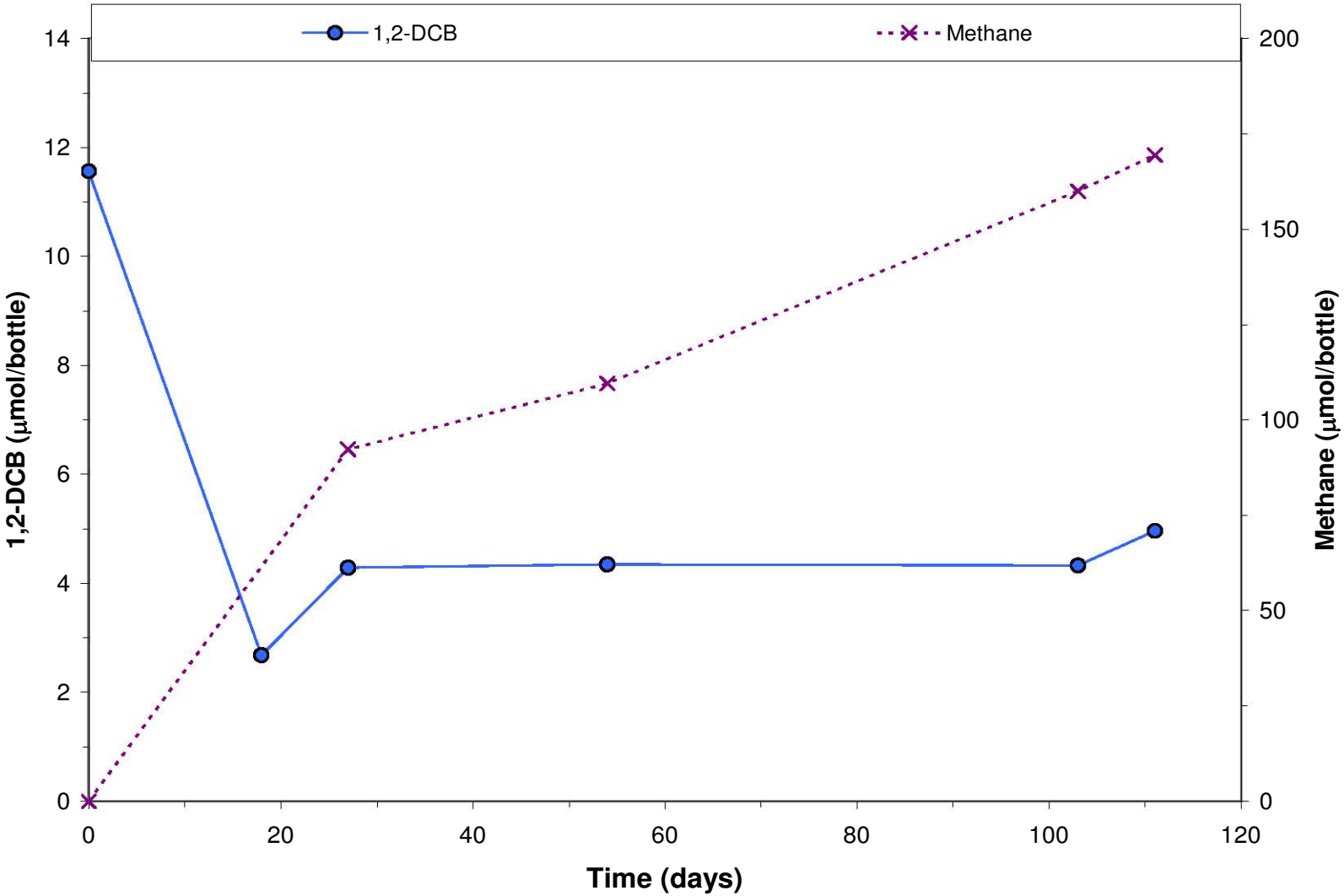

Figure 4.71 Results for one of the TEA Experiment 1,2-DCB bottles (1,2-DCB-1).

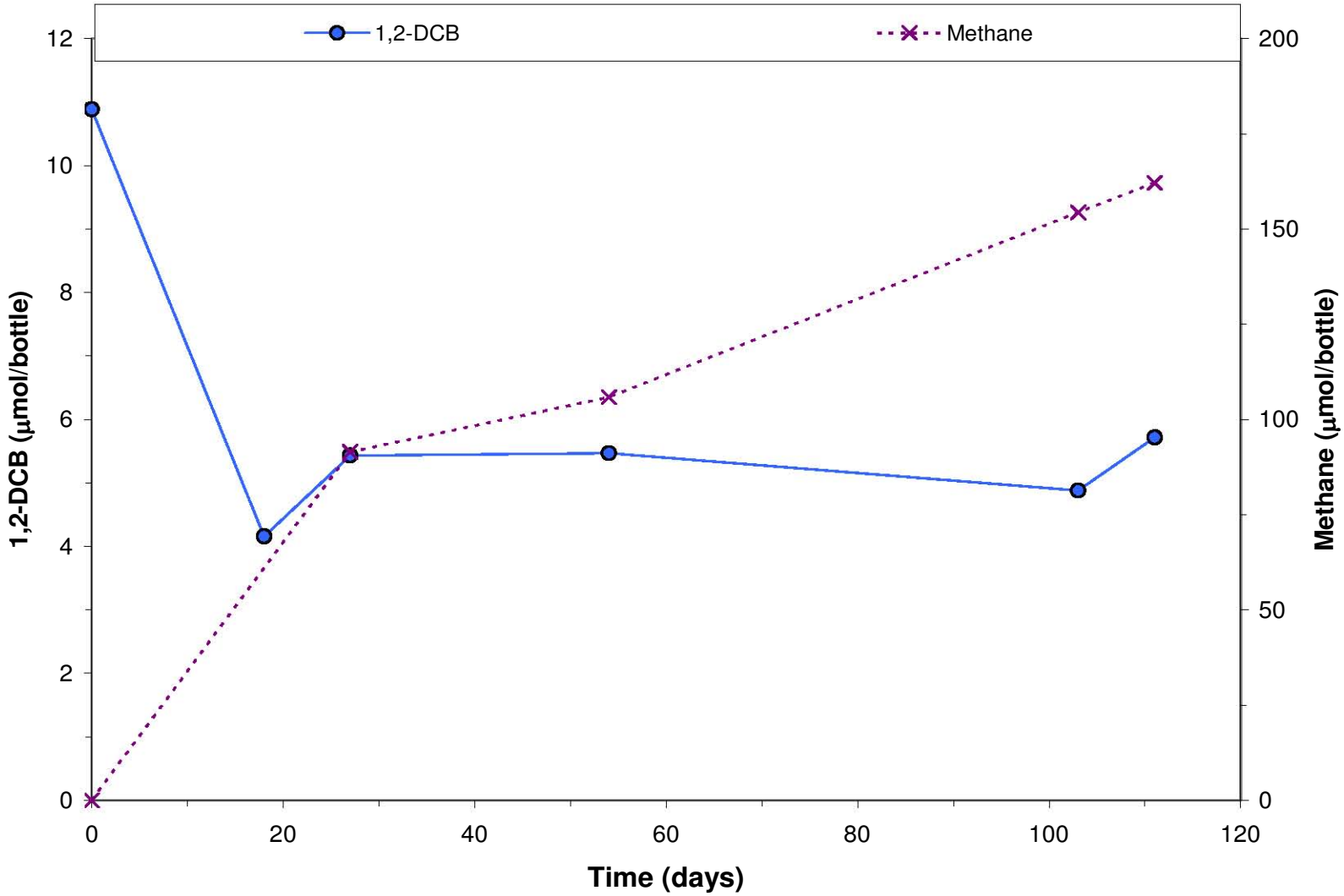

Figure 4.72 Results for one of the TEA Experiment 1,2-DCB bottles (1,2-DCB-2).

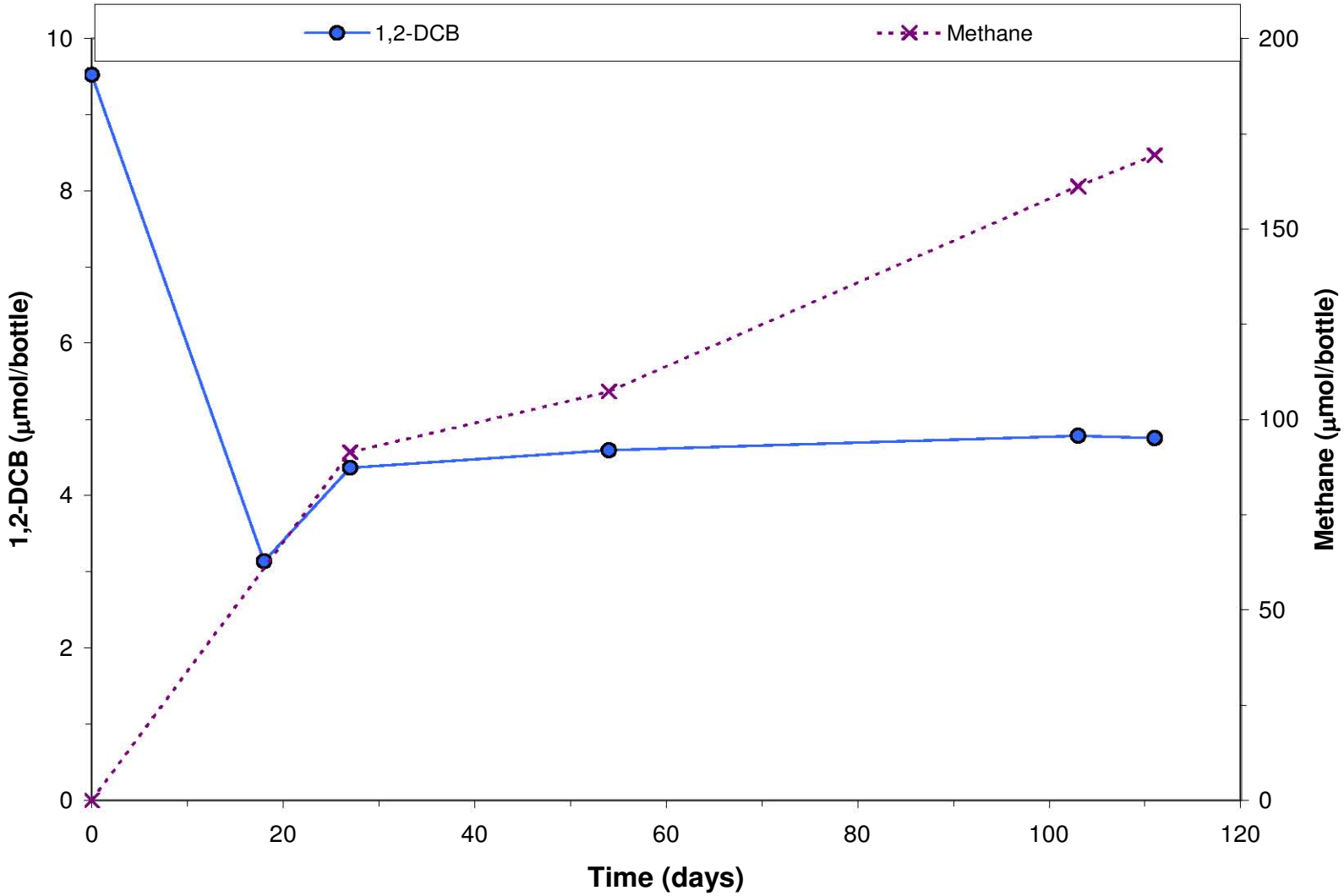

Figure 4.73 Results for one of the TEA Experiment 1,2-DCB bottles (1,2-DCB-3).

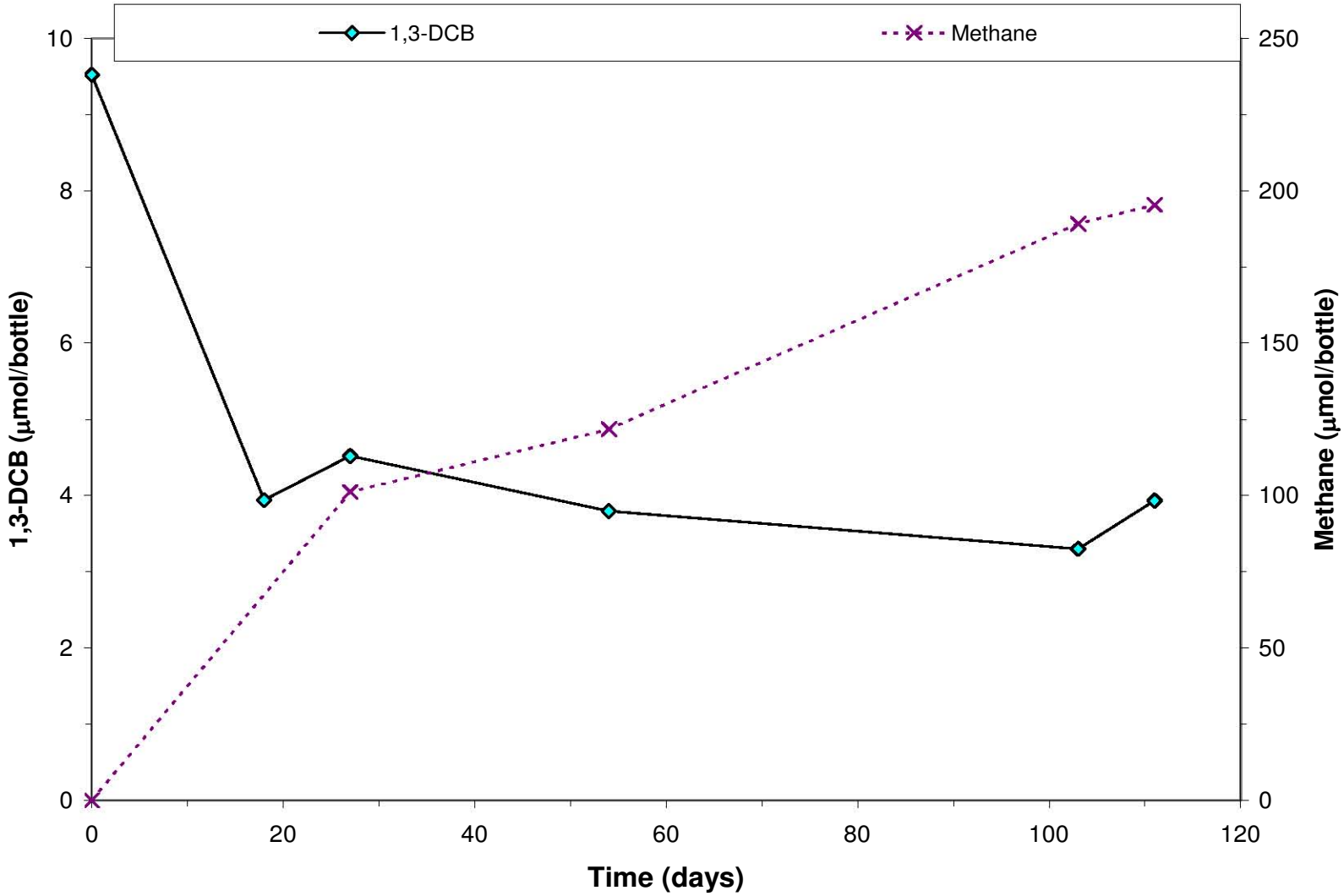

Figure 4.74 Results for one of the TEA Experiment 1,3-DCB bottles (1,3-DCB-1).

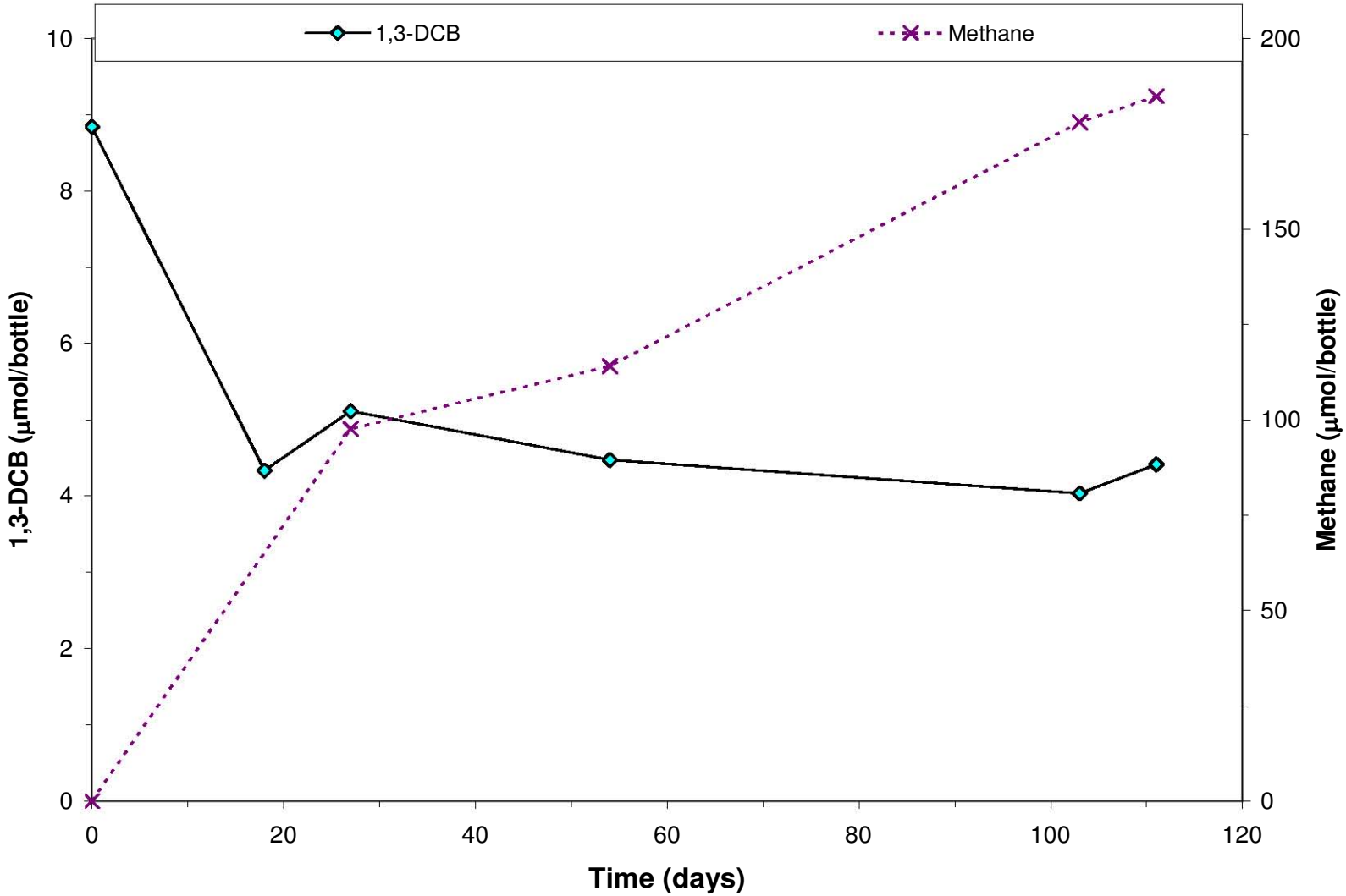

Figure 4.75 Results for one of the TEA Experiment 1,3-DCB bottles (1,3-DCB-2).

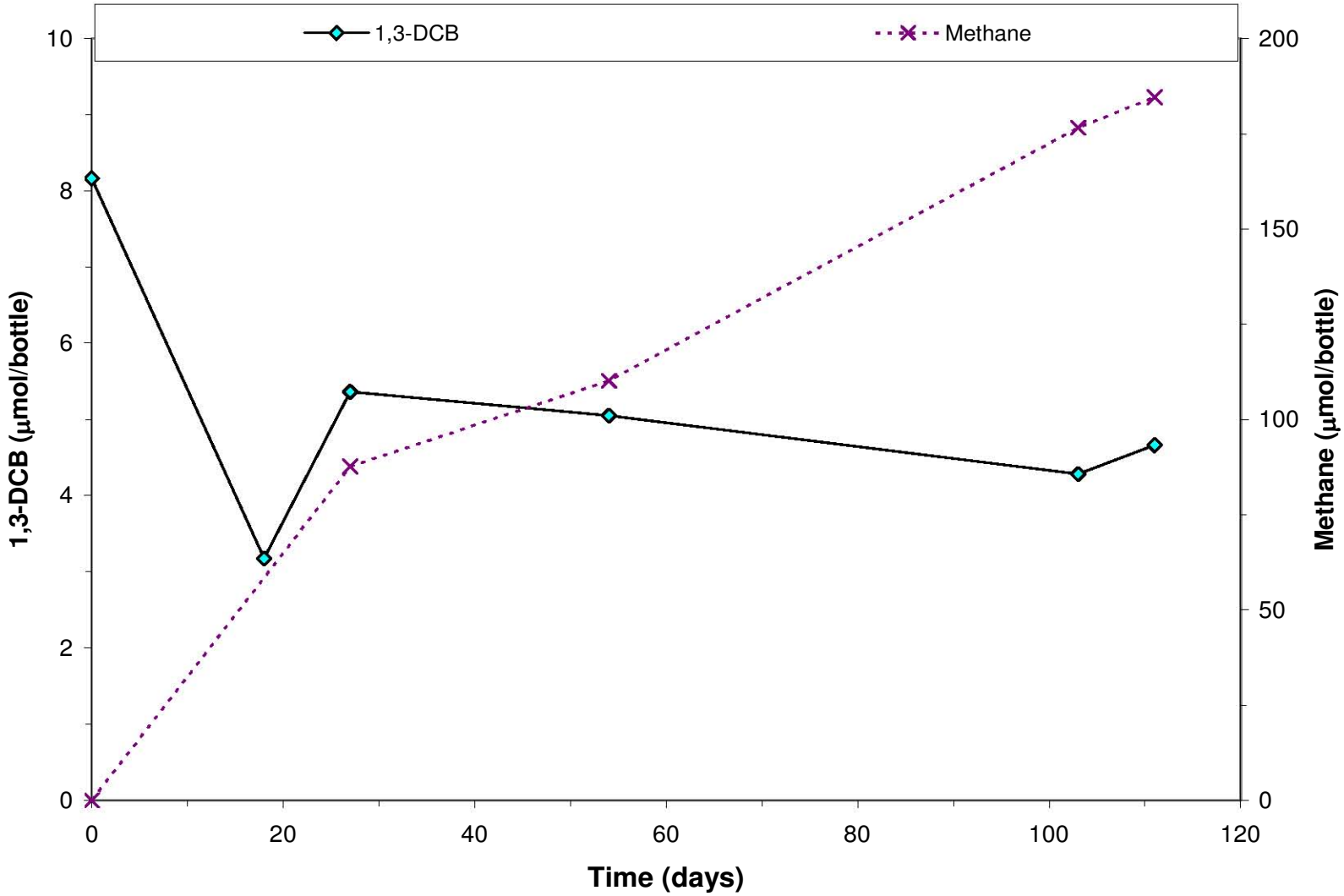

Figure 4.76 Results for one of the TEA Experiment 1,3-DCB bottles (1,3-DCB-3).

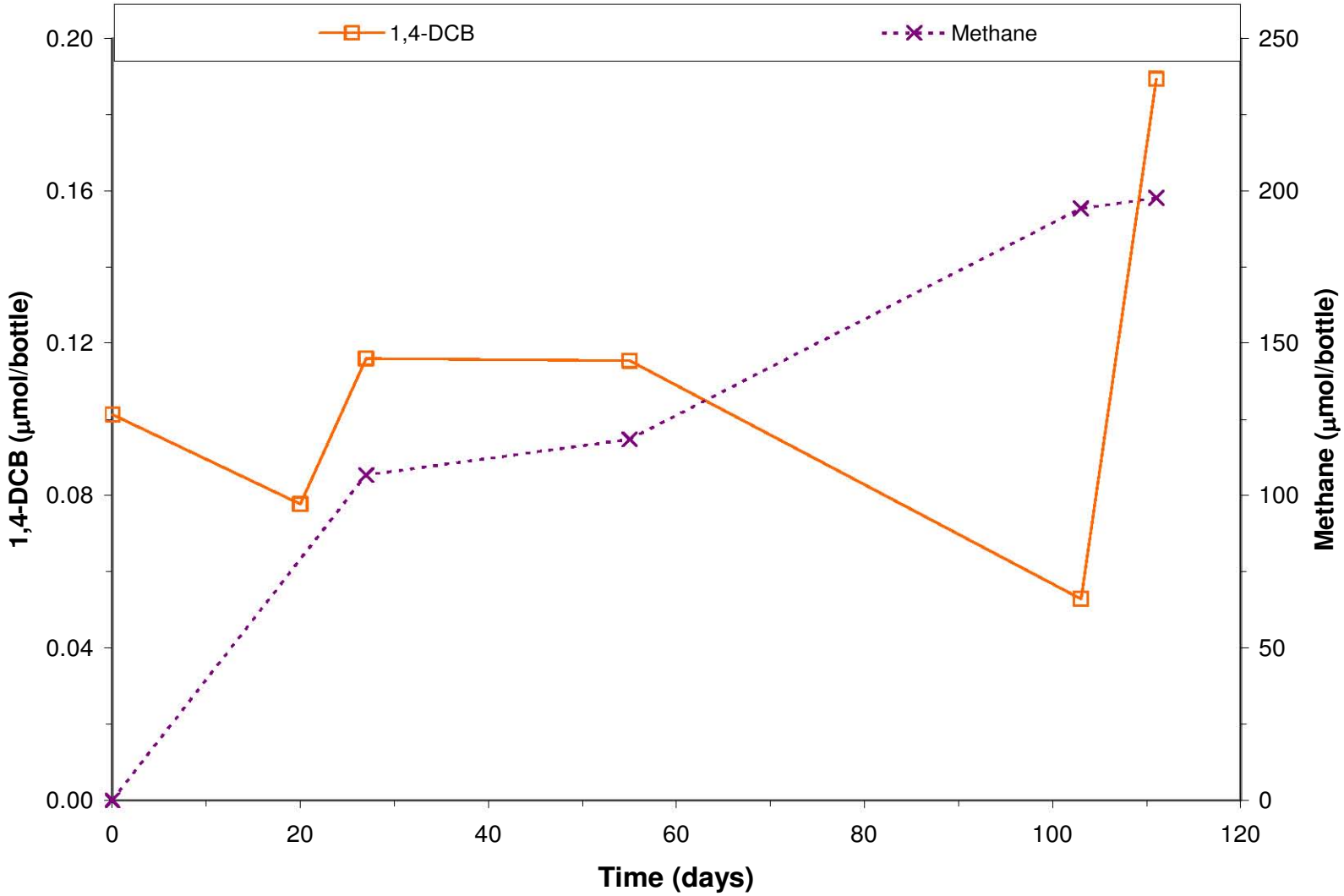

Figure 4.77 Results for one of the TEA Experiment 1,4-DCB bottles (1,4-DCB-1).

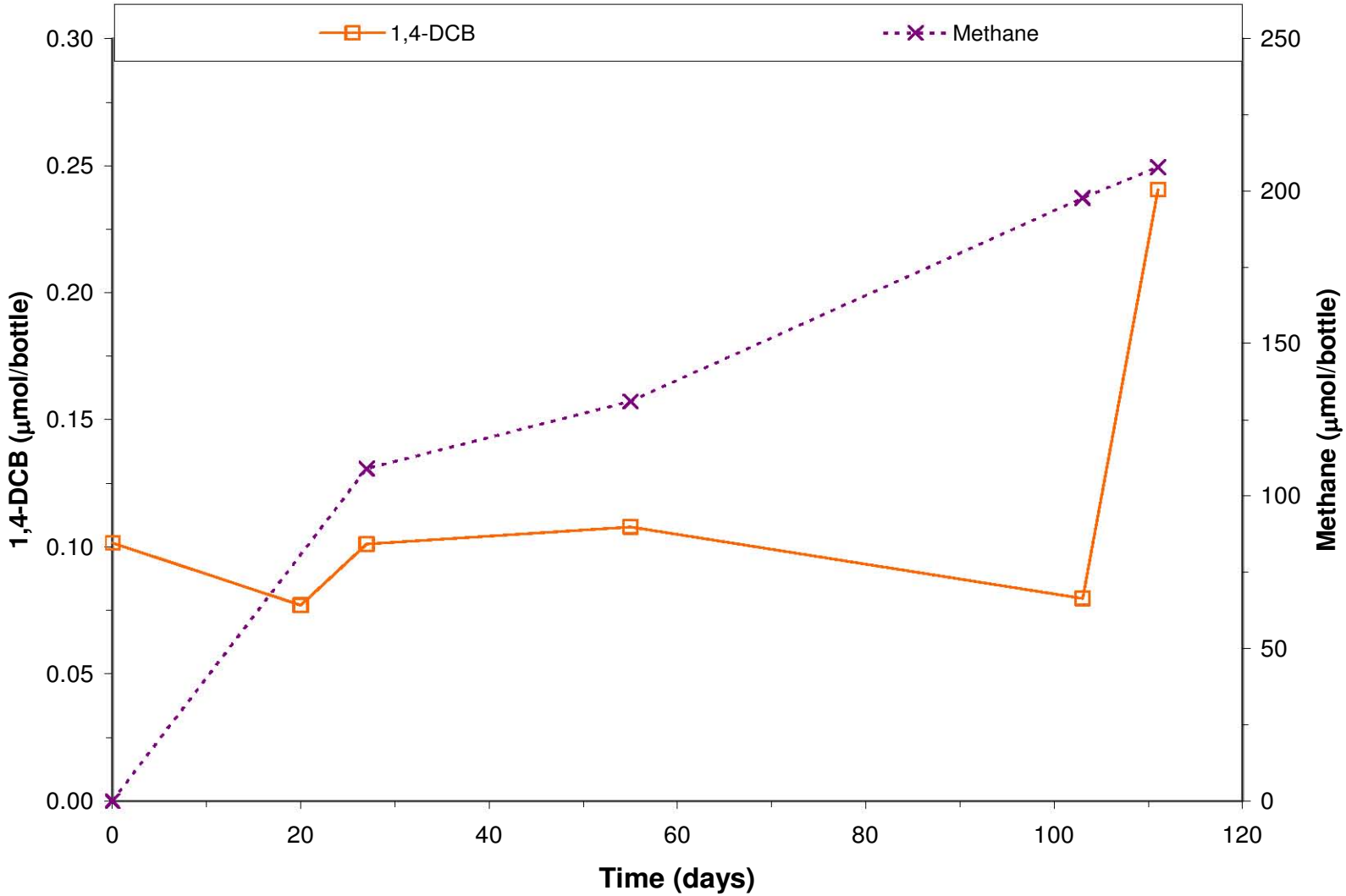

Figure 4.78 Results for one of the TEA Experiment 1,4-DCB bottles (1,4-DCB-2).

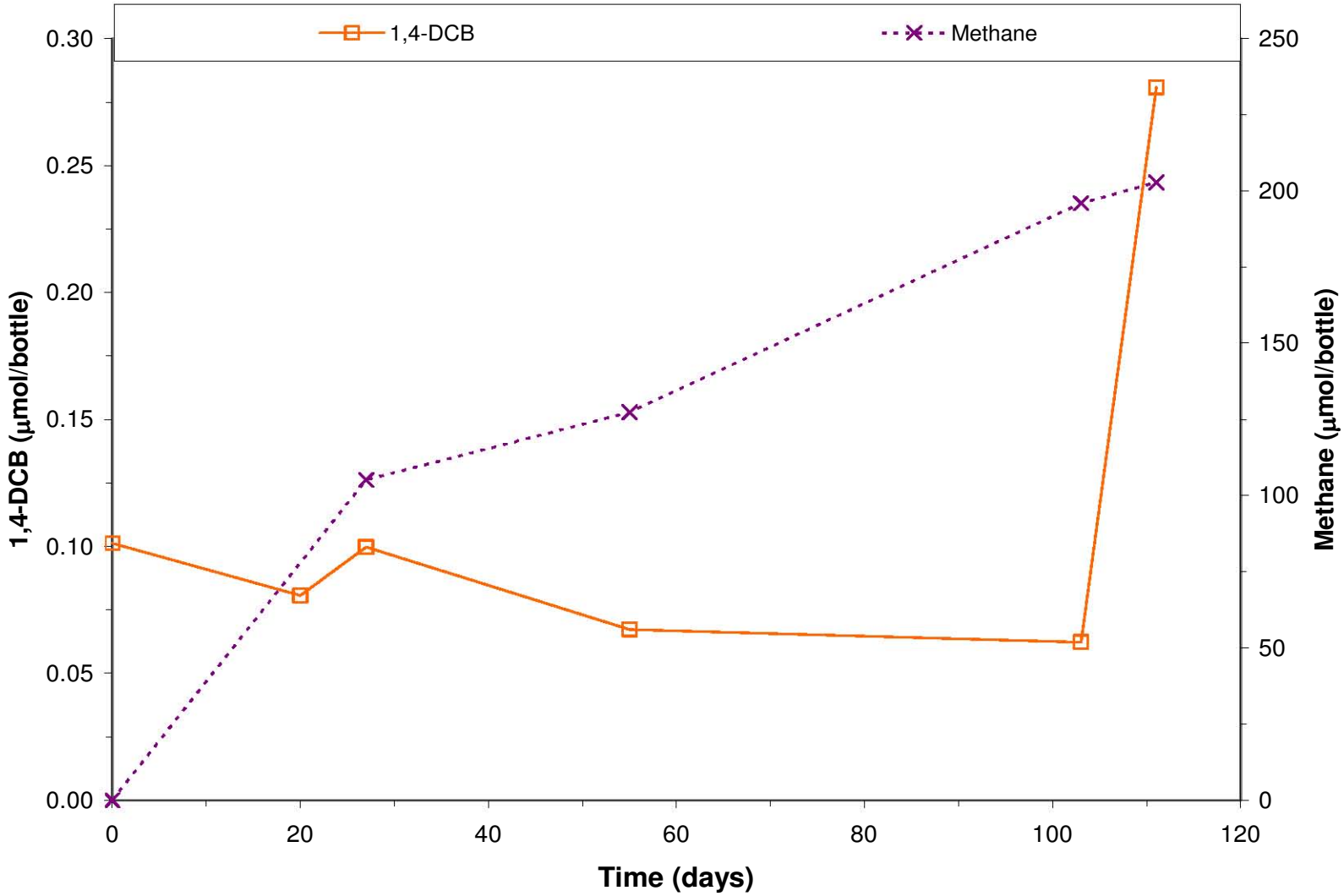

Figure 4.79 Results for one of the TEA Experiment 1,4-DCB bottles (1,4-DCB-3).

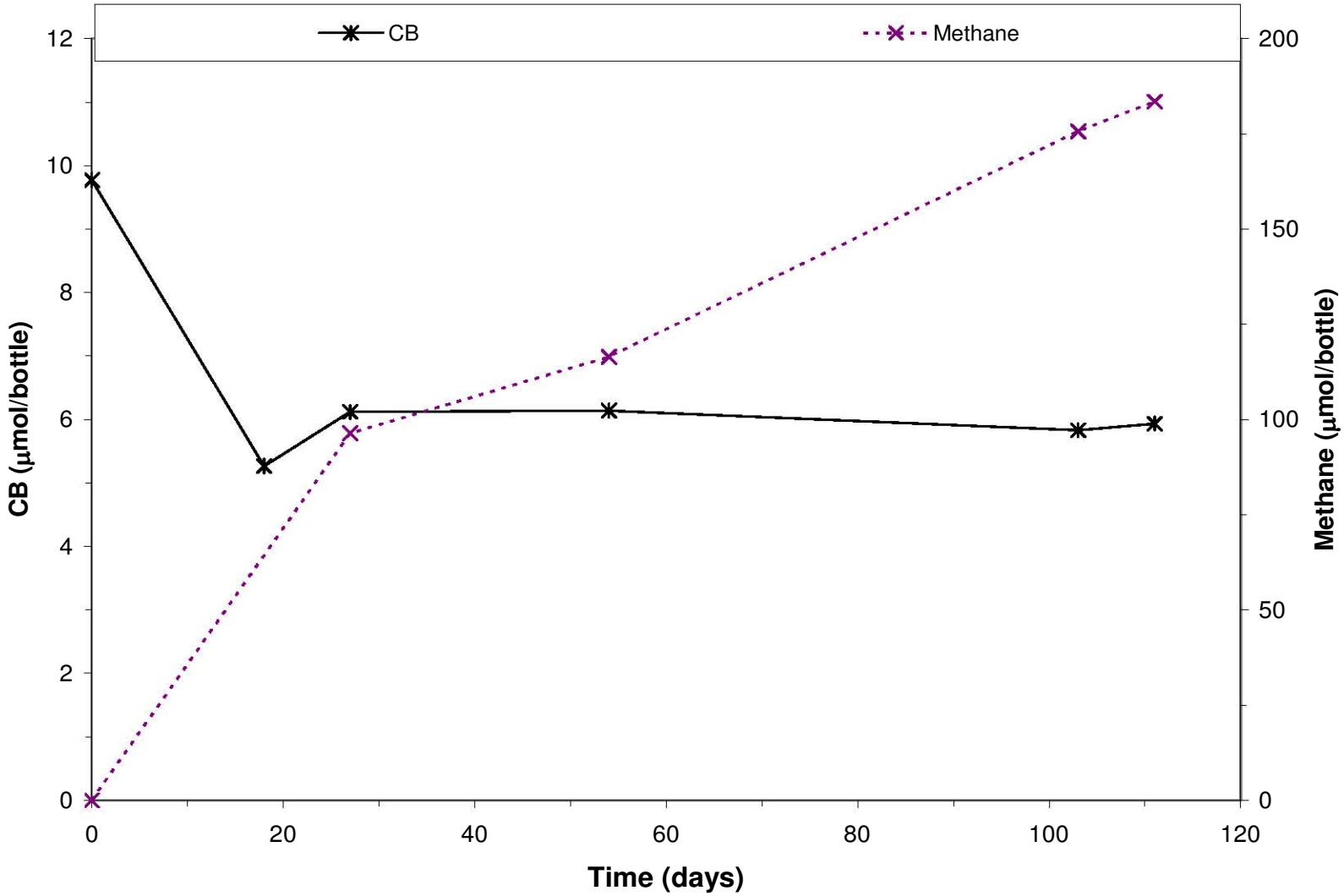

Figure 4.80 Results for one of the TEA Experiment CB bottles (CB-1).

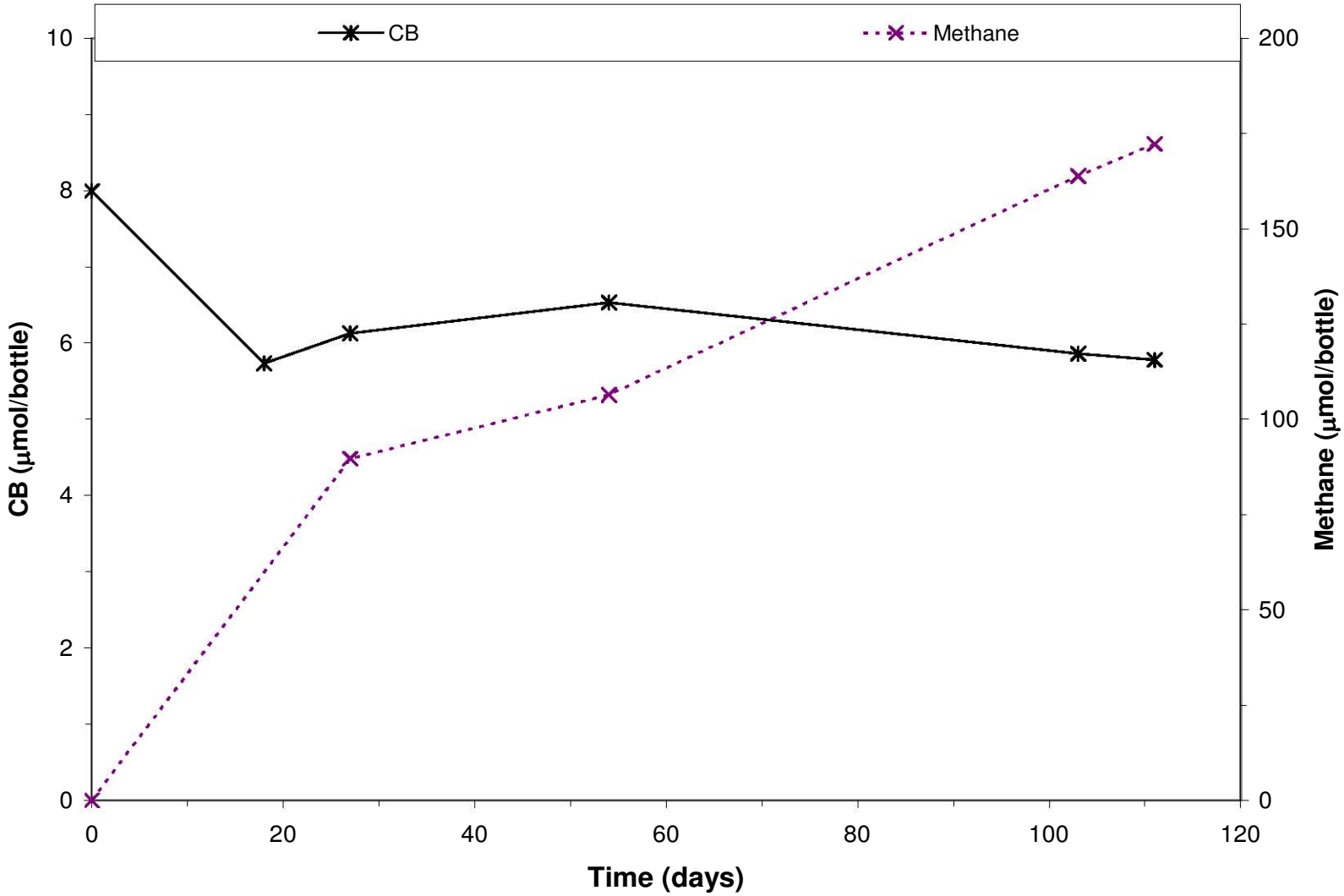

Figure 4.81 Results for one of the TEA Experiment CB bottles (CB-2).

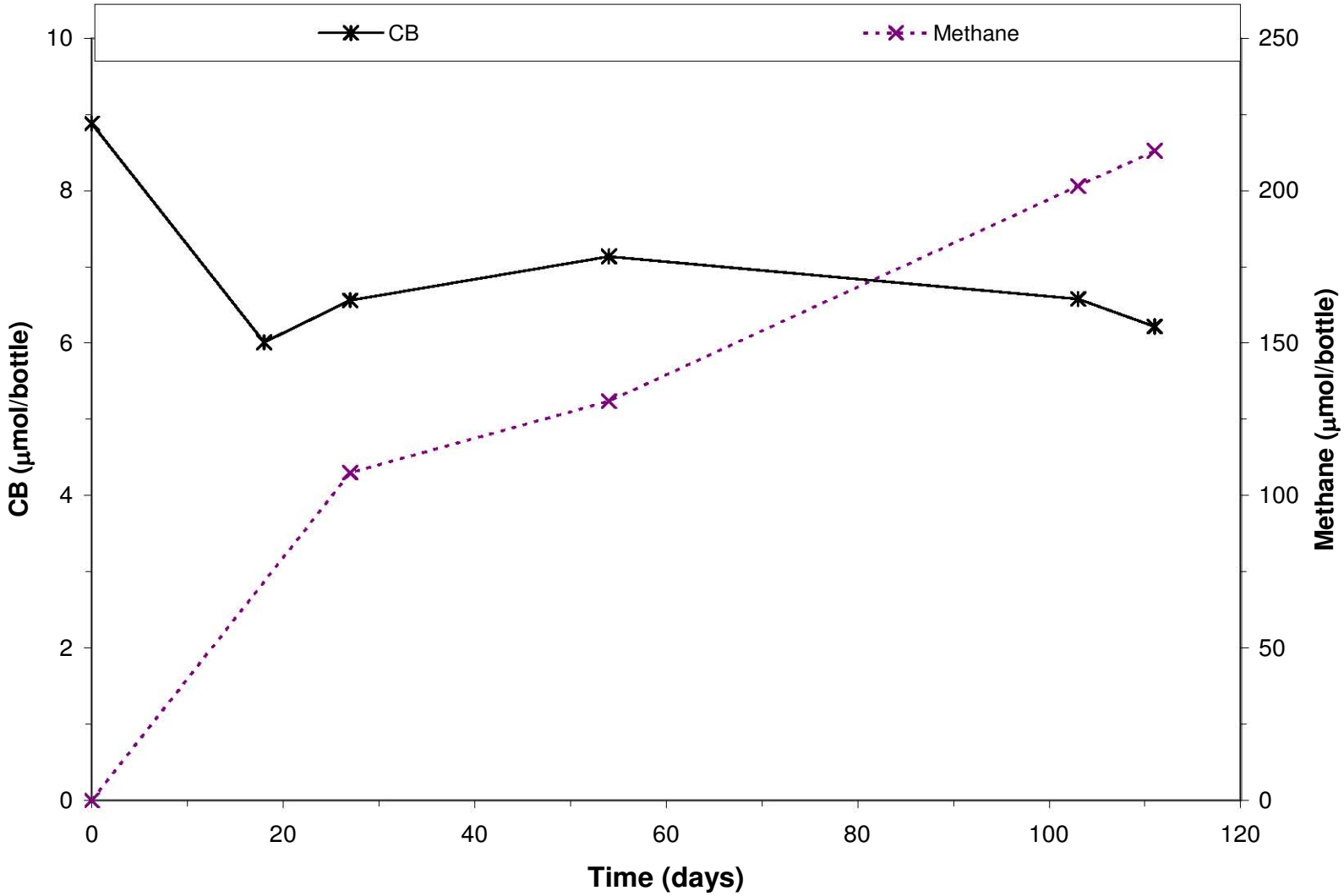

Figure 4.82 Results for one of the TEA Experiment CB bottles (CB-3).

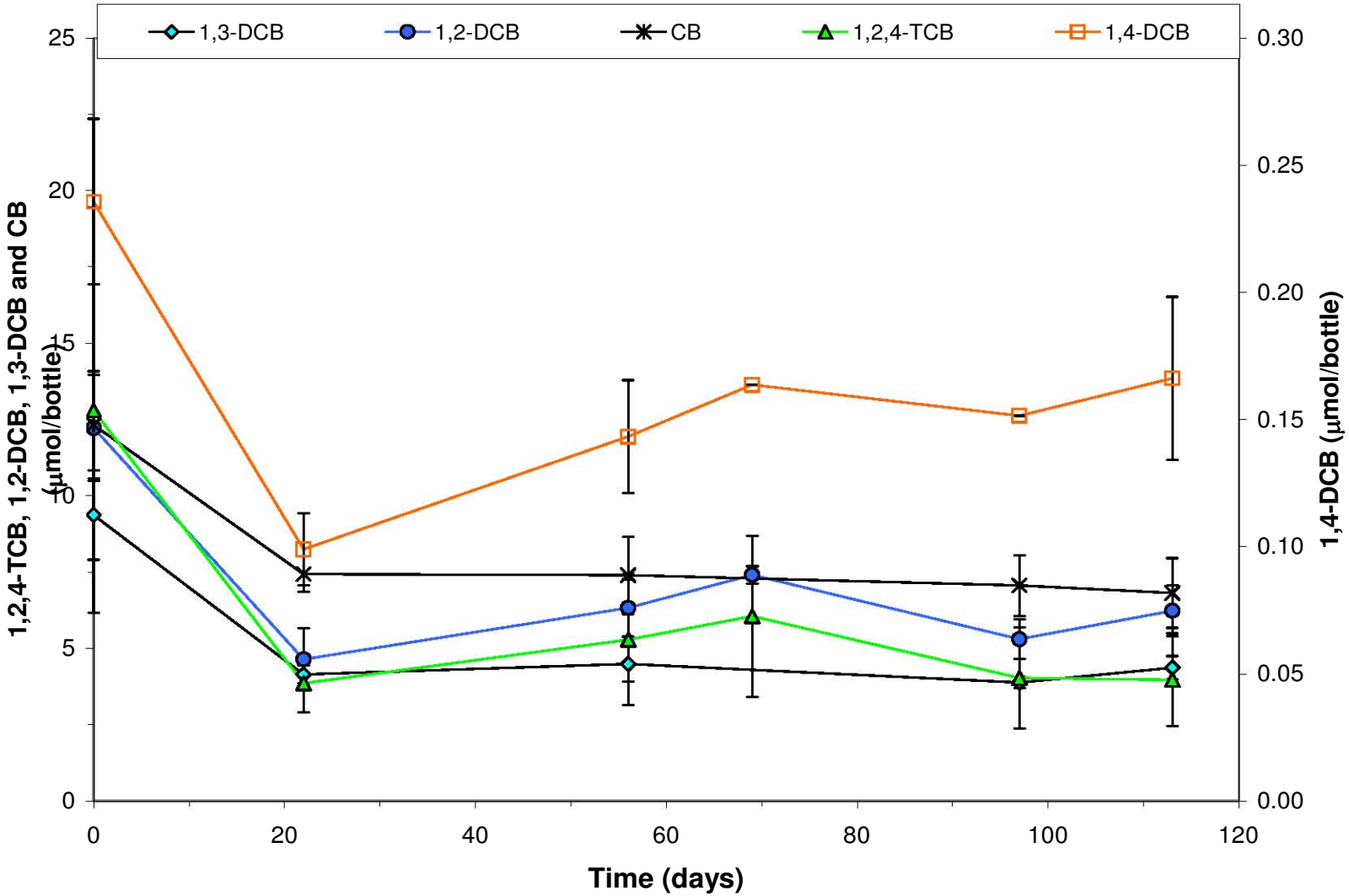

Figure 4.83 Results for the first set of TEA Experiment chlorinated benzenes WCs (average of triplicates). Error bars represent ± one standard deviation.

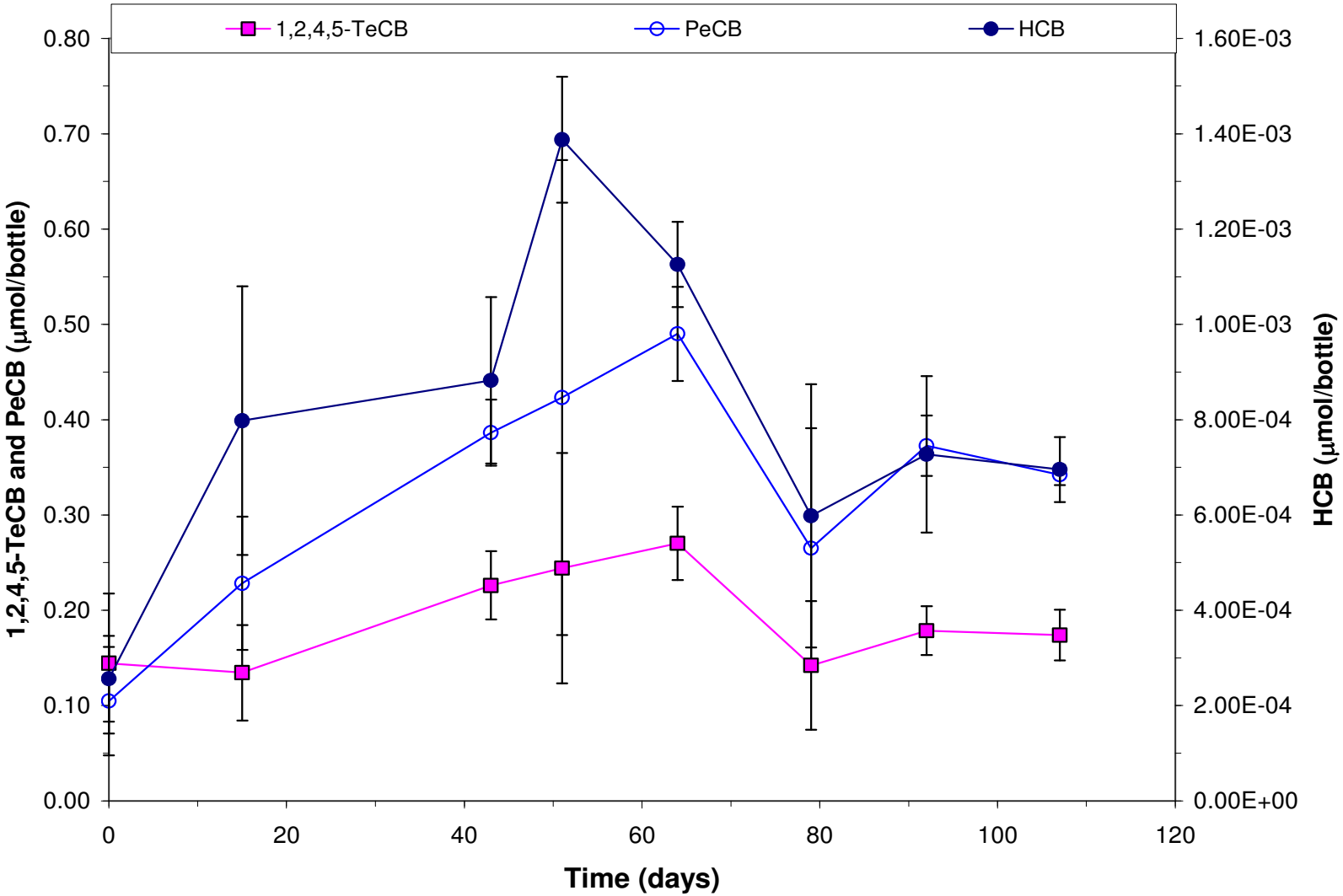

Figure 4.84 Results for the second set of TEA Experiment chlorinated benzenes WCs (average of triplicates). Error bars represent ± one standard deviation.

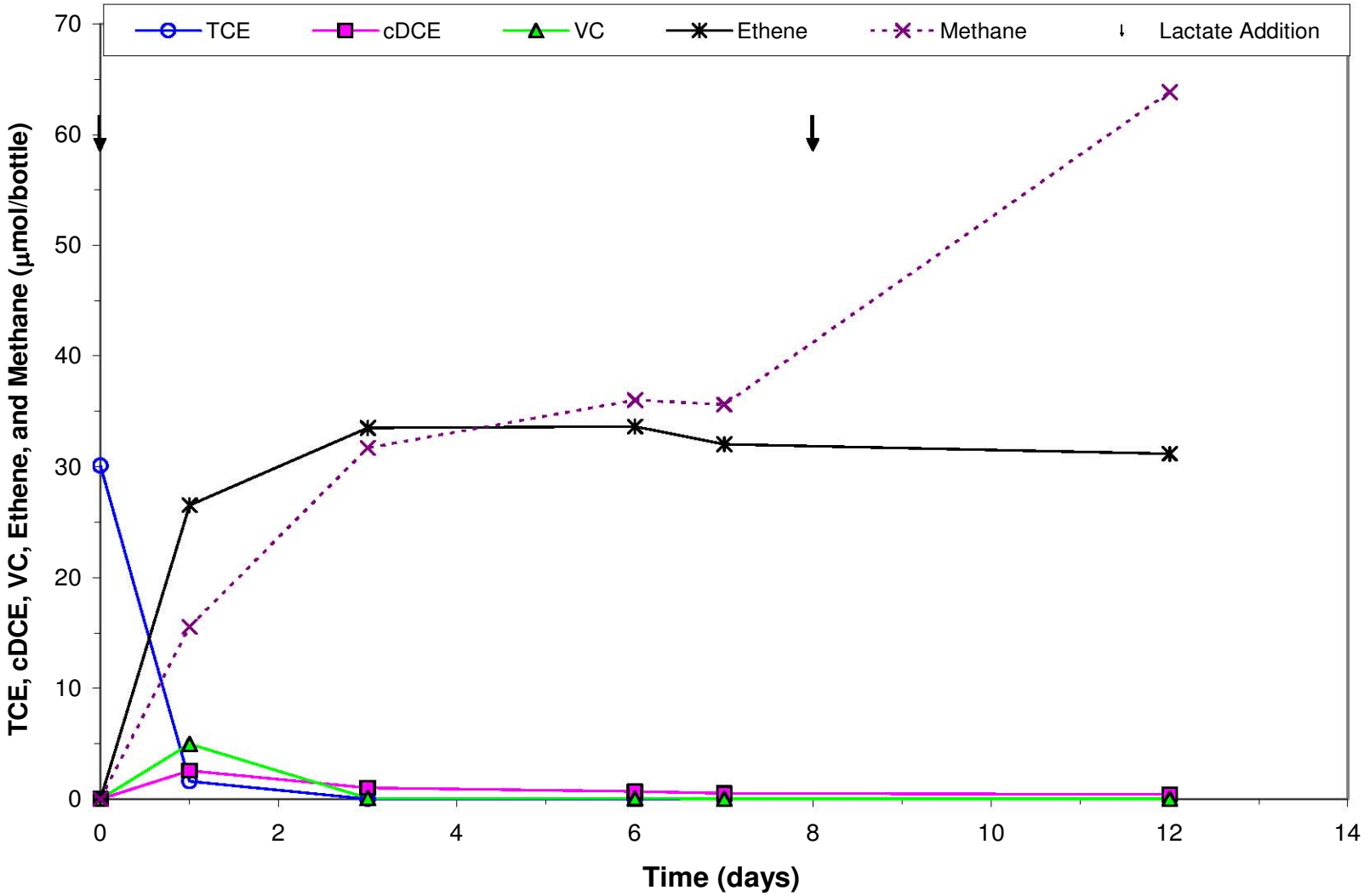

Figure 4.85 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 1 TCE only bottles (TCE only-1).

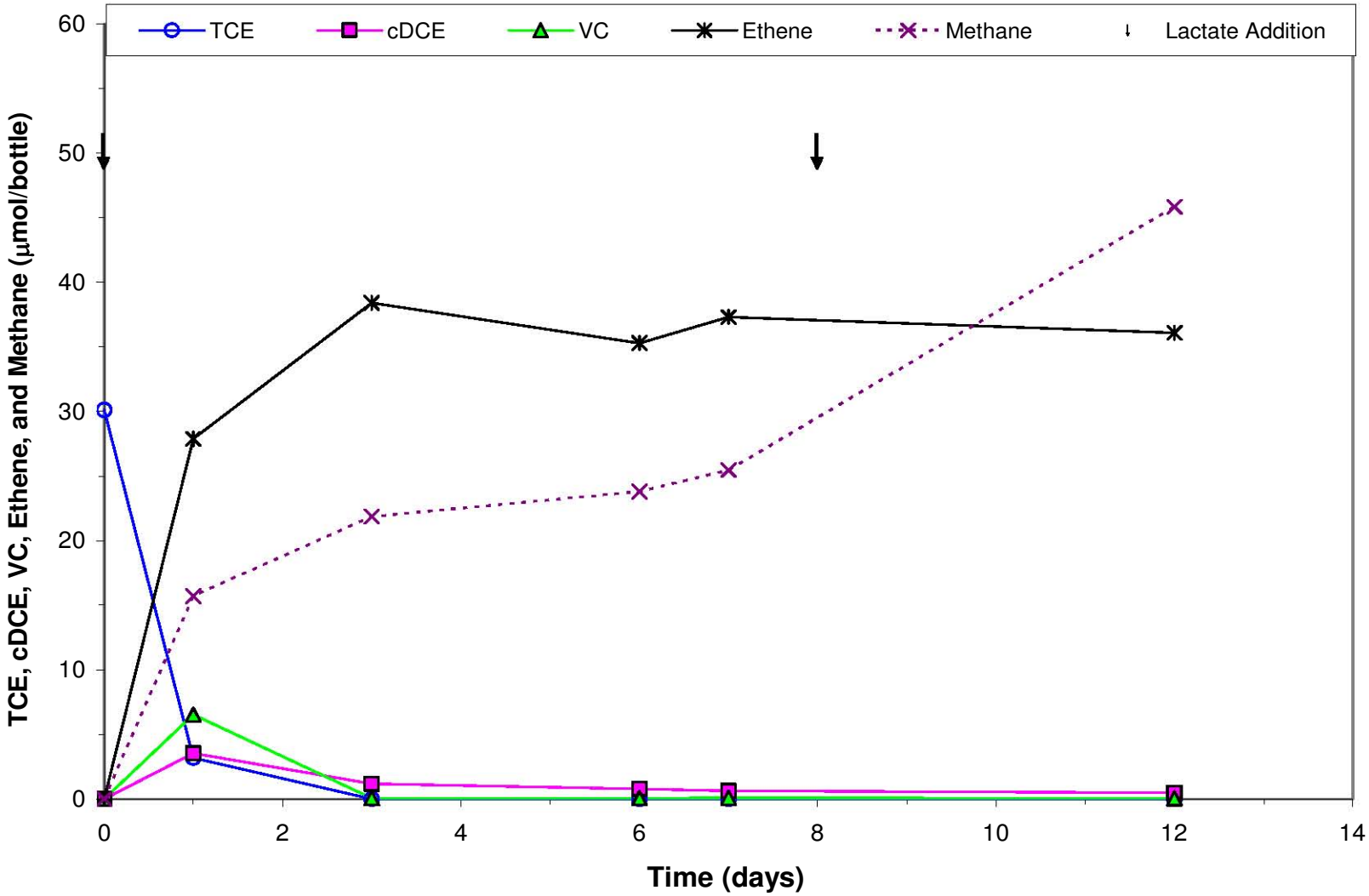

Figure 4.86 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 1 TCE only bottles (TCE only-2).

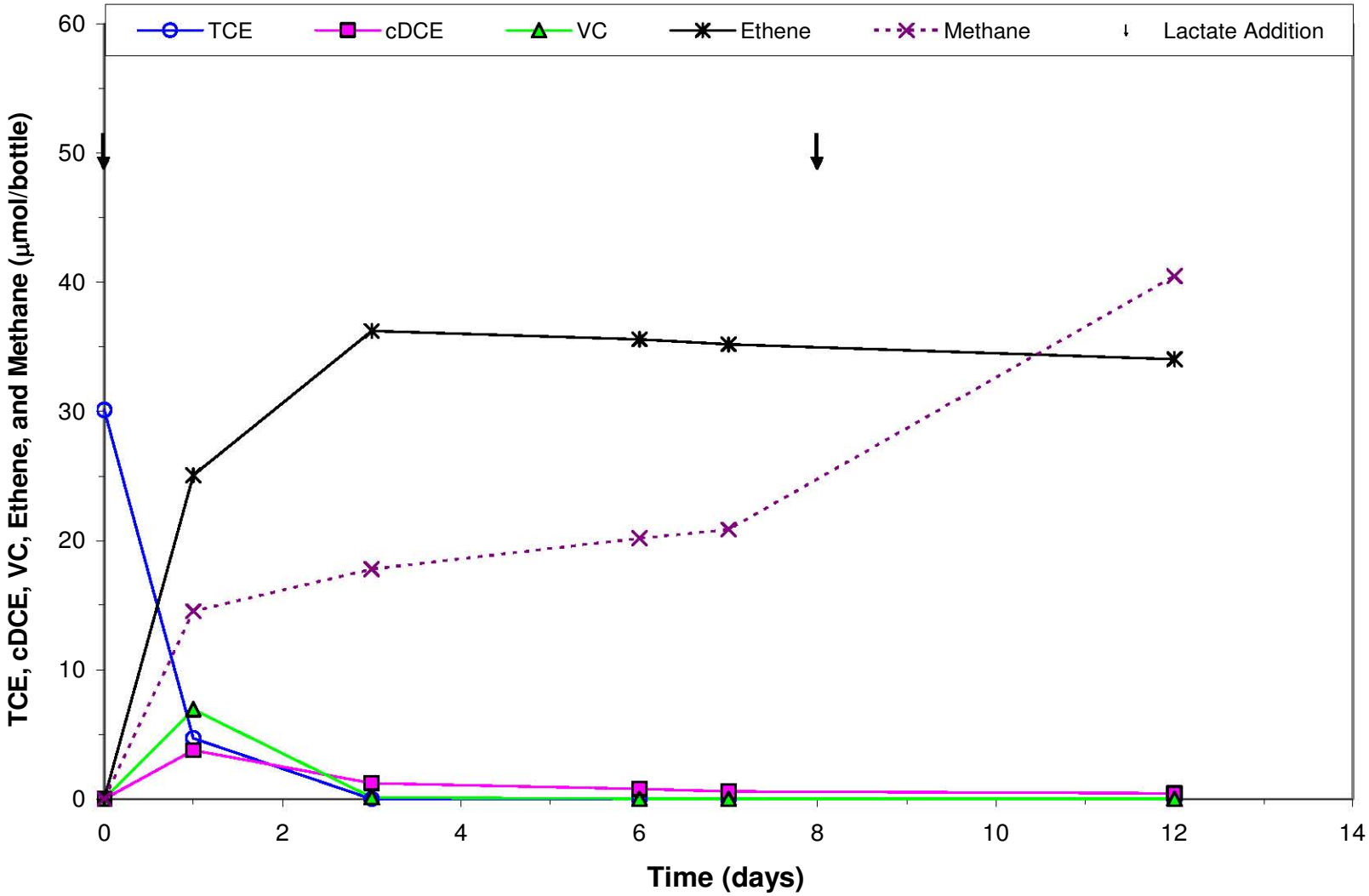

Figure 4.87 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 1 TCE only bottles (TCE only-3).

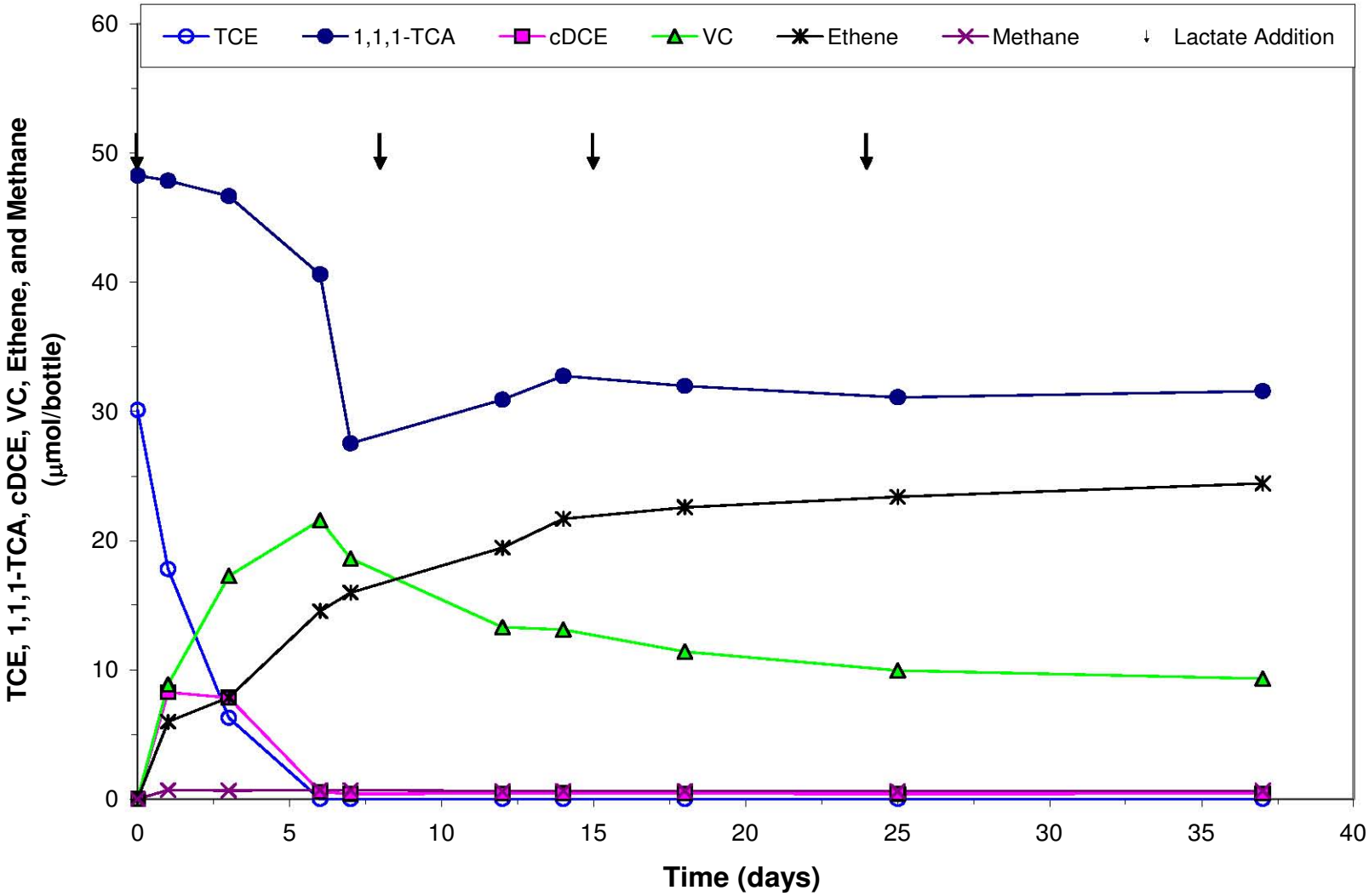

Figure 4.88 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 1 TCE + 1,1,1-TCA bottles (TCE + 1,1,1-TCA-1).

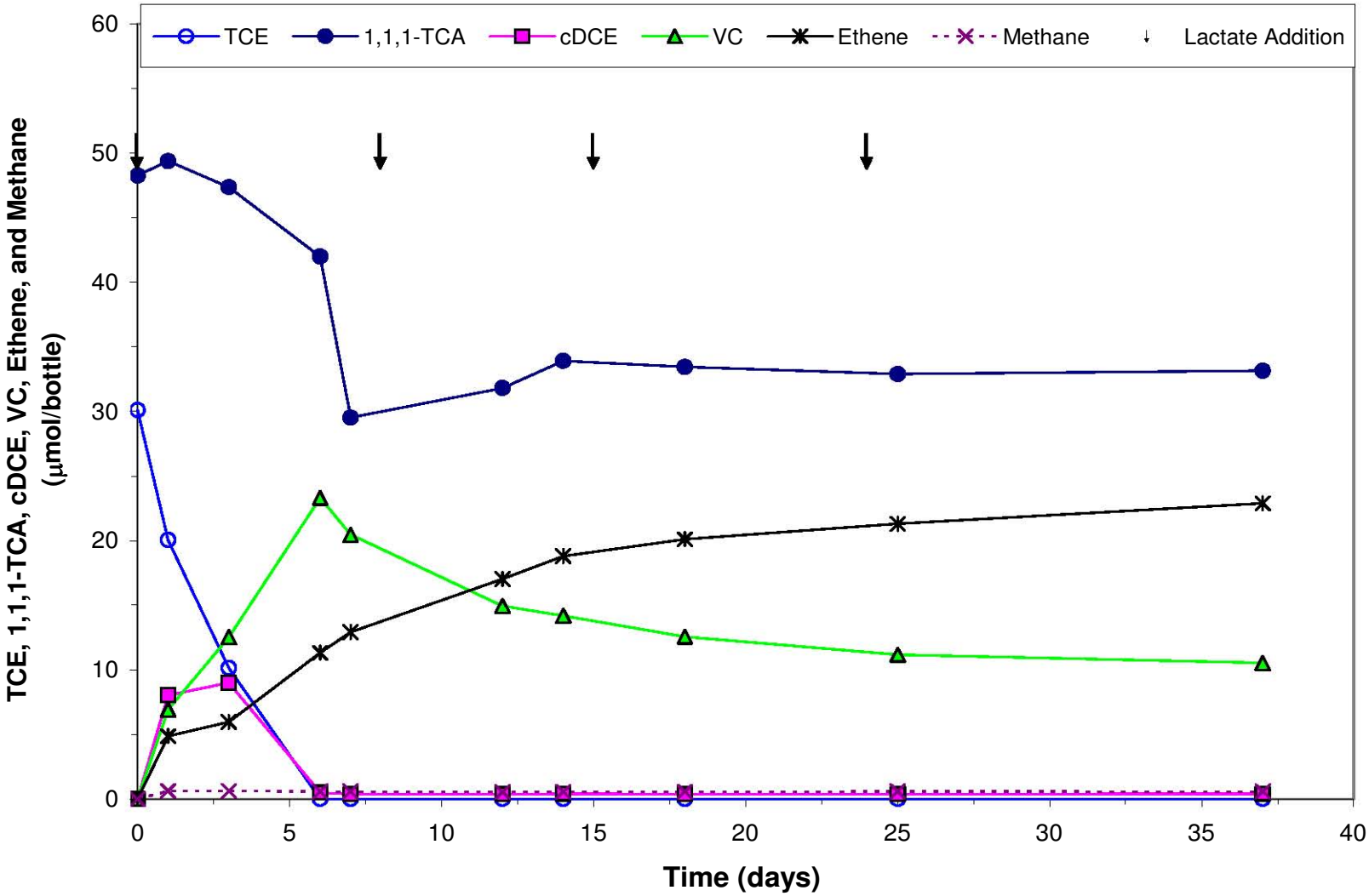

Figure 4.89 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 1 TCE + 1,1,1-TCA bottles (TCE + 1,1,1-TCA-2).

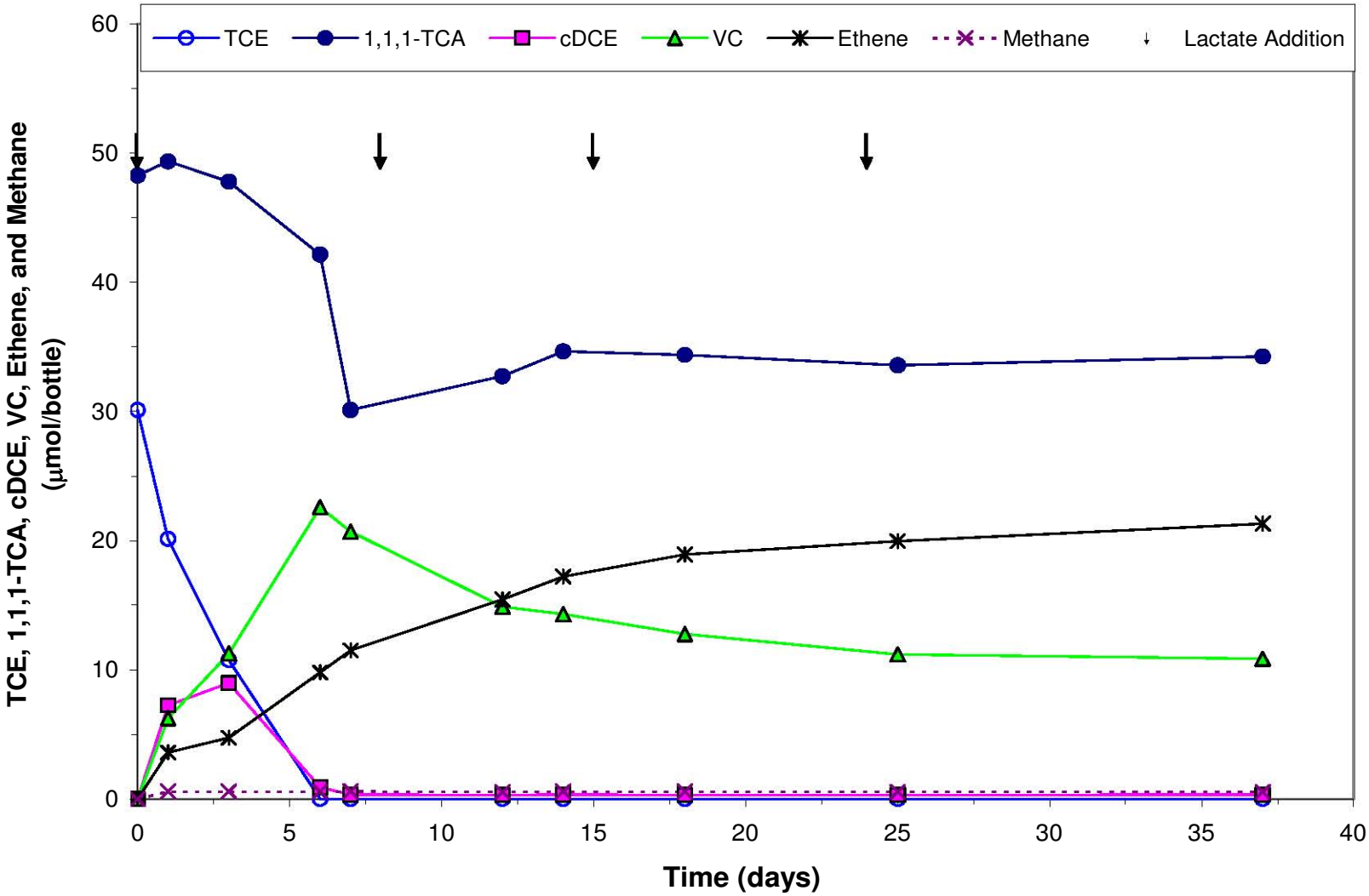

Figure 4.90 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 1 TCE + 1,1,1-TCA bottles (TCE + 1,1,1-TCA-3).

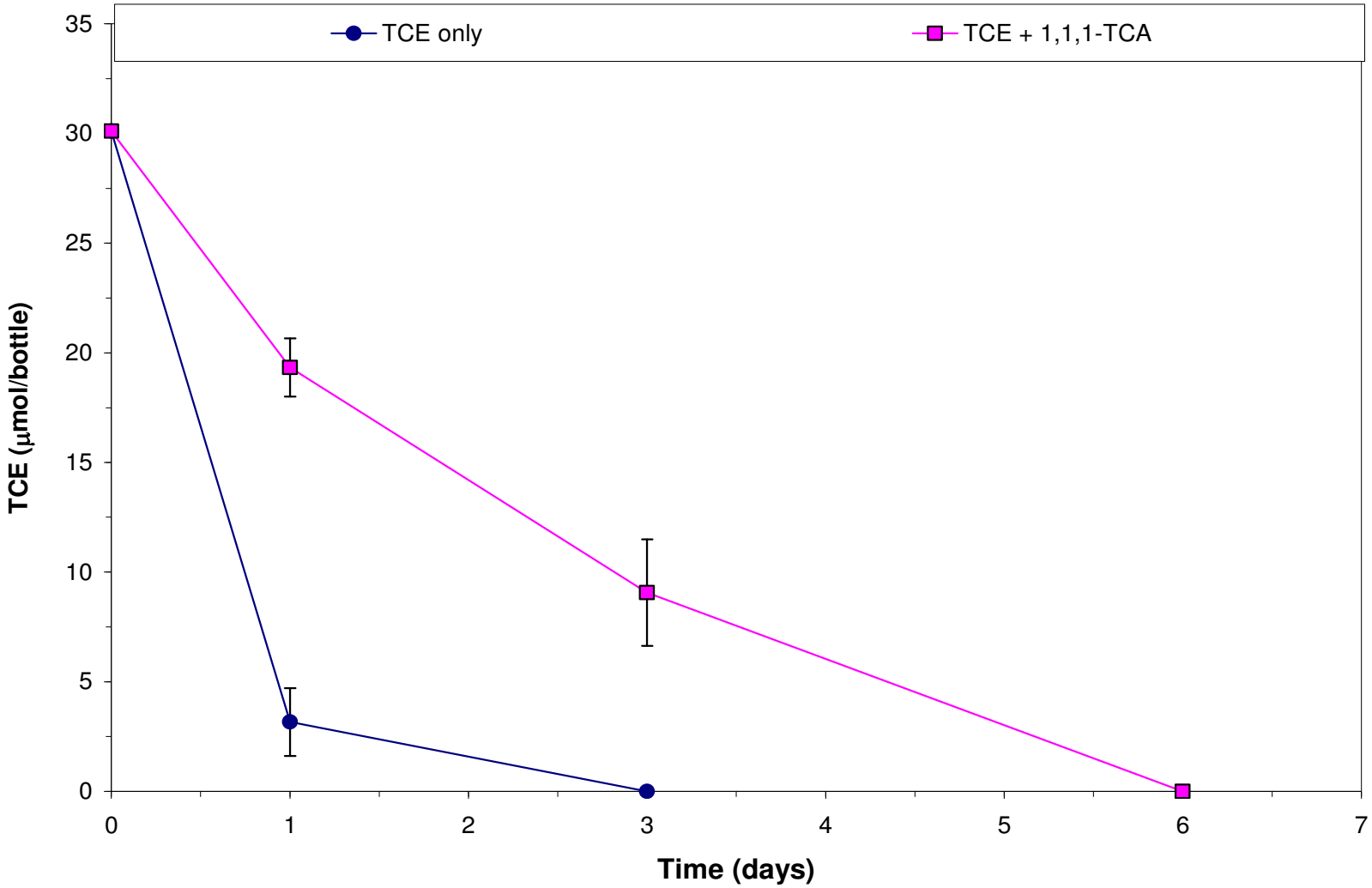

Figure 4.91 Comparison of the effect of 300 µM 1,1,1-TCA on reductive dechlorination of TCE (averages of triplicates). Error bars represent ± one standard deviation.

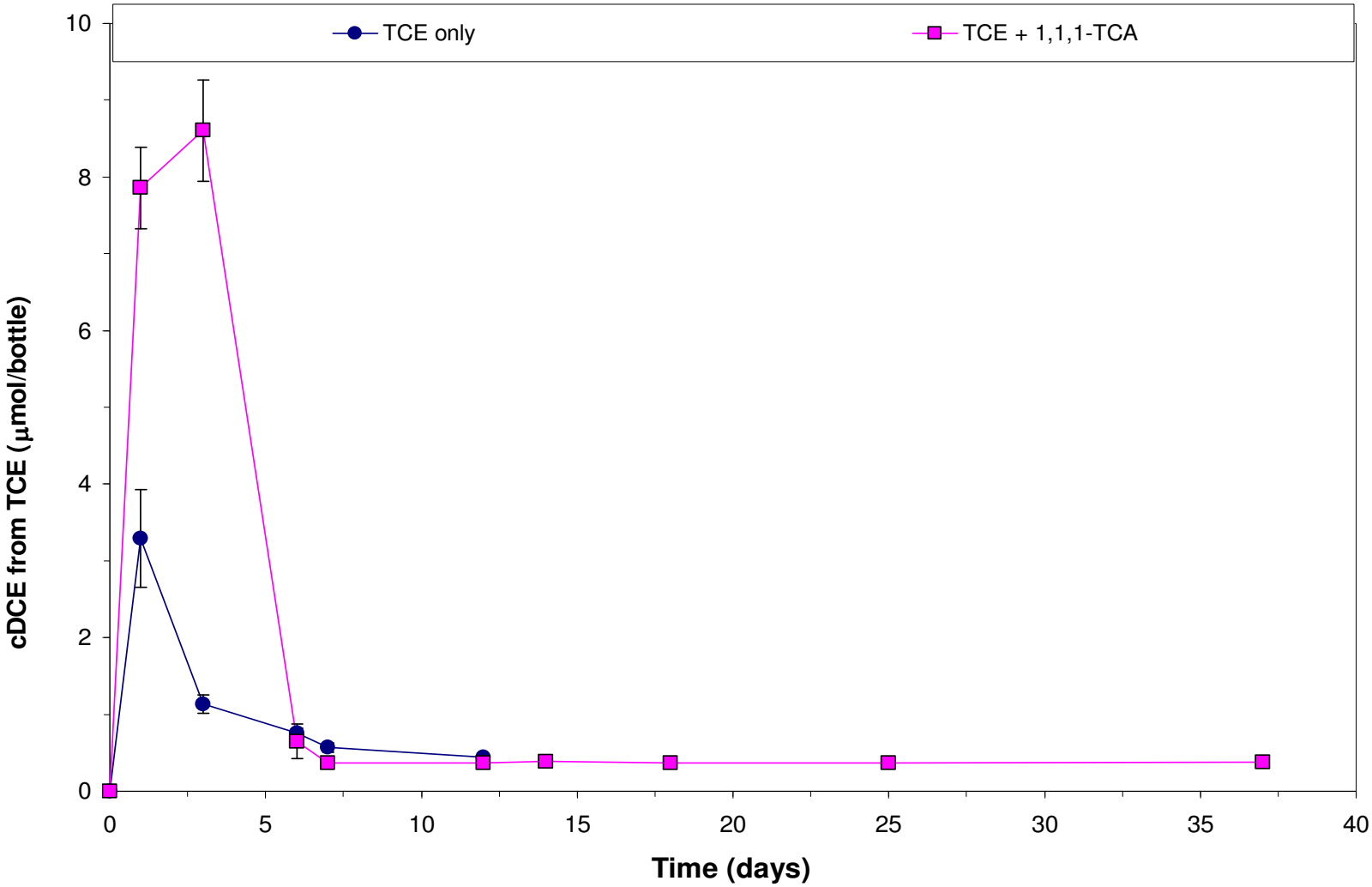

Figure 4.92 Comparison of the effect of 300 µM 1,1,1-TCA on reductive dechlorination of cDCE (averages of triplicates). Error bars represent ± one standard deviation.

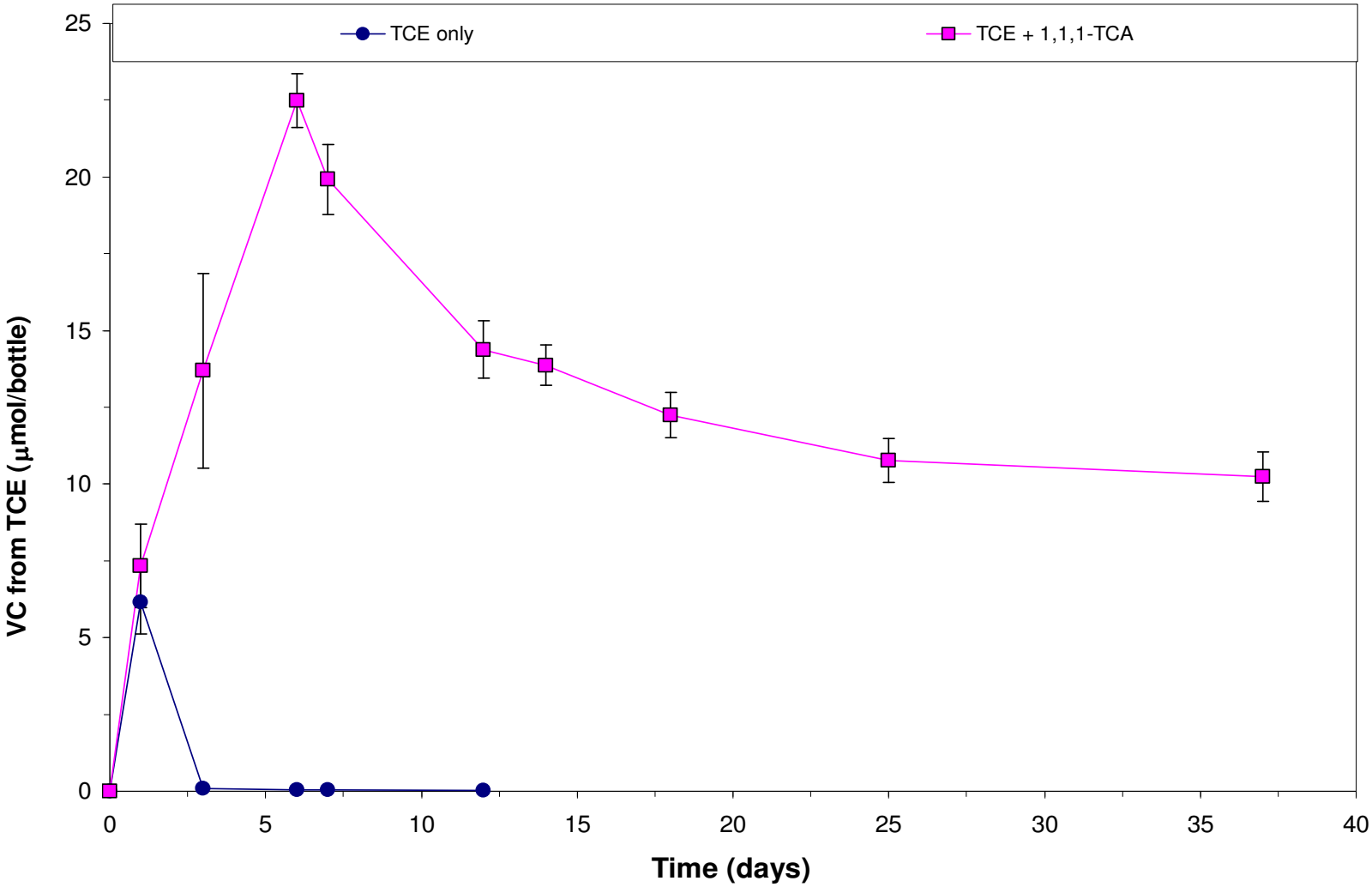

Figure 4.93 Comparison of the effect of 300 µM 1,1,1-TCA on reductive dechlorination of VC (averages of triplicates). Error bars represent ± one standard deviation.

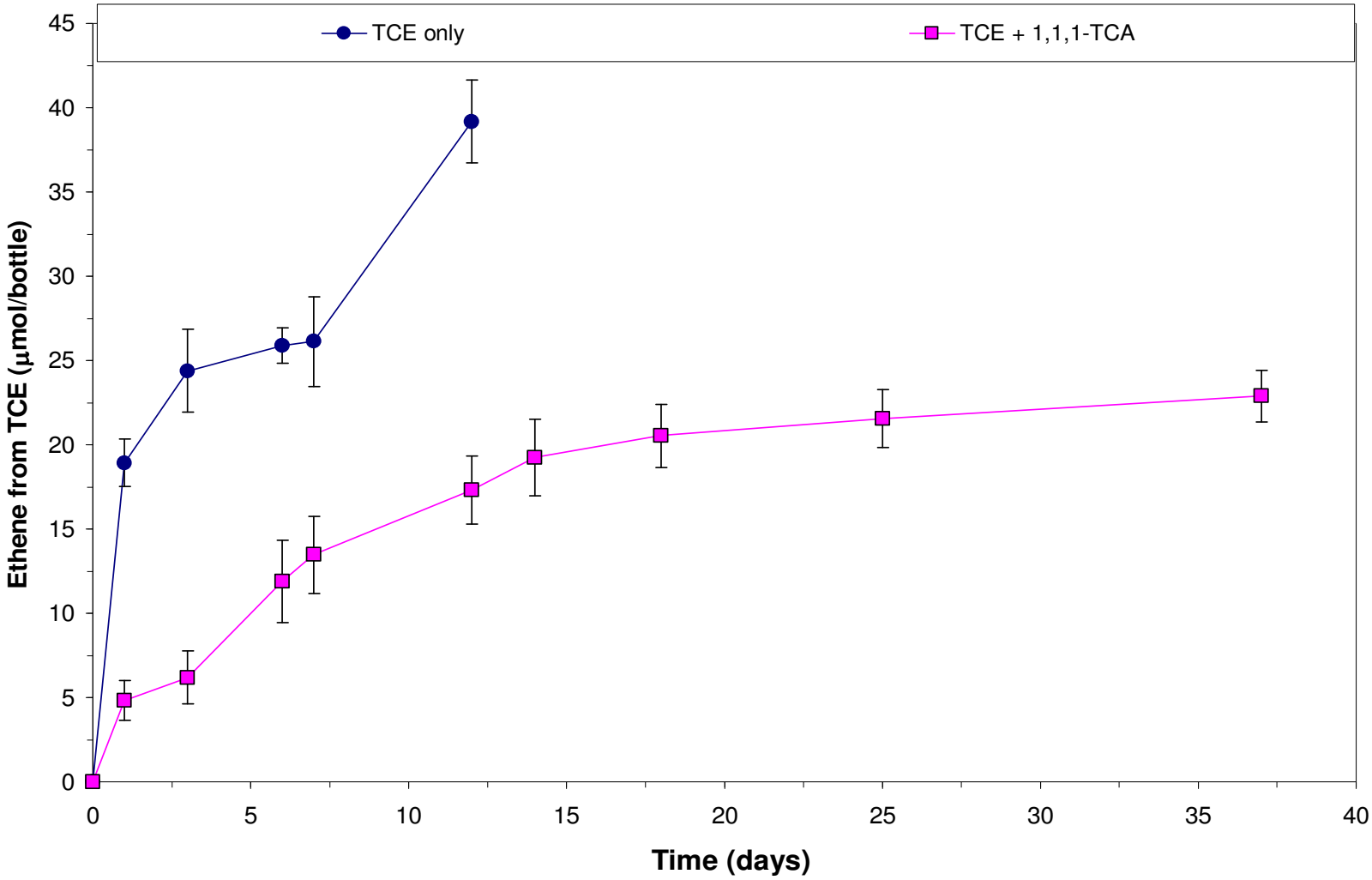

Figure 4.94 Comparison of the effect of 300 µM 1,1,1-TCA on production of ethene (averages of triplicates). Error bars represent ± one standard deviation.

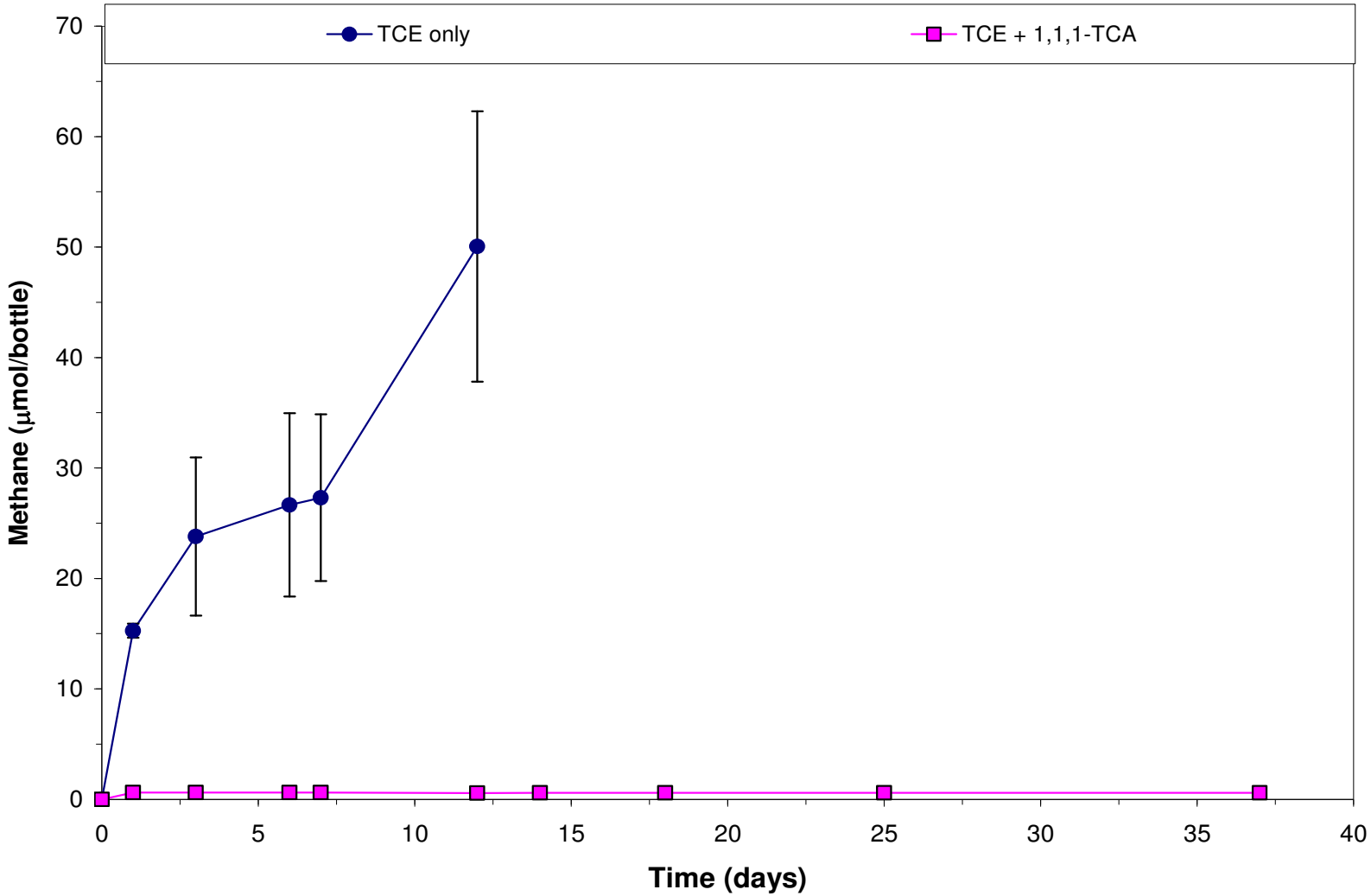

Figure 4.95 Comparison of the effect of 300 µM 1,1,1-TCA on production of methane (averages of triplicates). Error bars represent ± one standard deviation.

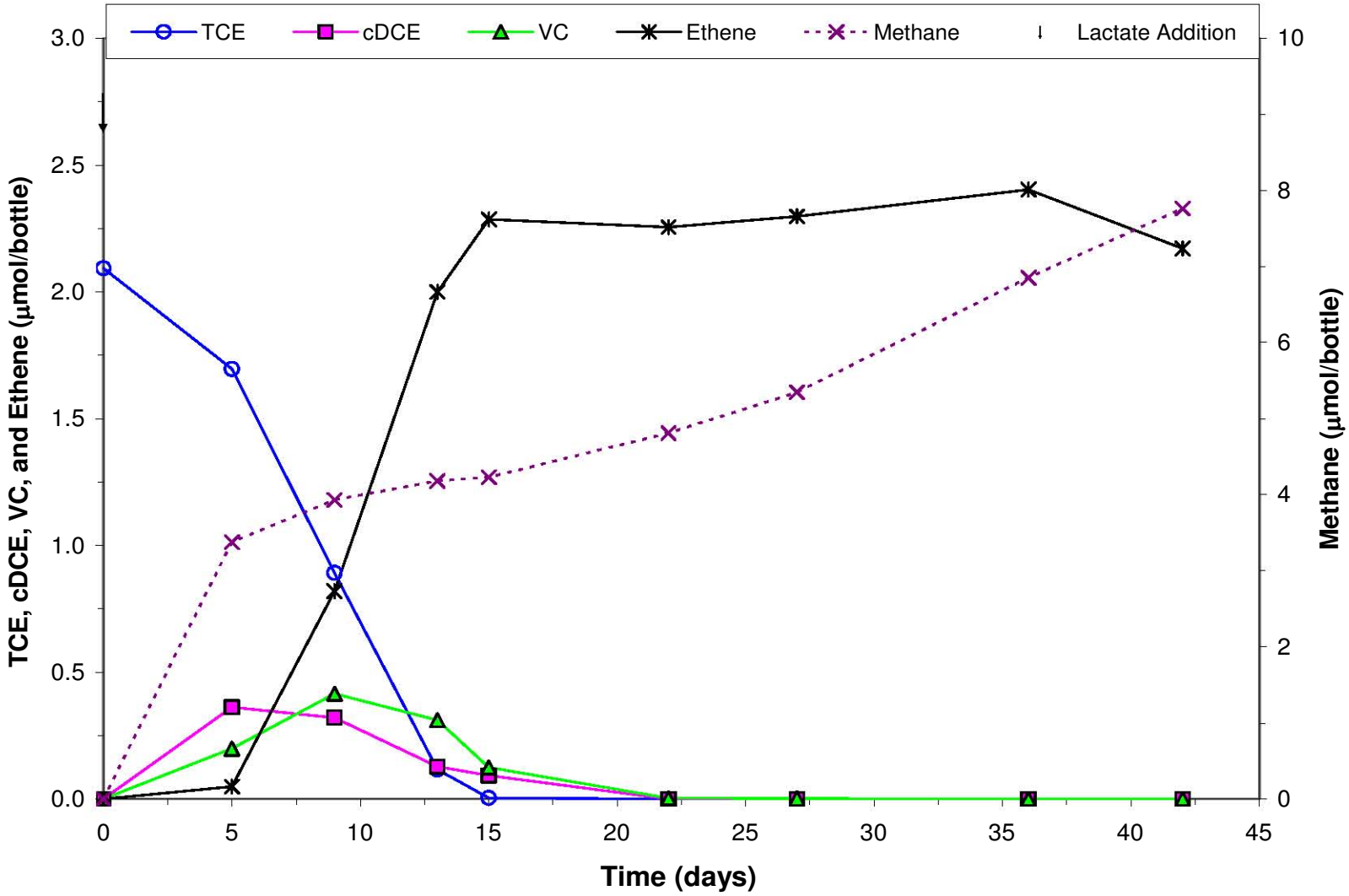

Figure 4.96 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 2 TCE only bottles (TCE only-1).

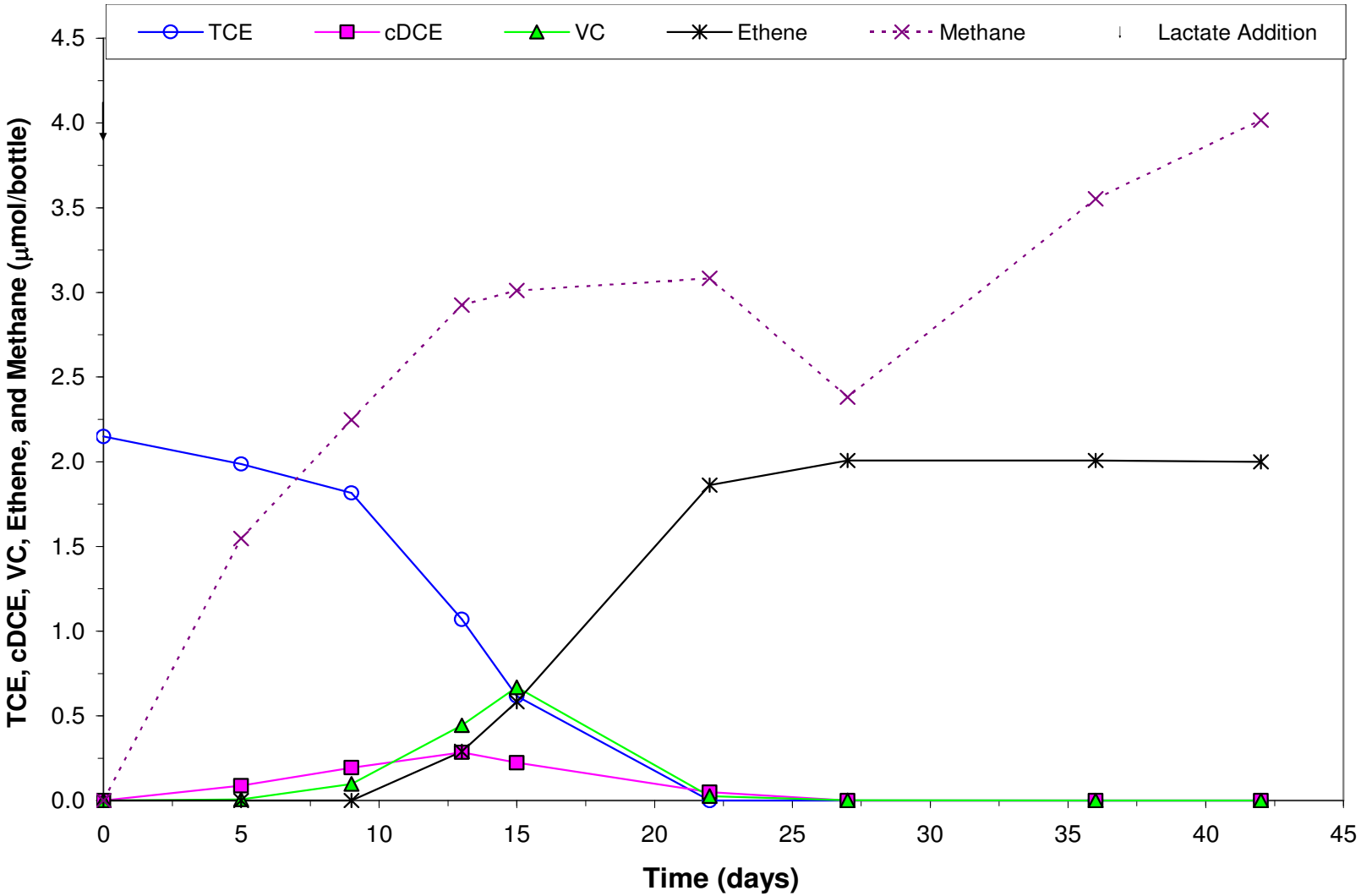

Figure 4.97 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 2 TCE only bottles (TCE only-2).

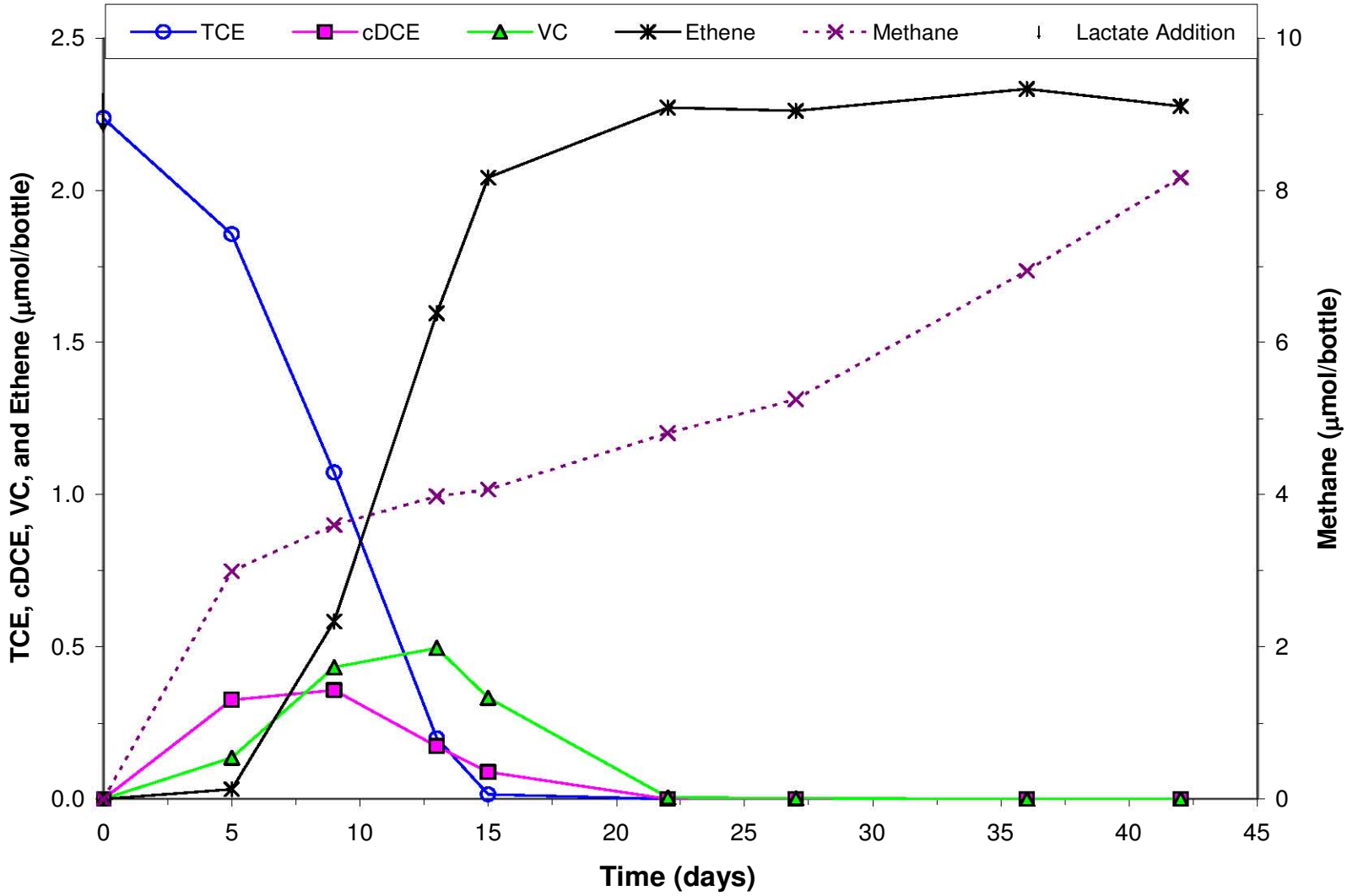

Figure 4.98 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 2 TCE only bottles (TCE only-3).

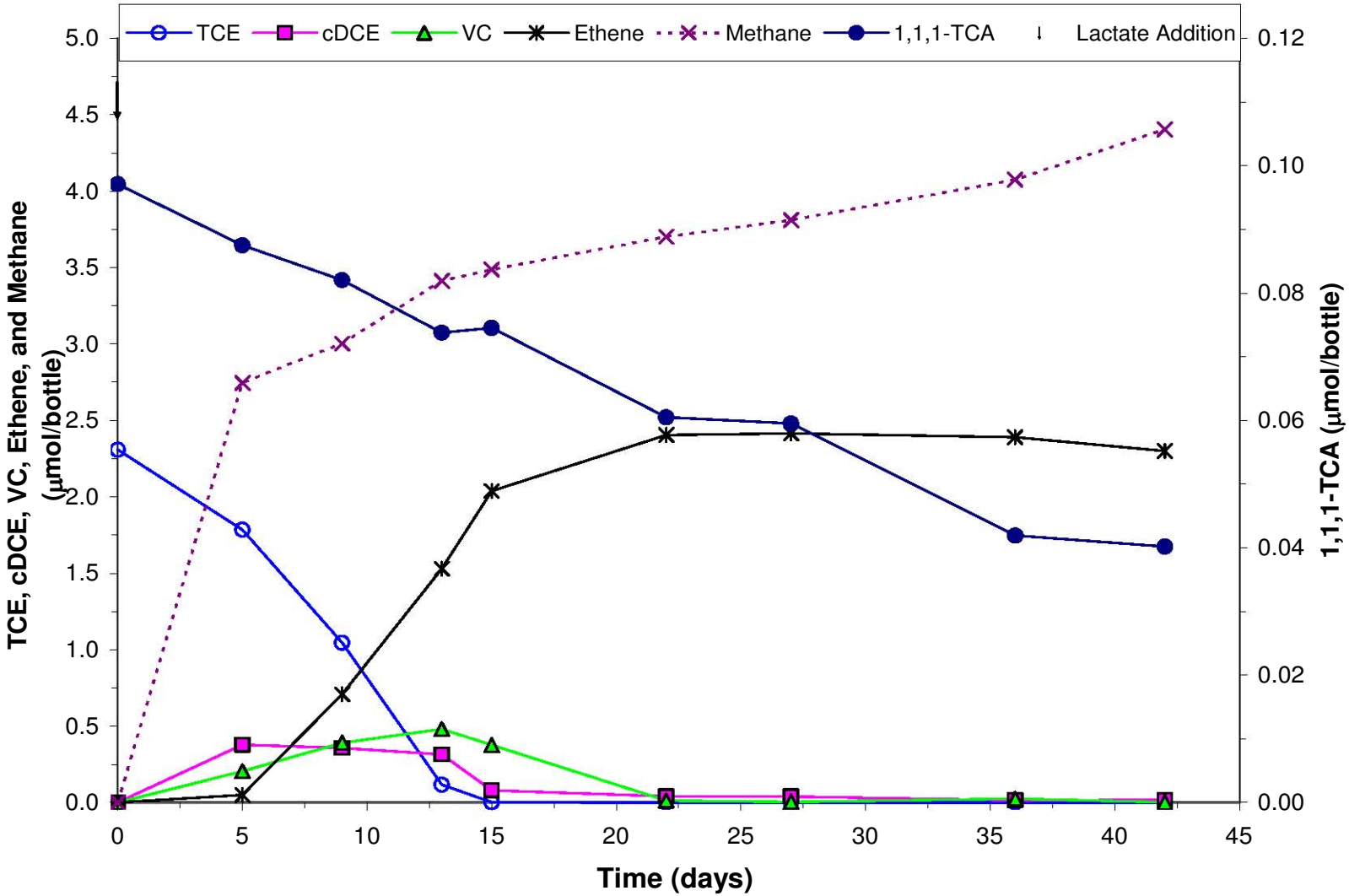

Figure 4.99 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 2 31 µM TCE + 0.7 µM 1,1,1-TCA bottles (31 µM TCE + 0.7 µM 1,1,1-TCA-1).

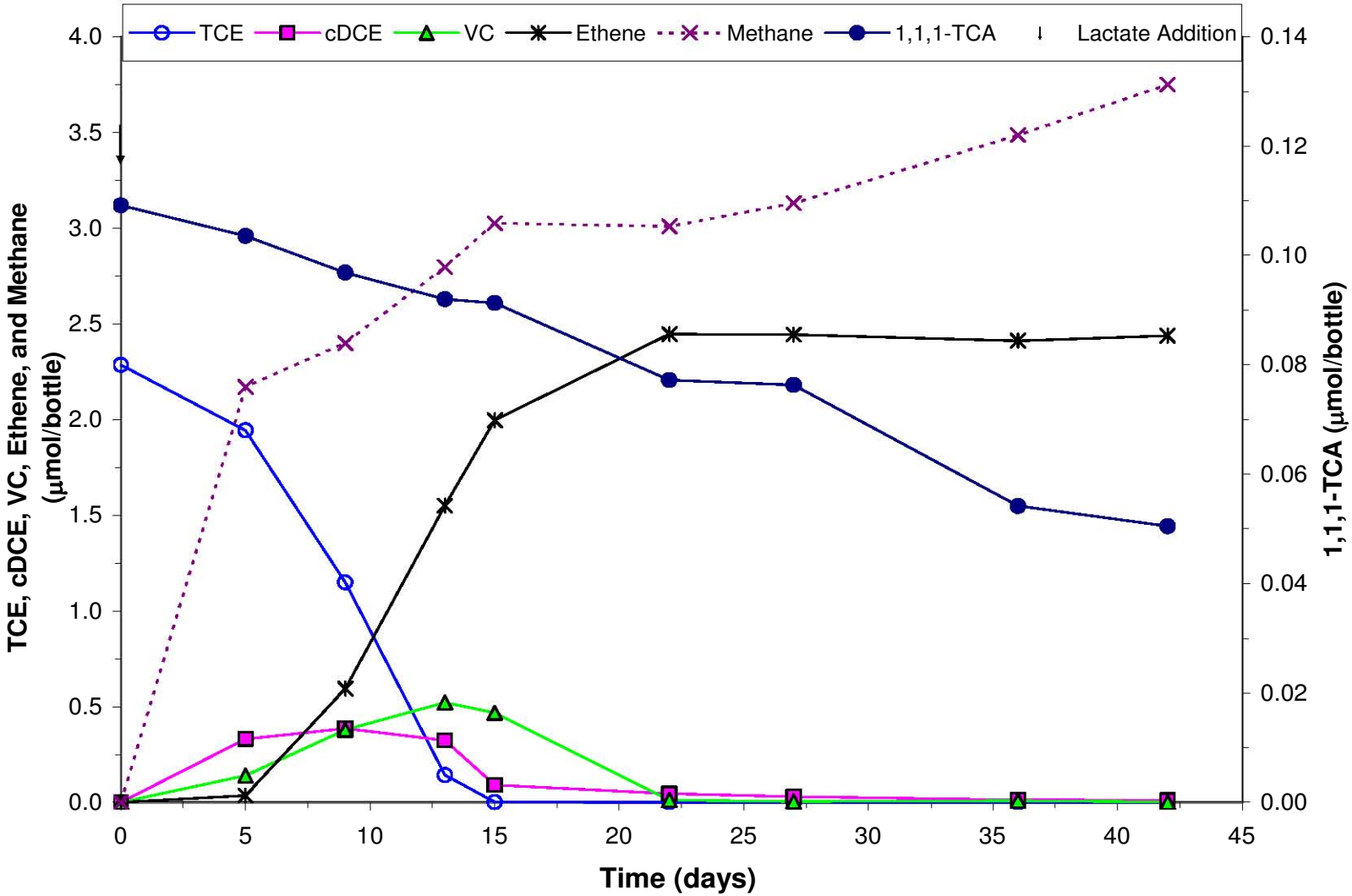

Figure 4.100 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 2 31 µM TCE + 0.7 µM 1,1,1-TCA bottles (31 µM TCE + 0.7 µM 1,1,1-TCA-2).

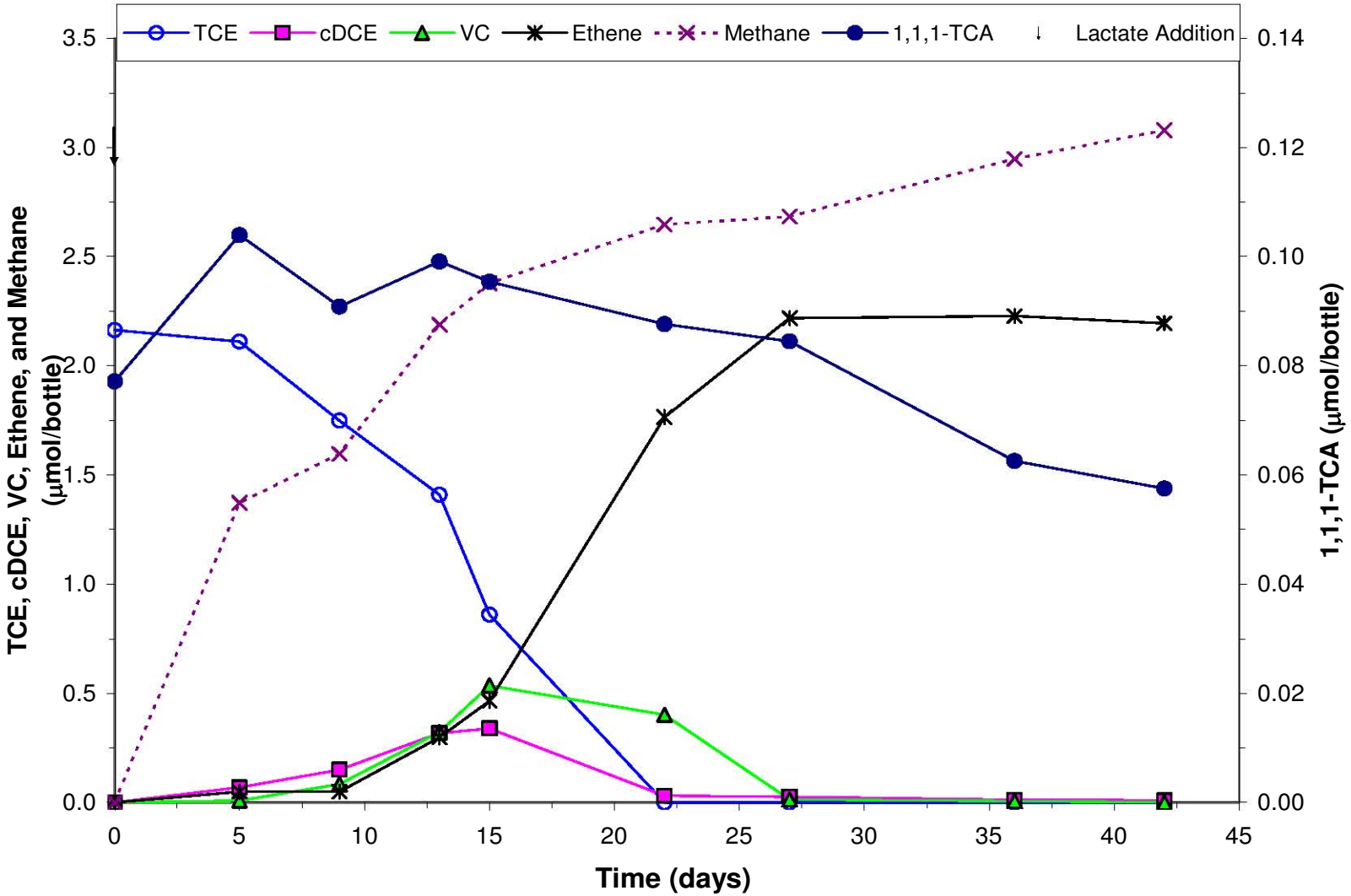

Figure 4.101 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 2 31 µM TCE + 0.7 µM 1,1,1-TCA bottles (31 µM TCE + 0.7 µM 1,1,1-TCA-3).

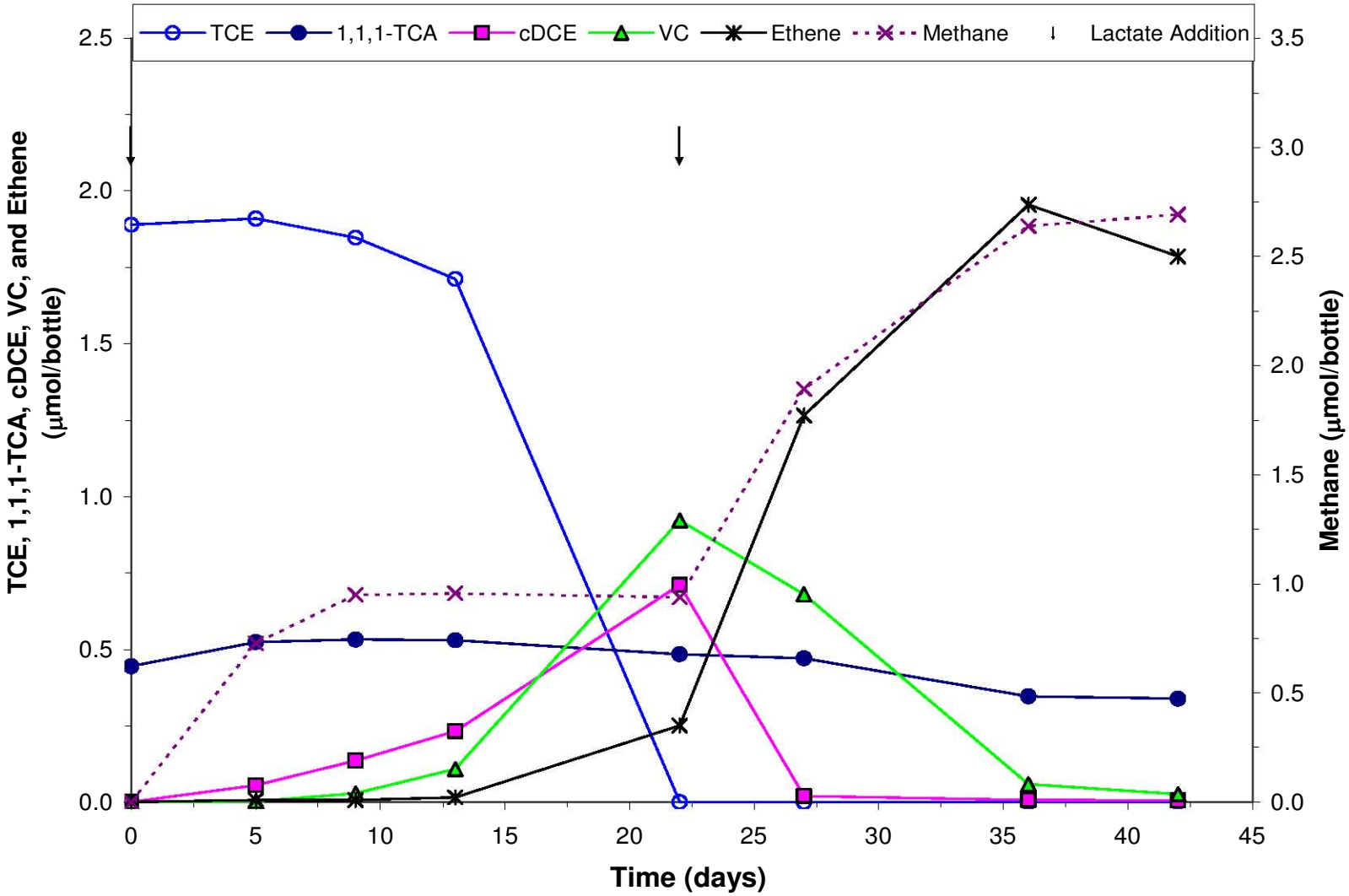

Figure 4.102 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 2 31 µM TCE + 3.6 µM 1,1,1-TCA bottles (31 µM TCE + 3.6 µM 1,1,1-TCA-1).

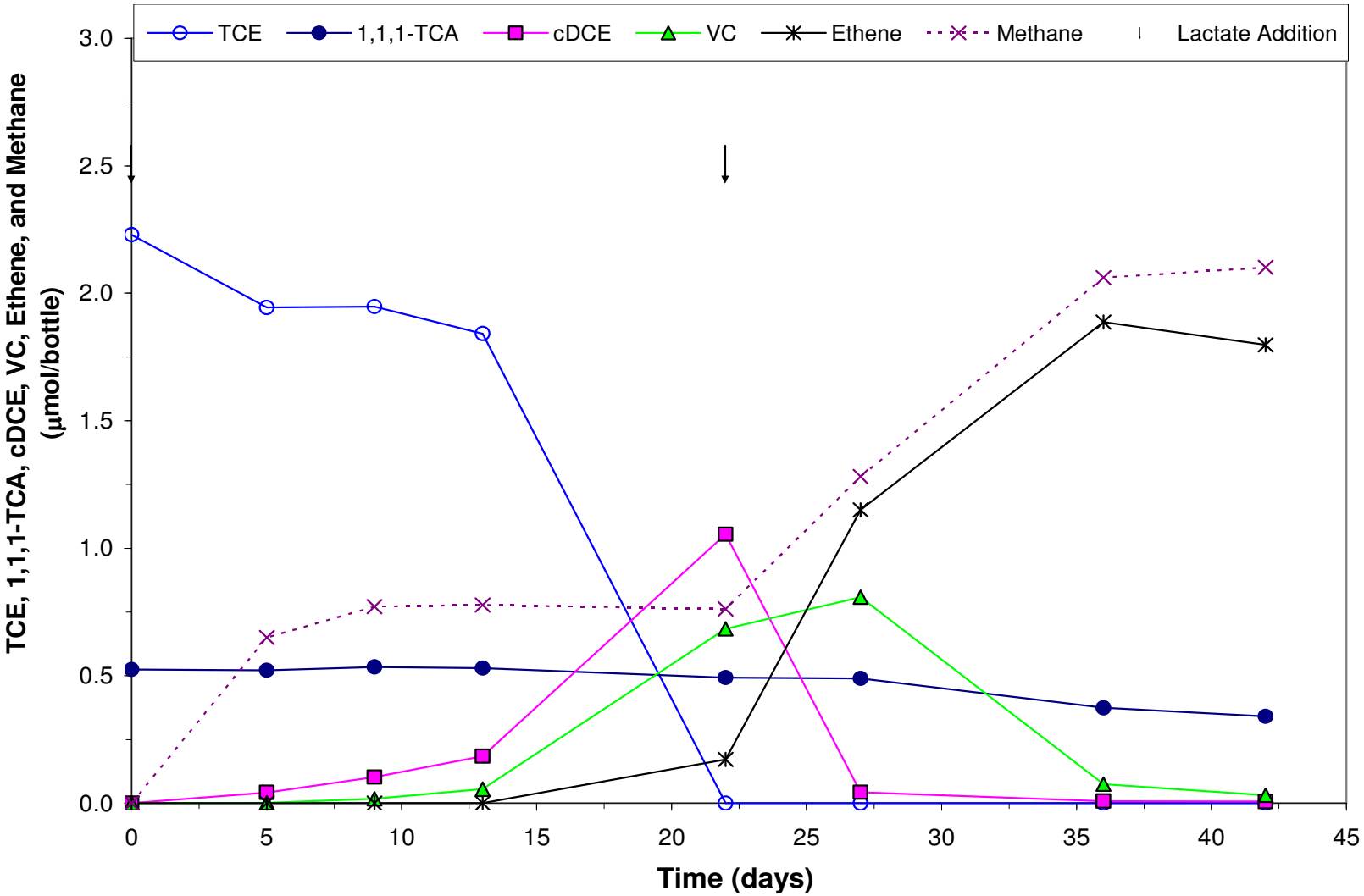

Figure 4.103 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 2 31 µM TCE + 3.6 µM 1,1,1-TCA bottles (31 µM TCE + 3.6 µM 1,1,1-TCA-2).

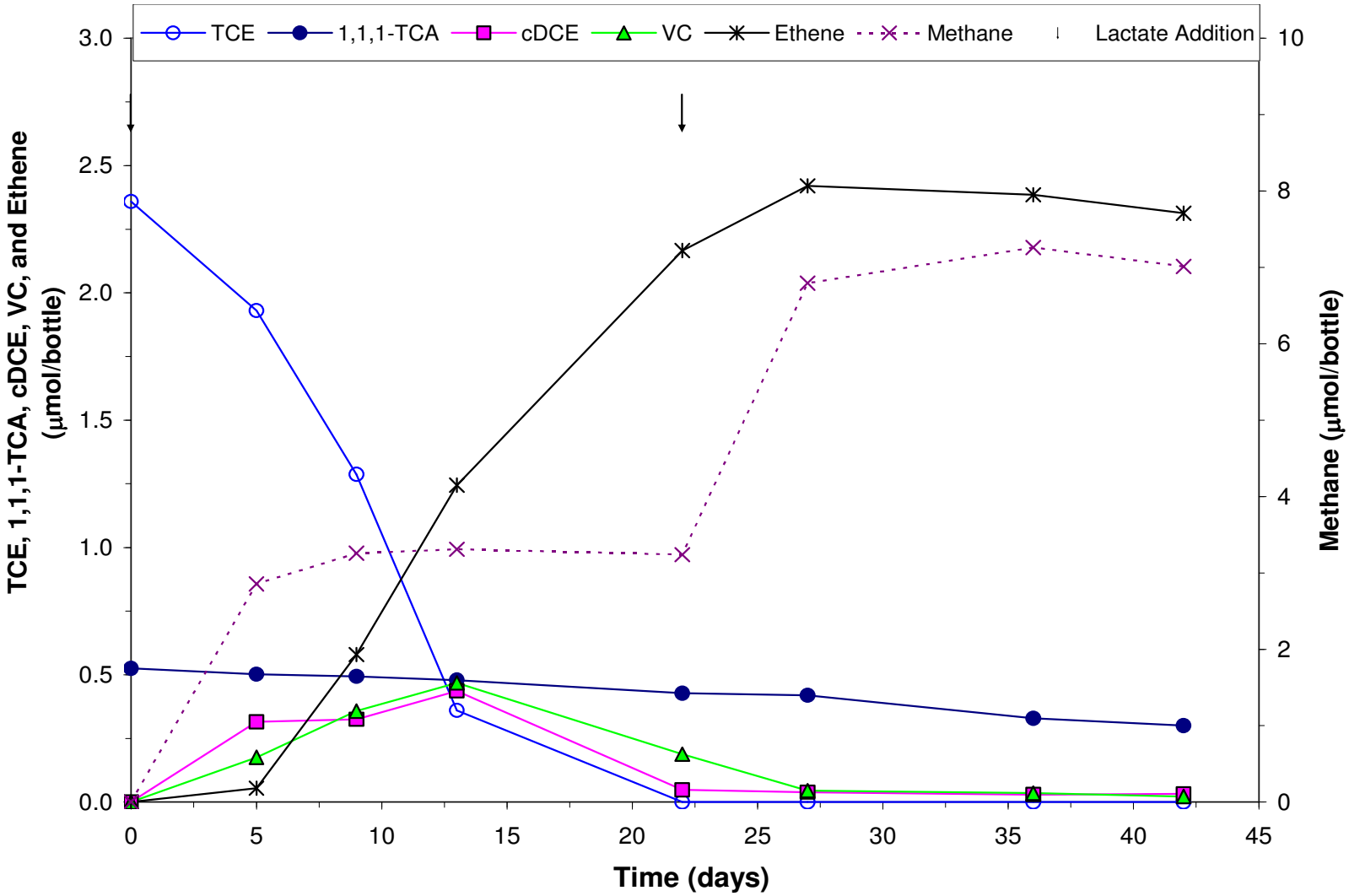

Figure 4.104 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 2 31 µM TCE + 3.6 µM 1,1,1-TCA bottles (31 µM TCE + 3.6 µM 1,1,1-TCA-3).

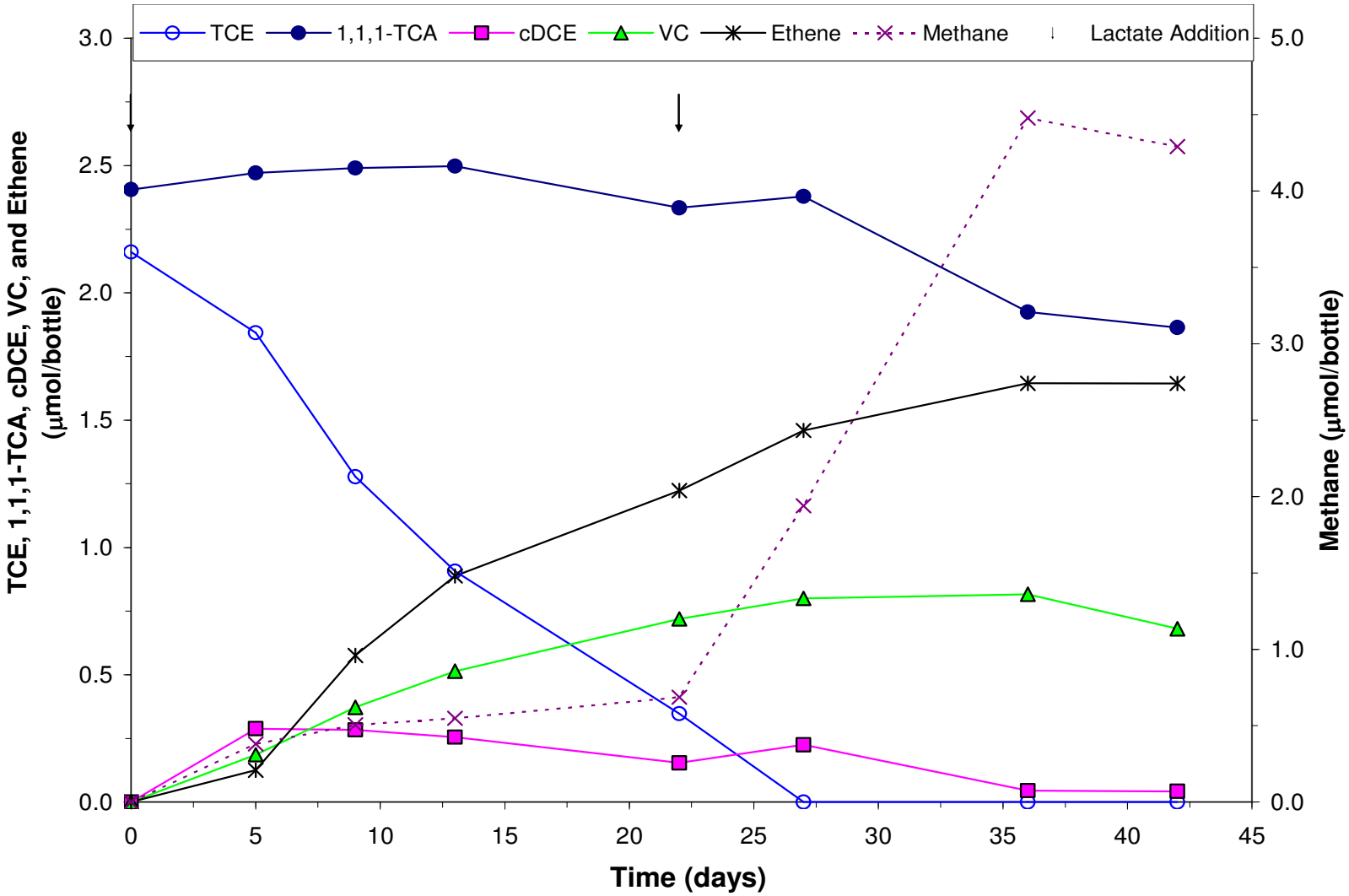

Figure 4.105 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 2 31 µM TCE + 18 µM 1,1,1-TCA bottles (31 µM TCE + 18 µM 1,1,1-TCA-1).

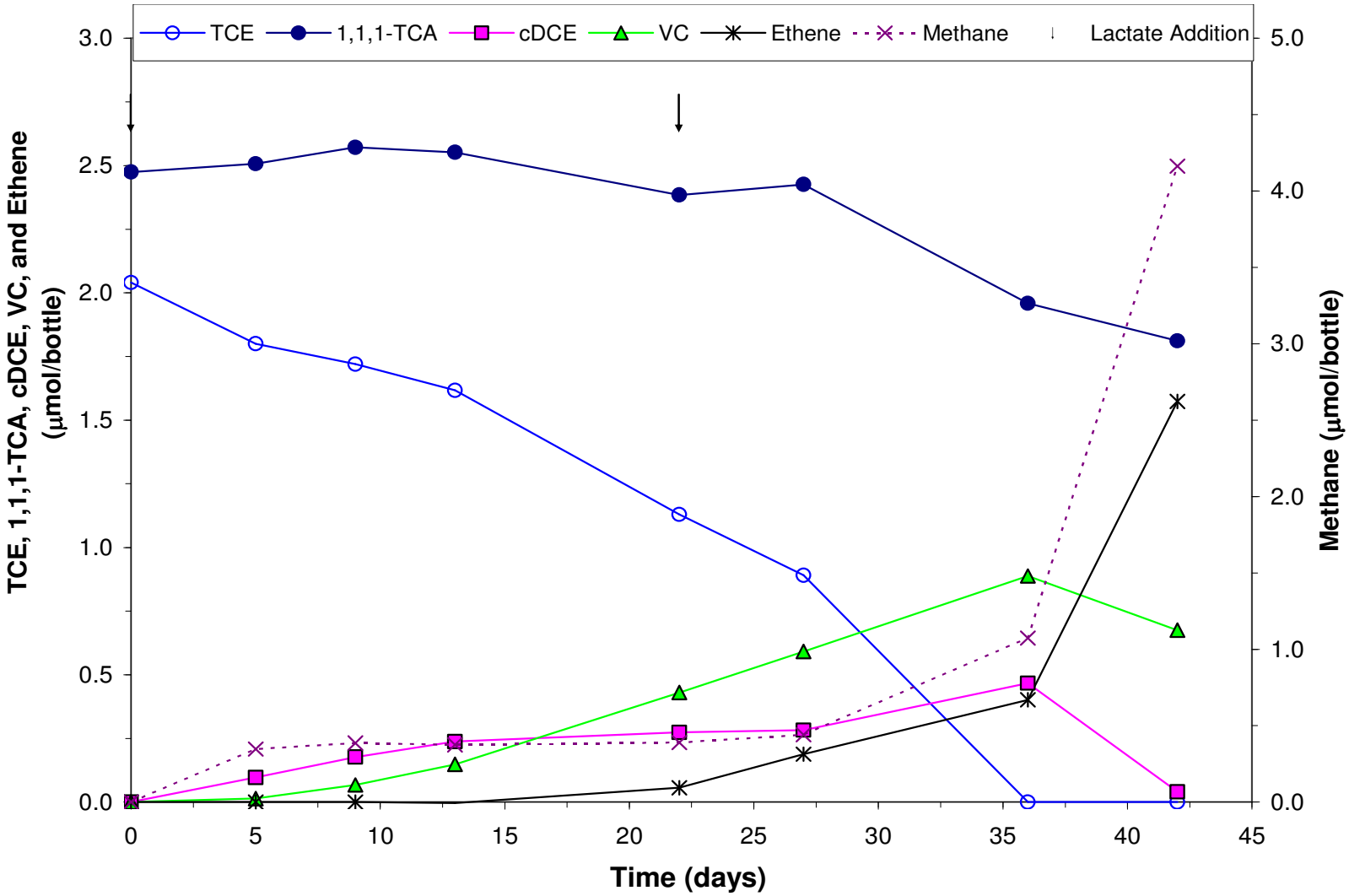

Figure 4.106 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 2 31 µM TCE + 18 µM 1,1,1-TCA bottles (31 µM TCE + 18 µM 1,1,1-TCA-2).

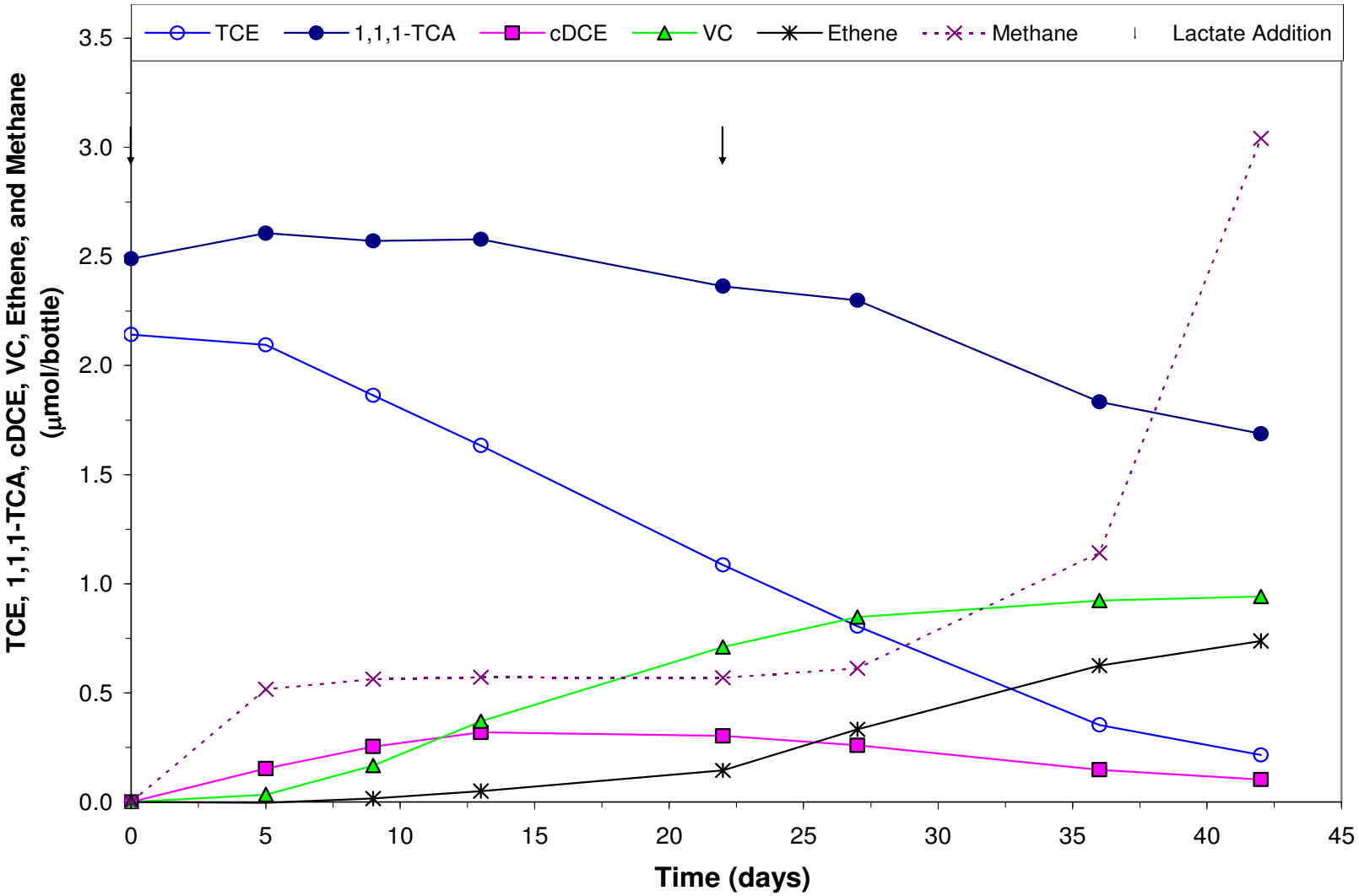

Figure 4.107 Results for one of the 1,1,1-TCA Inhibition Experiment Phase 2 31 µM TCE + 18 µM 1,1,1-TCA bottles (31 µM TCE + 18 µM 1,1,1-TCA-3).

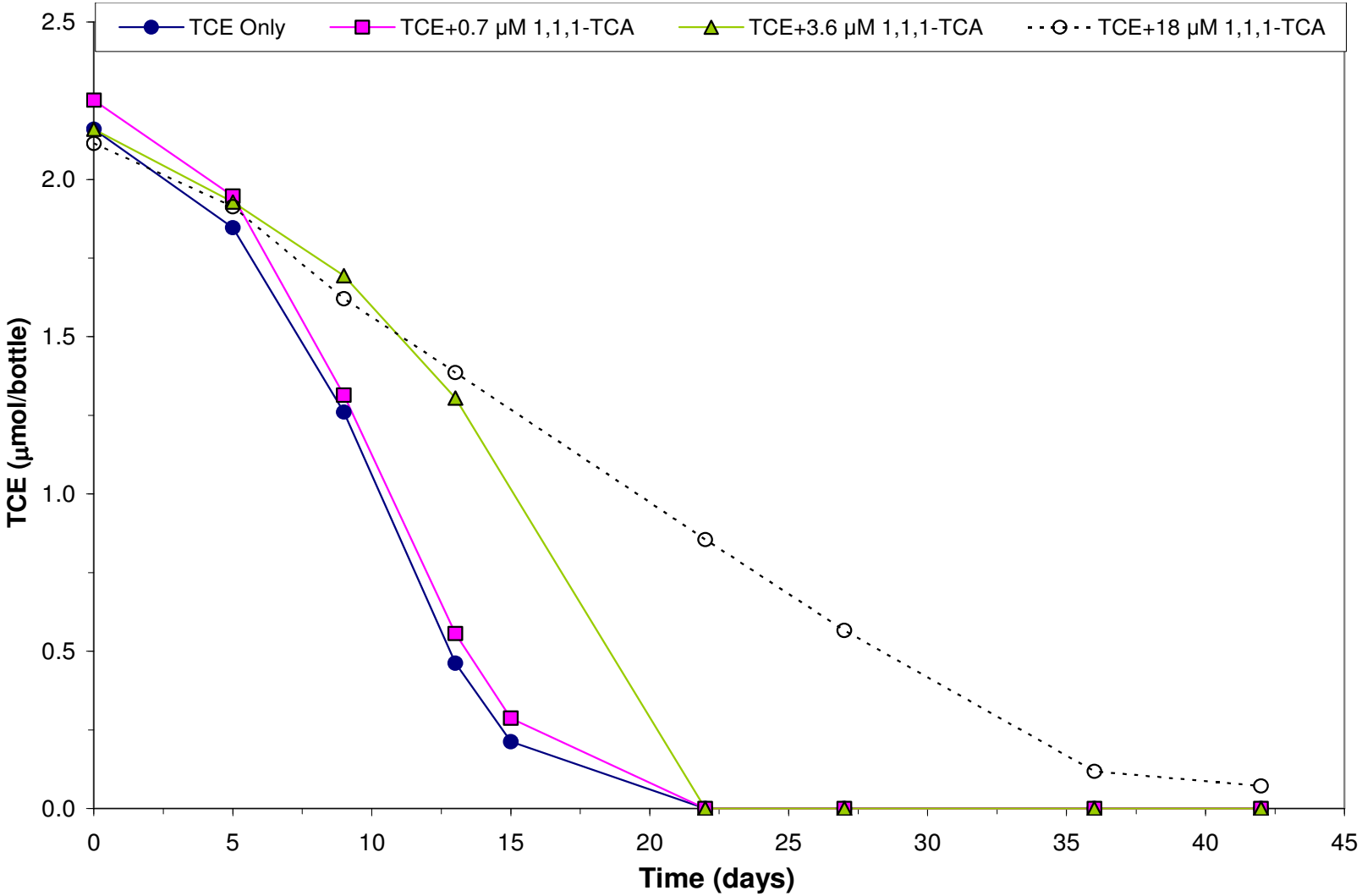

Figure 4.108 Comparison of the effect of 1,1,1-TCA on reductive dechlorination of TCE at all levels (averages of triplicates).

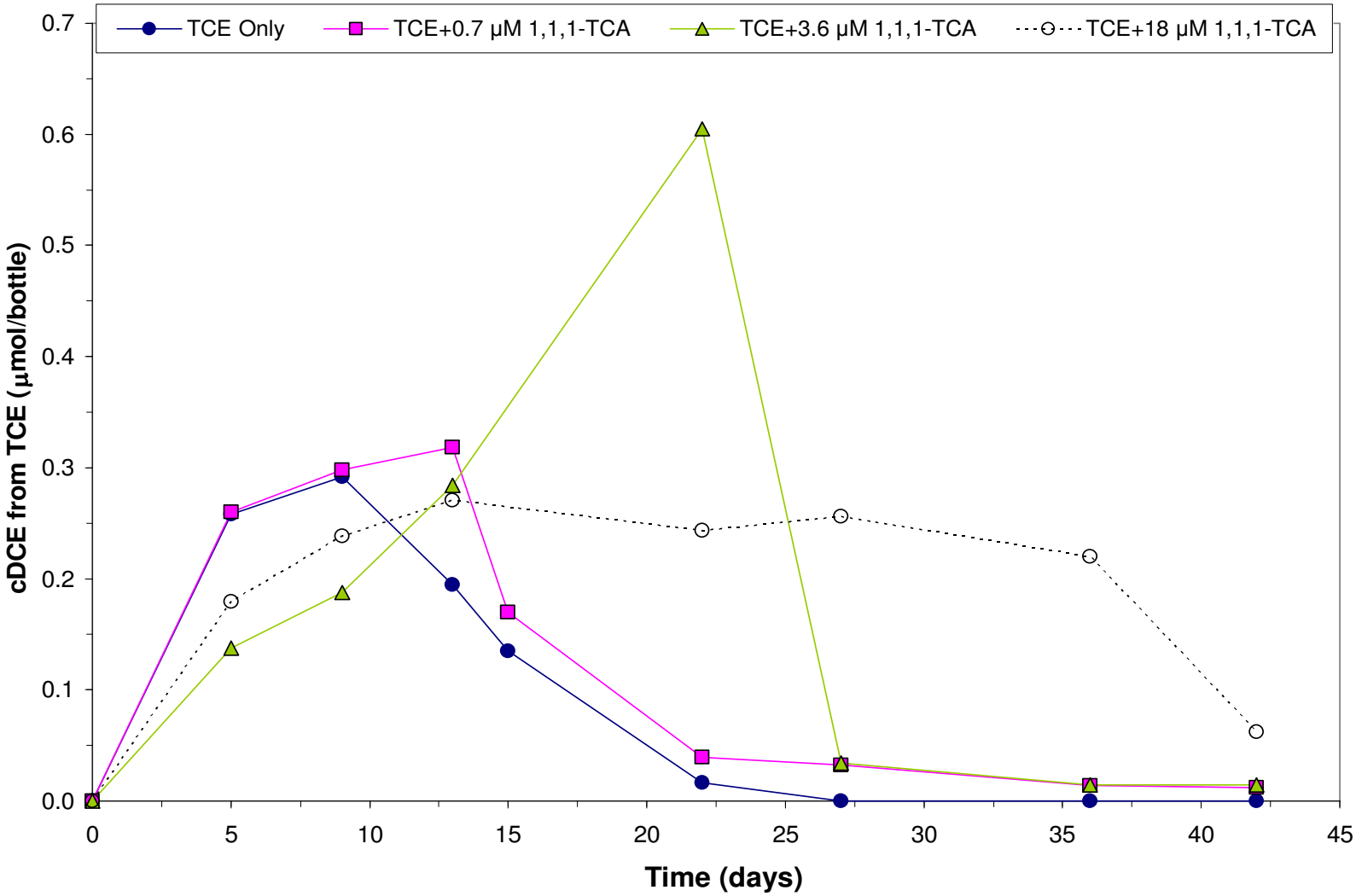

Figure 4.109 Comparison of the effect of 1,1,1-TCA on reductive dechlorination of cDCE at all levels (averages of triplicates).

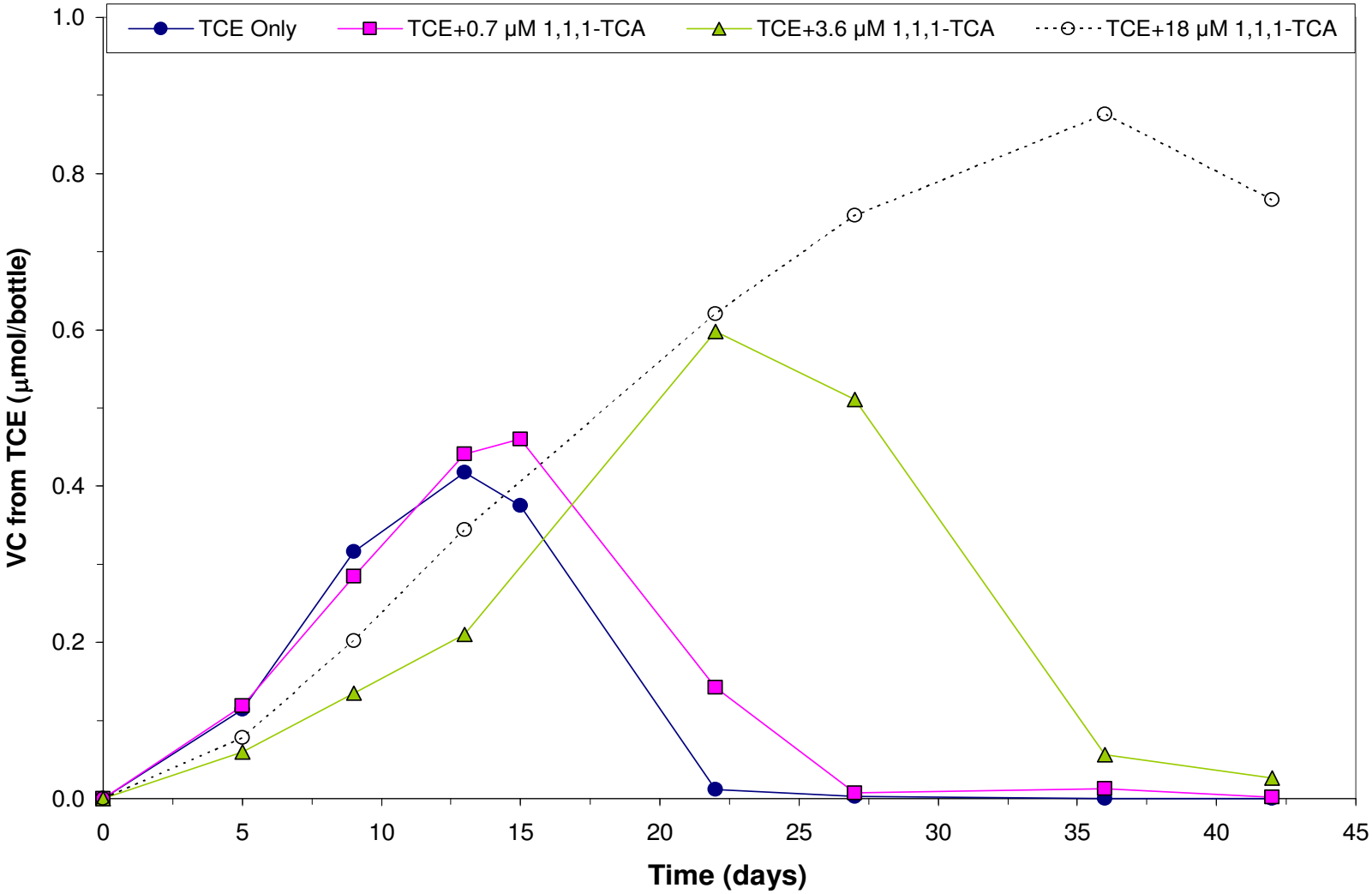

Figure 4.110 Comparison of the effect of 1,1,1-TCA on reductive dechlorination of VC at all levels (averages of triplicates).

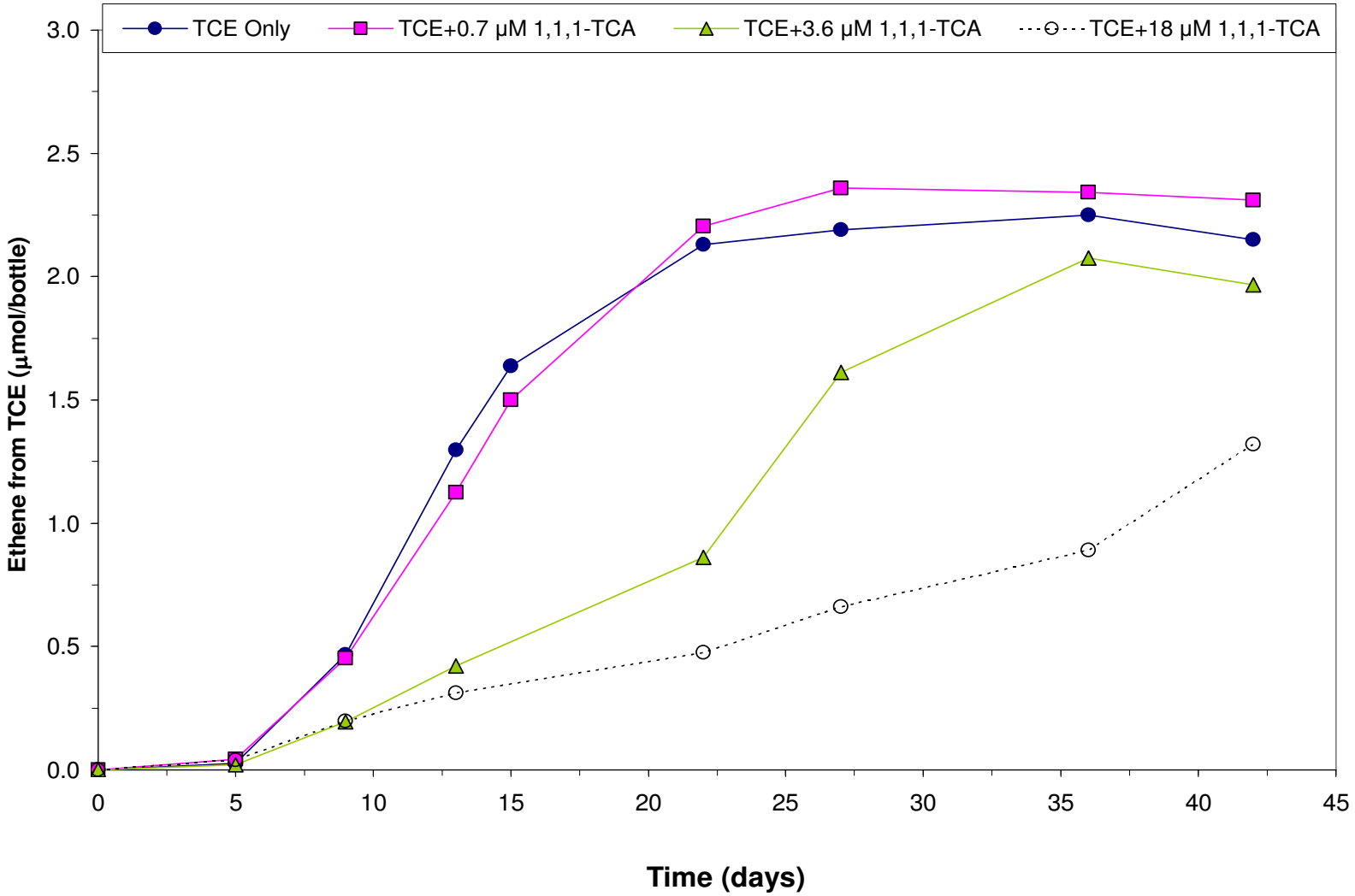

Figure 4.111 Comparison of the effect of 1,1,1-TCA on production of ethene at all levels (averages of triplicates).

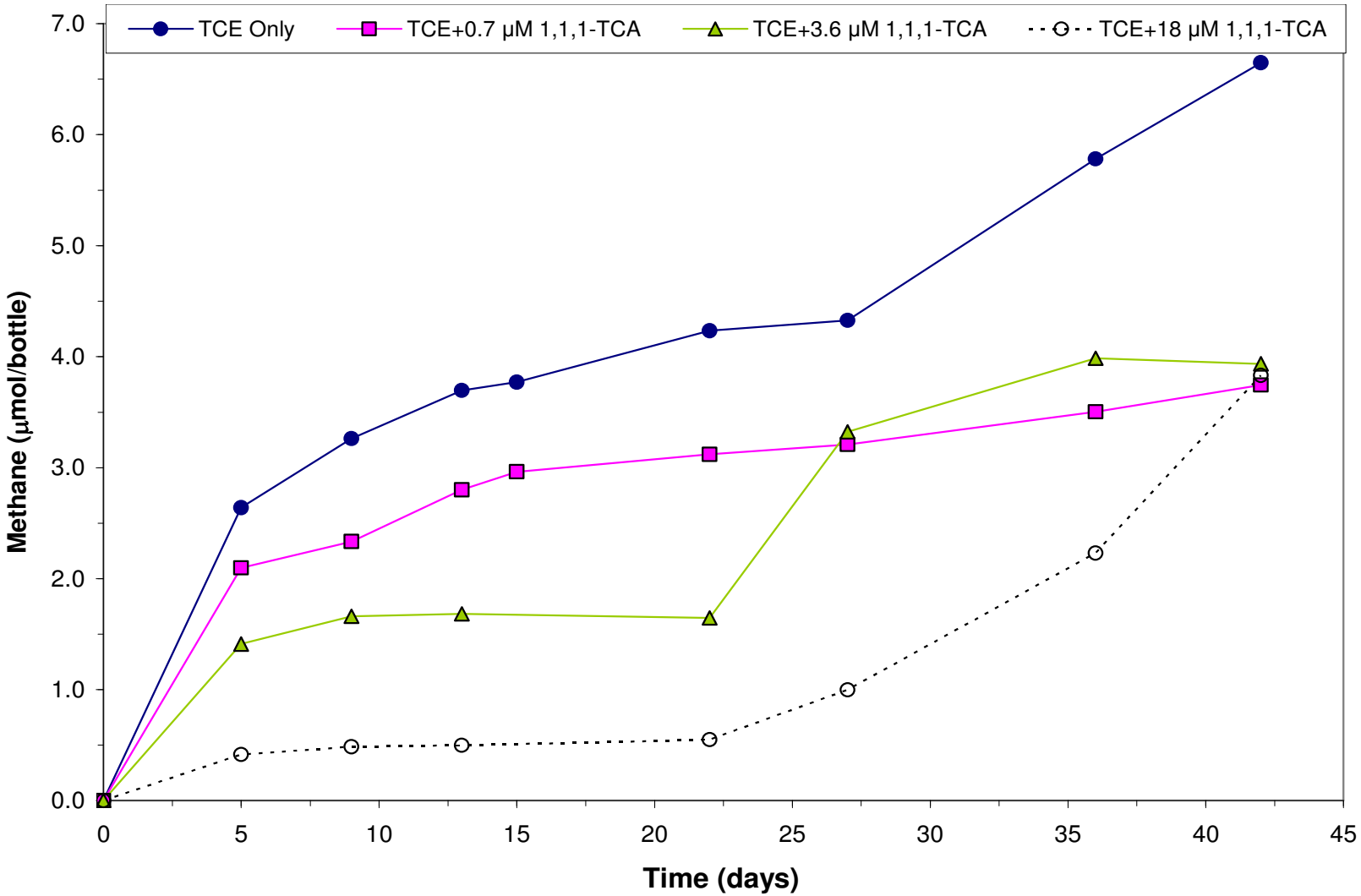

Figure 4.112 Comparison of the effect of 1,1,1-TCA on production of methane at all levels (averages of triplicates).

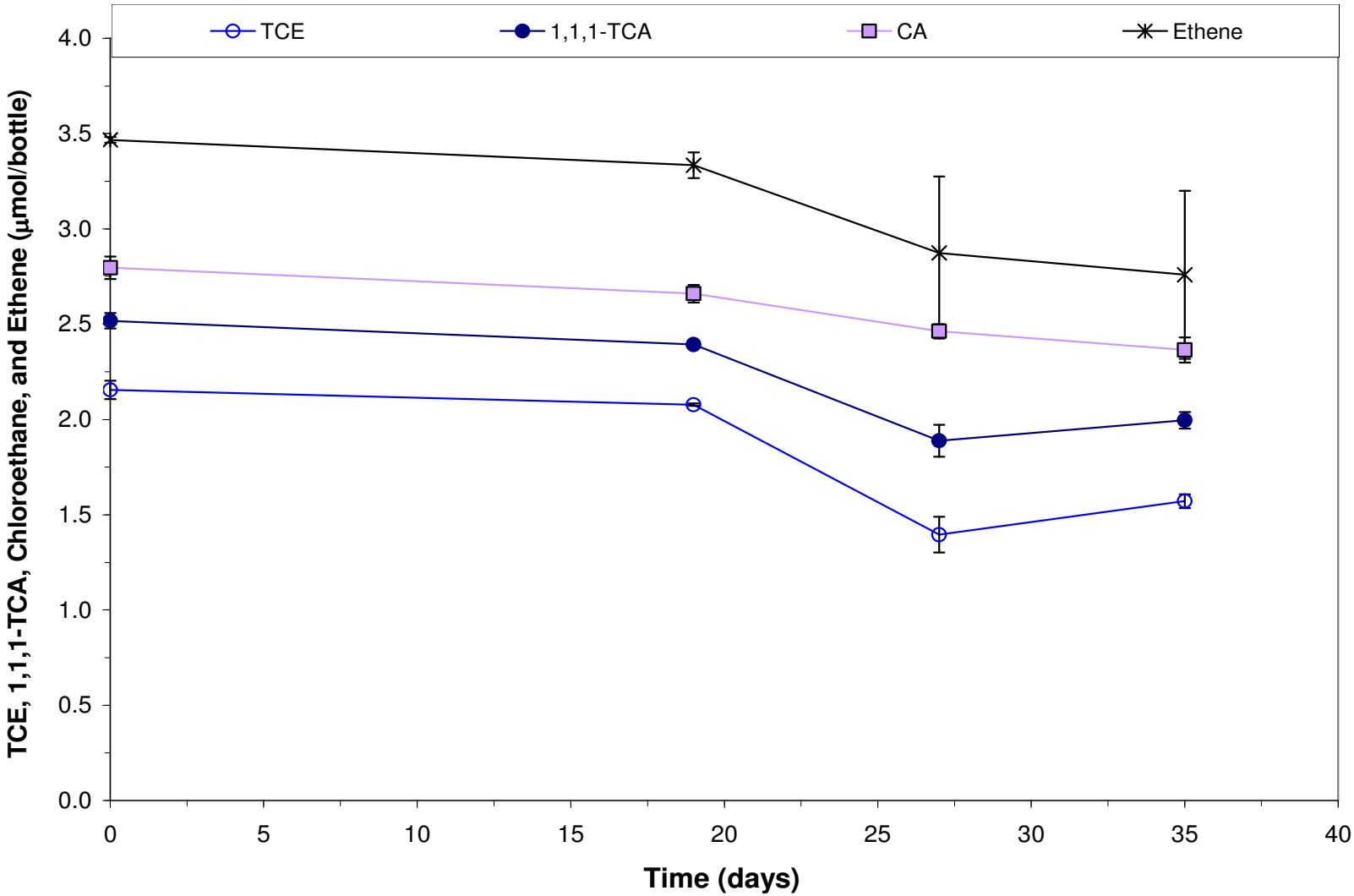

Figure 4.113 Results for the 1,1,1-TCA Inhibition Experiment Phase 2 WCs (average of triplicates). Error bars represent ± one standard deviation.

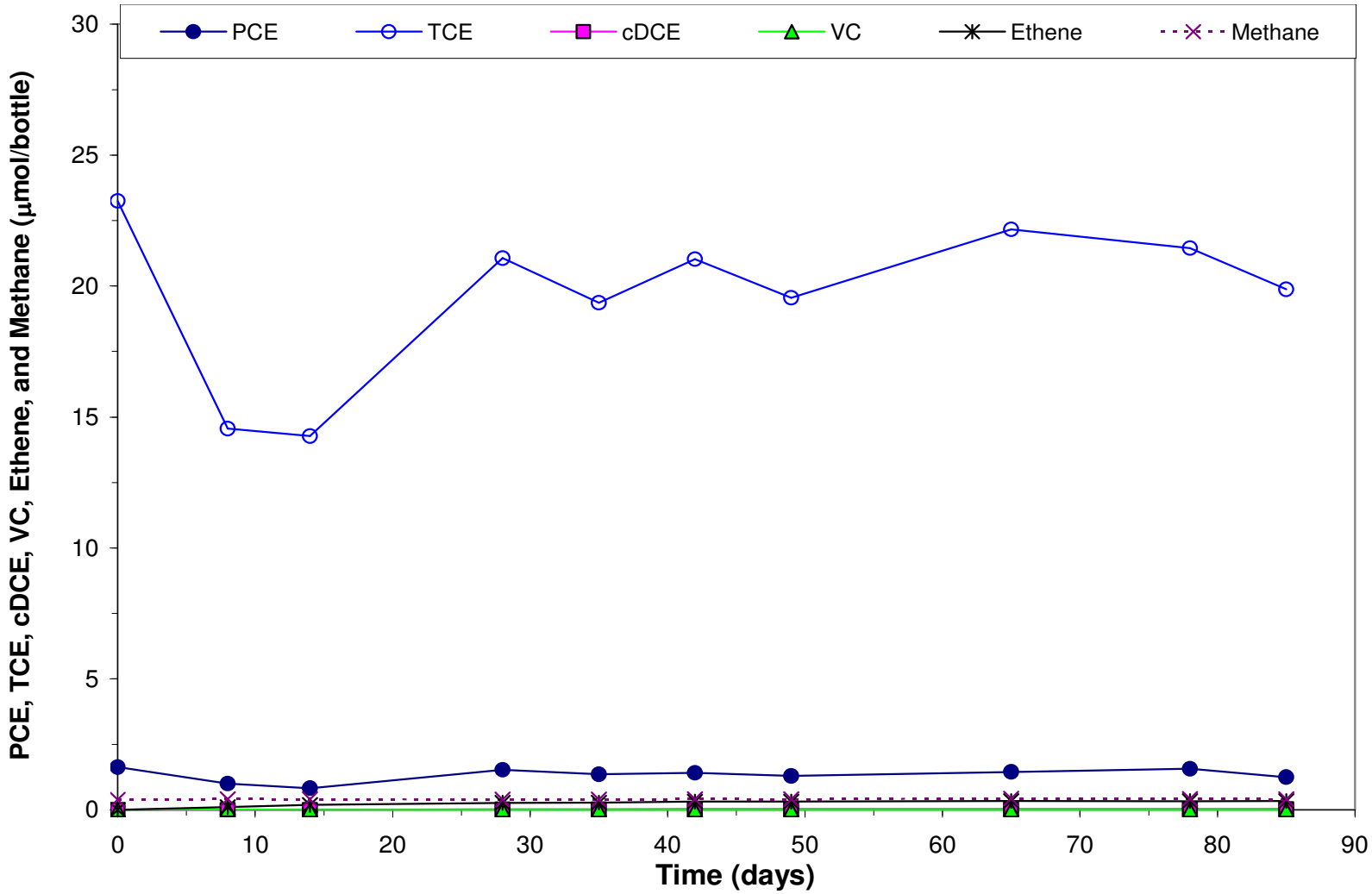

Figure 4.114 Results for one of the lactate only-amended bottles (lactate only-1).

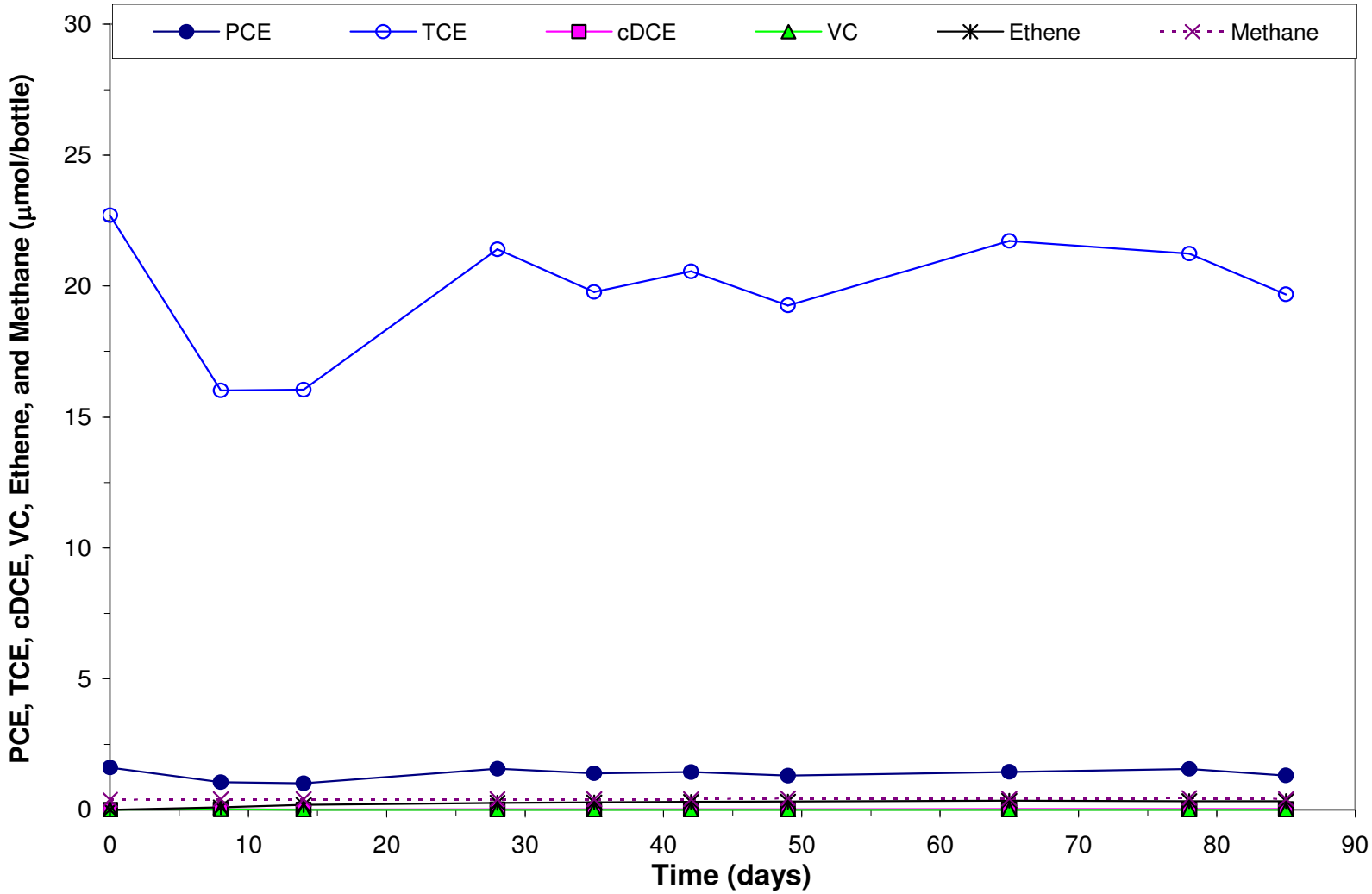

Figure 4.115 Results for one of the lactate only-amended bottles (lactate only-2).

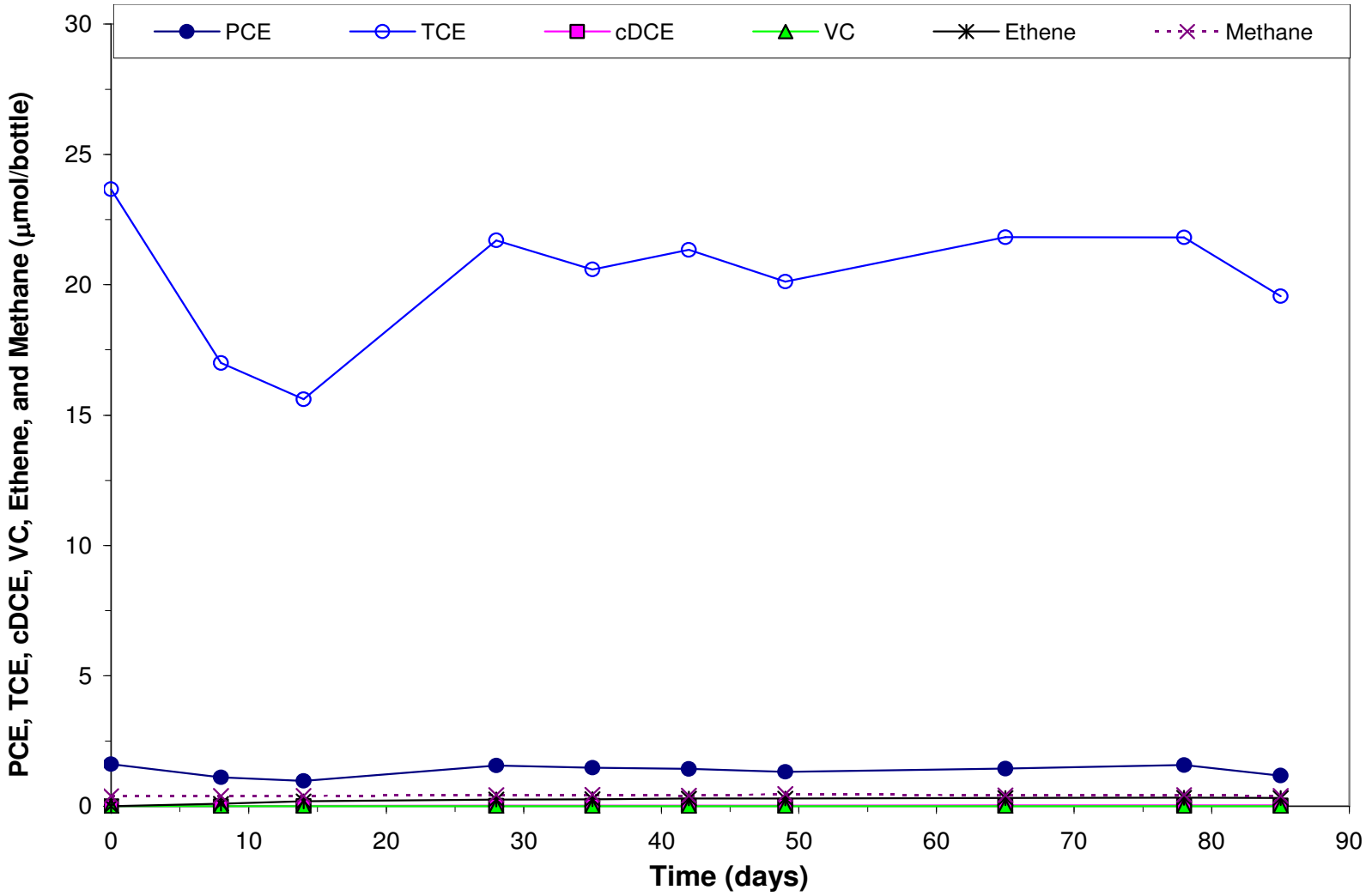

Figure 4.116 Results for one of the lactate only-amended bottles (lactate only-3).

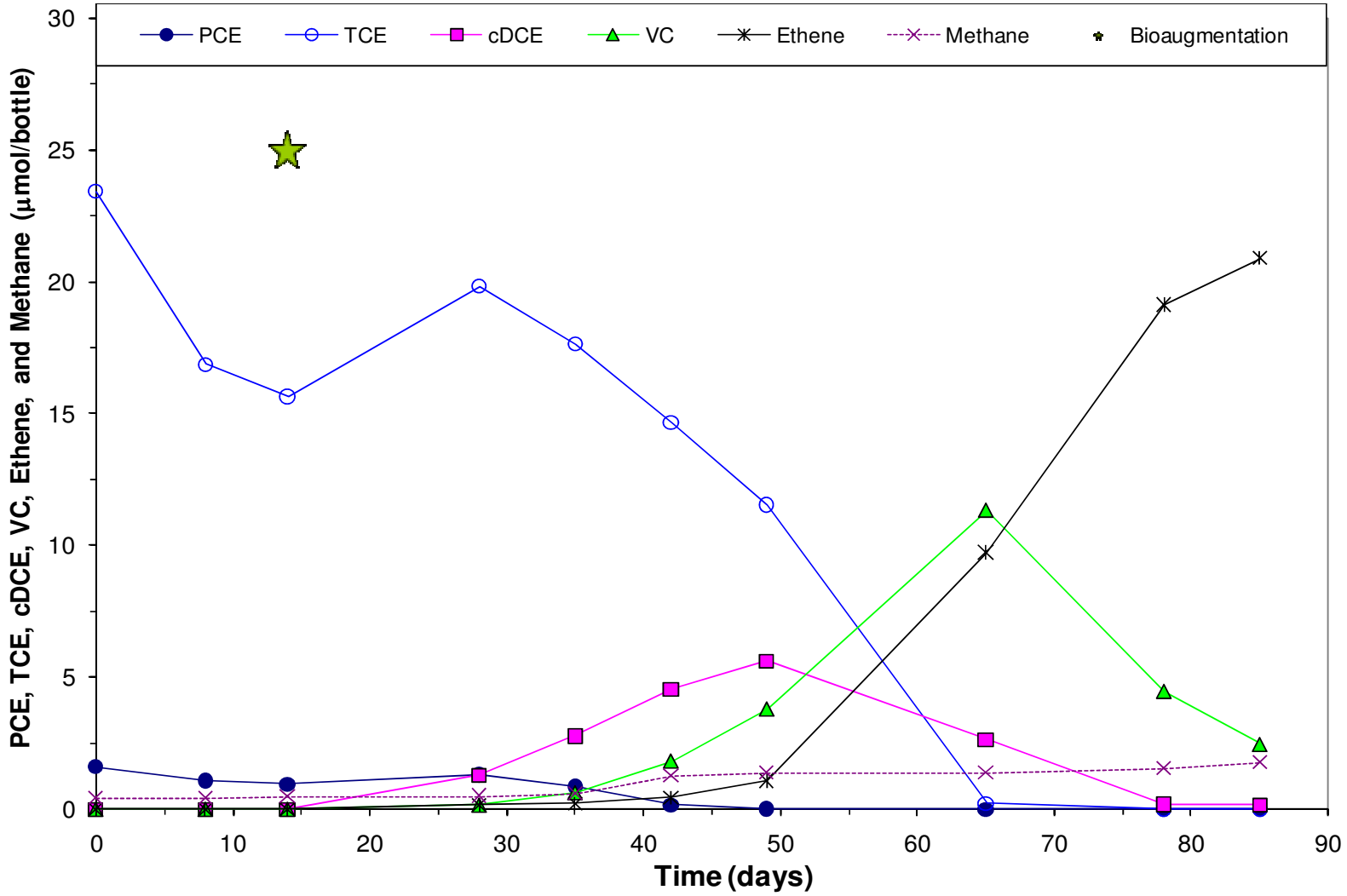

Figure 4.117 Results for one of the lactate + bioaugmentation-amended bottles (bio + lactate-1).

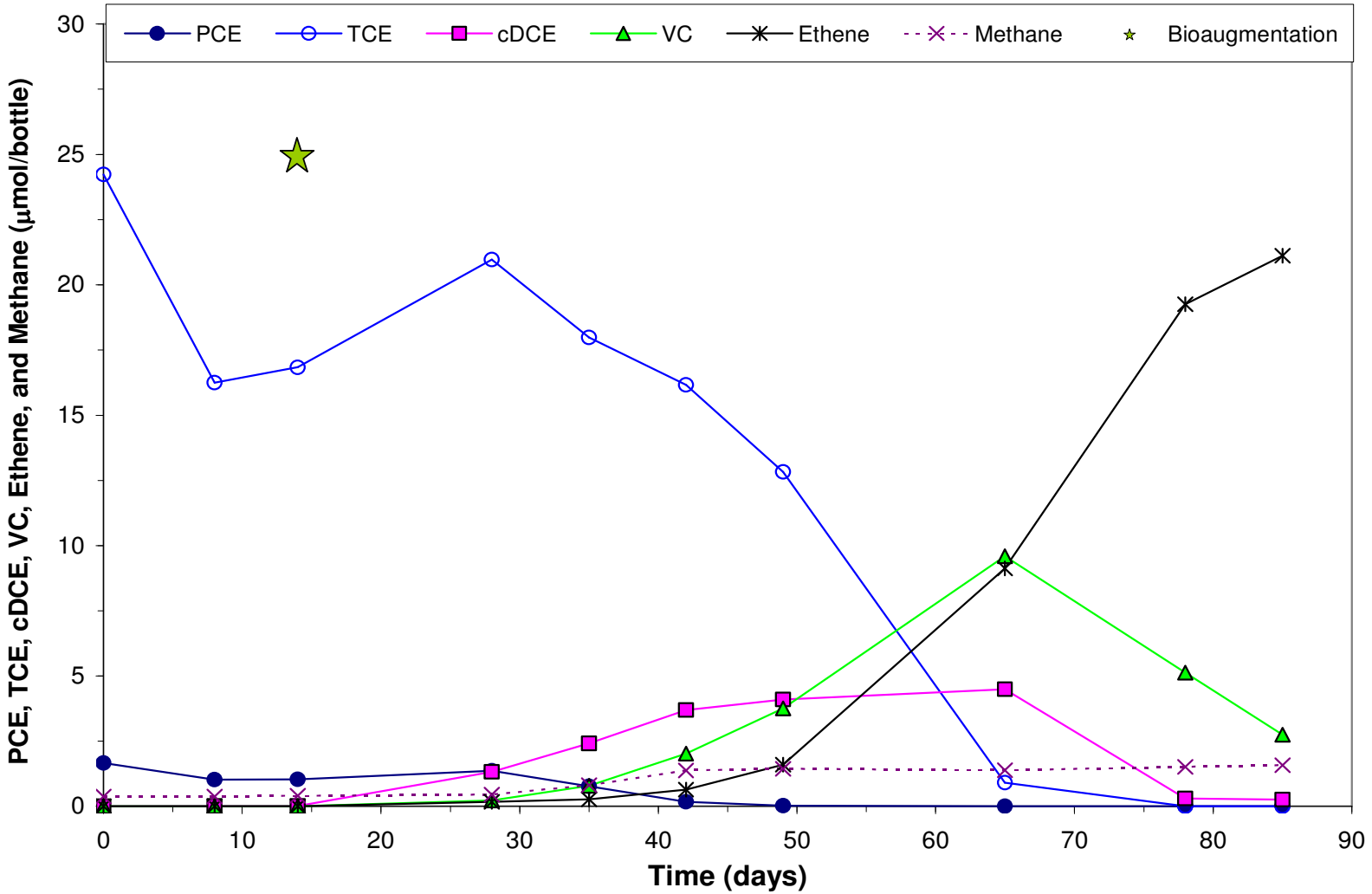

Figure 4.118 Results for one of the lactate + bioaugmentation-amended bottles (bio + lactate-2).

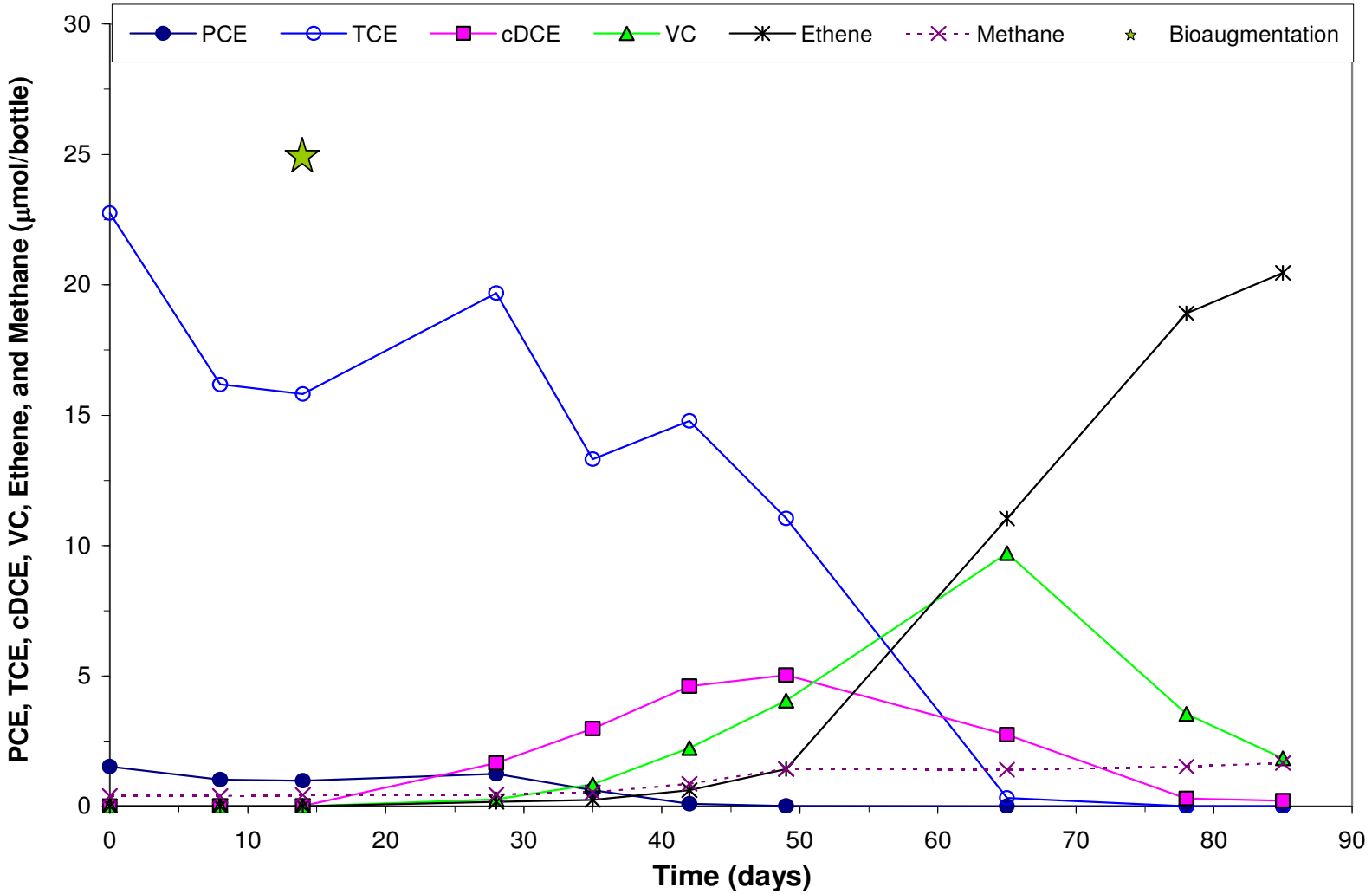

Figure 4.119 Results for one of the lactate + bioaugmentation-amended bottles (bio + lactate-3).

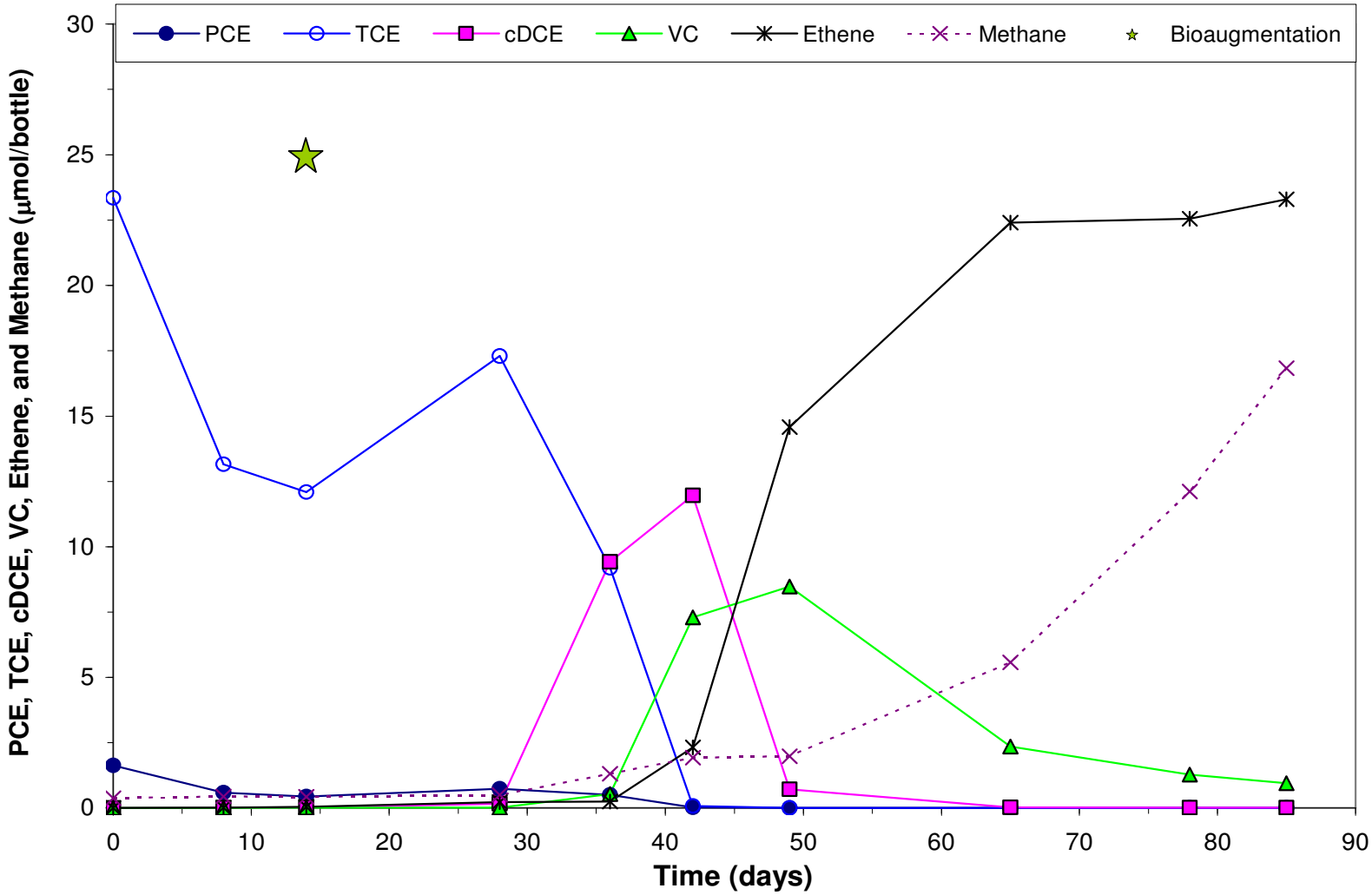

Figure 4.120 Results for one of the EOS® 450 + bioaugmentation-amended bottles (bio + EOS® 450-1).

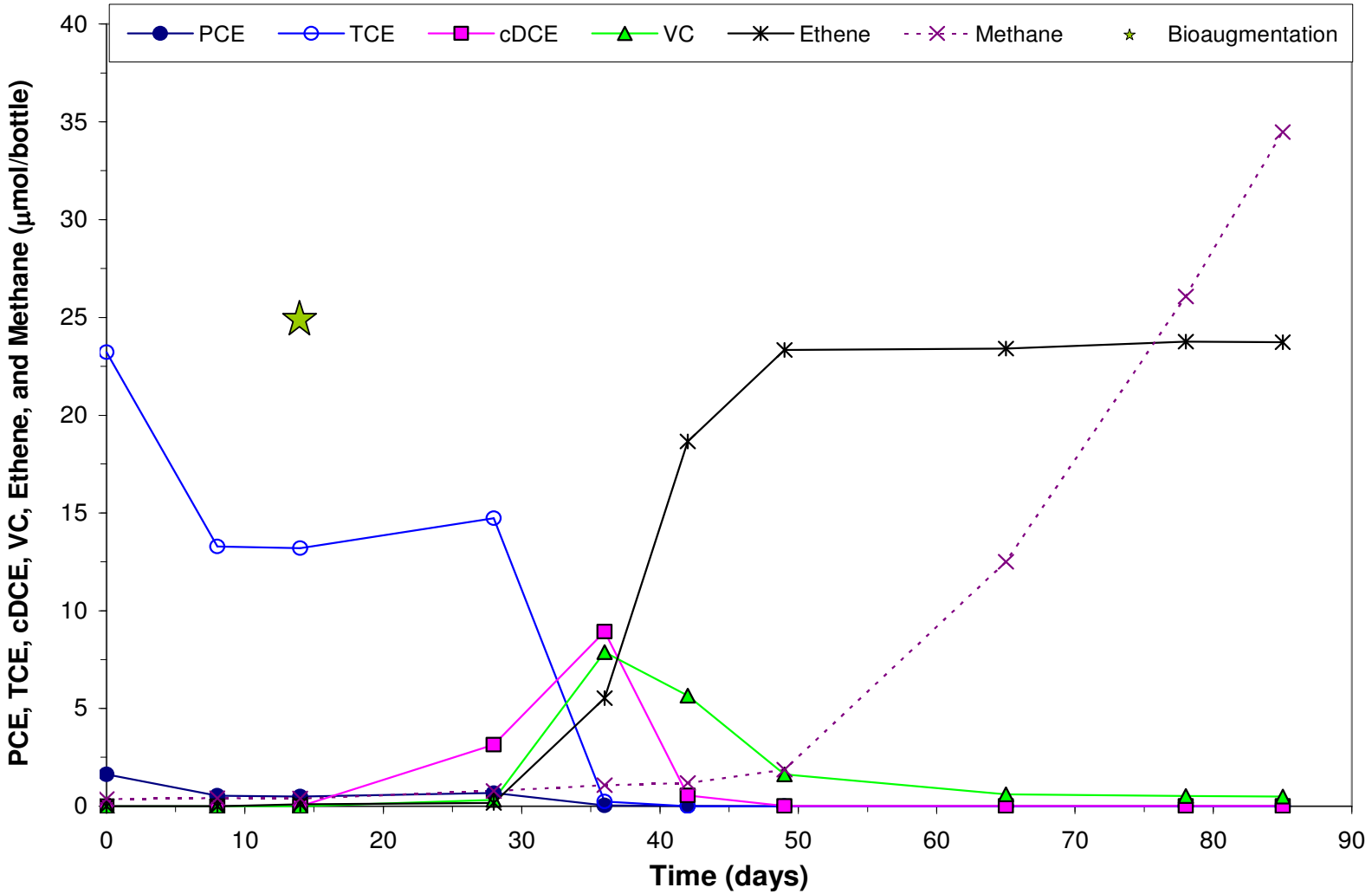

Figure 4.121 Results for one of the EOS® 450 + bioaugmentation-amended bottles (bio + EOS® 450-2).

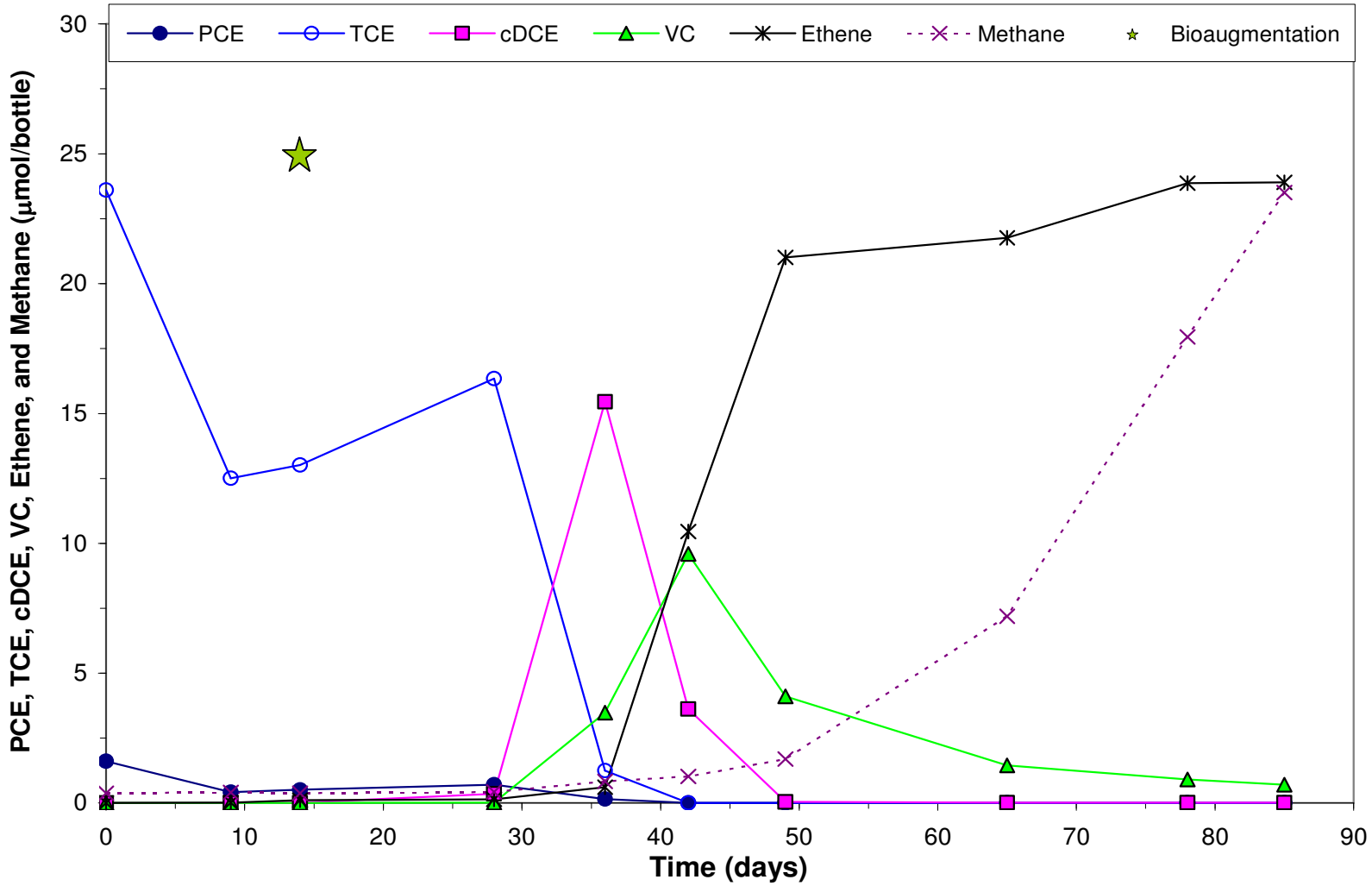

Figure 4.122 Results for one of the EOS® 450 + bioaugmentation-amended bottles (bio + EOS® 450-3).

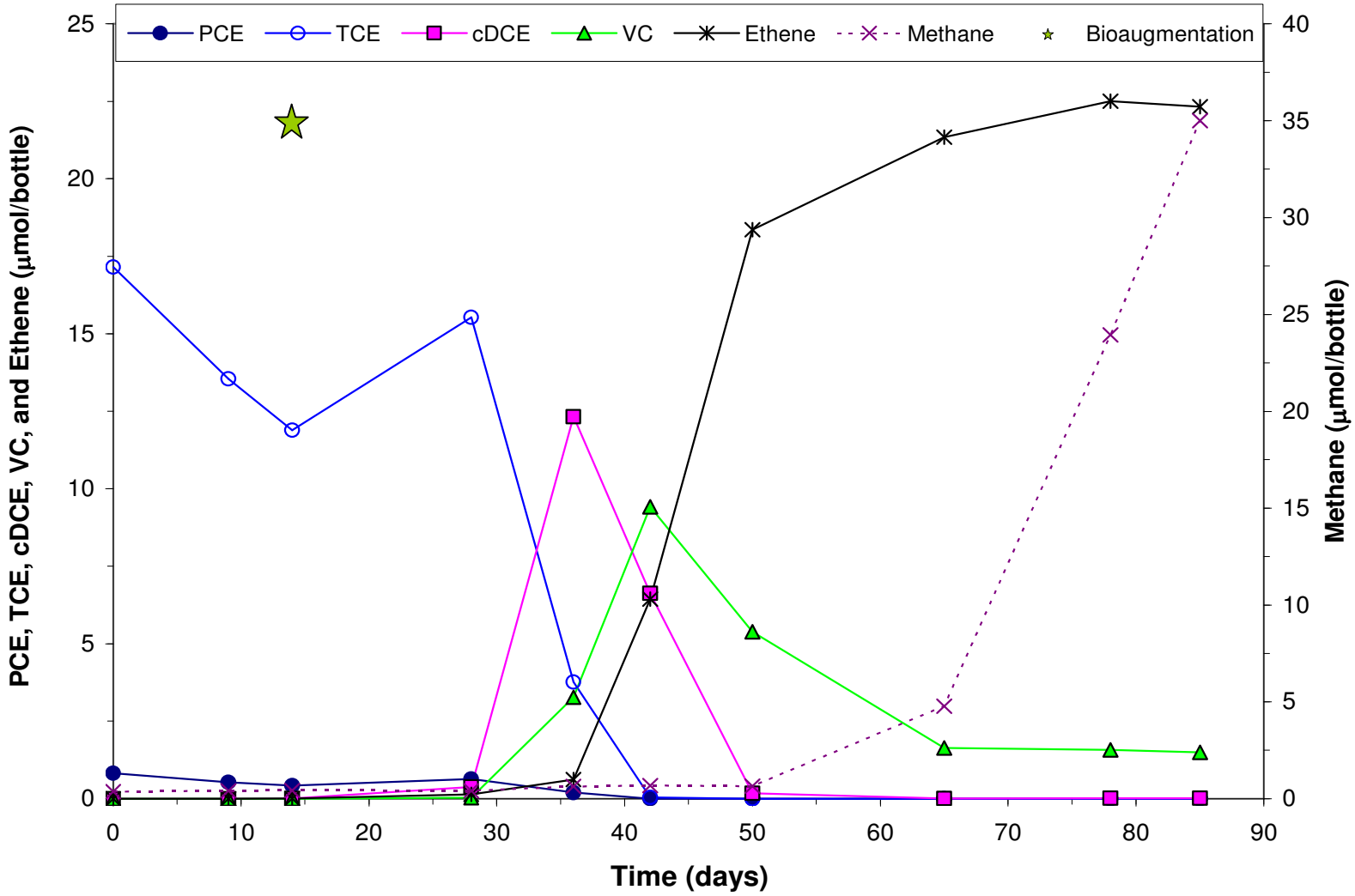

Figure 4.123 Results for one of the EOS® 598 + bioaugmentation-amended bottles (bio + EOS® 598-1).

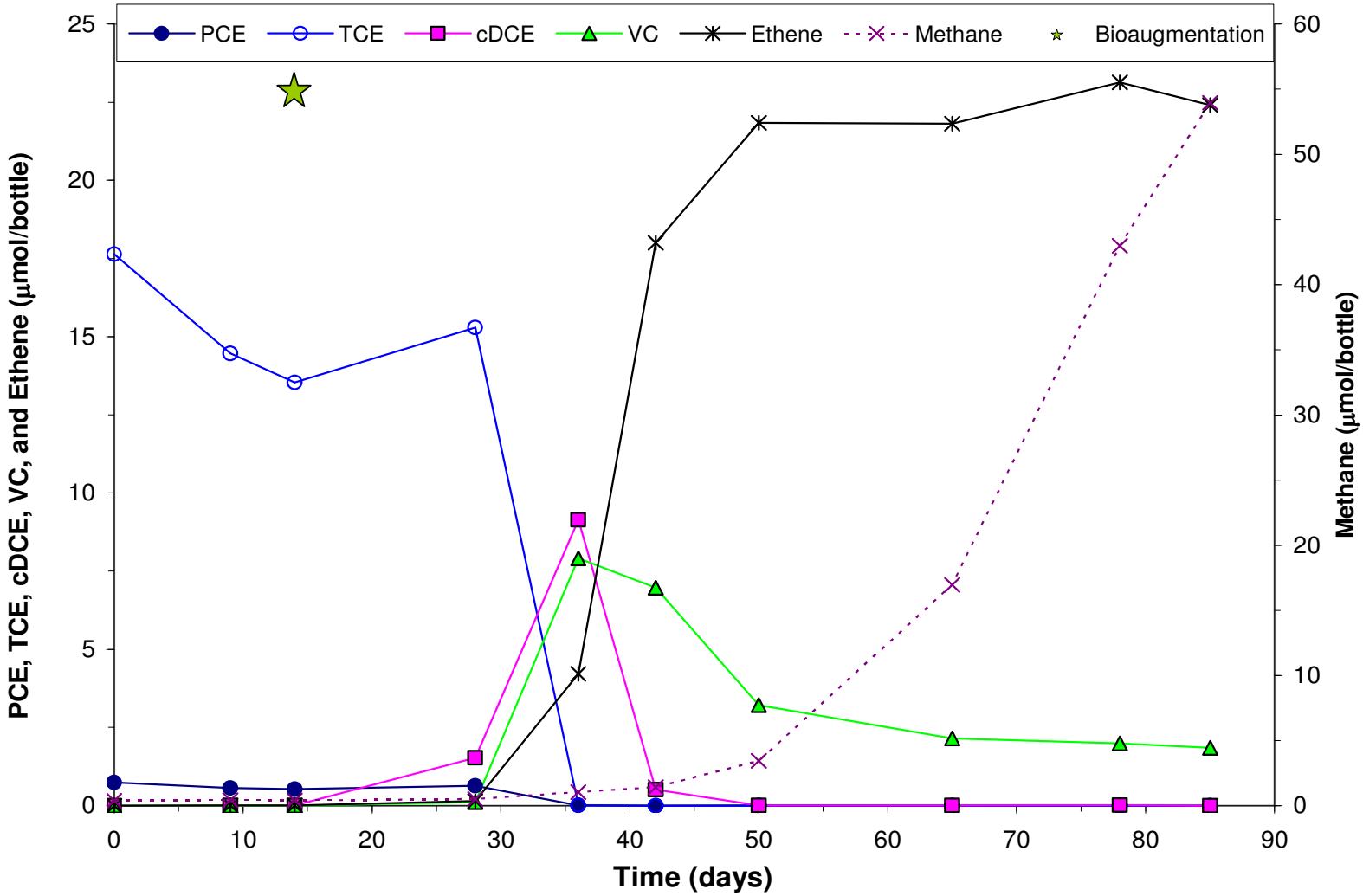

Figure 4.124 Results for one of the EOS® 598 + bioaugmentation-amended bottles (bio + EOS® 598-2).

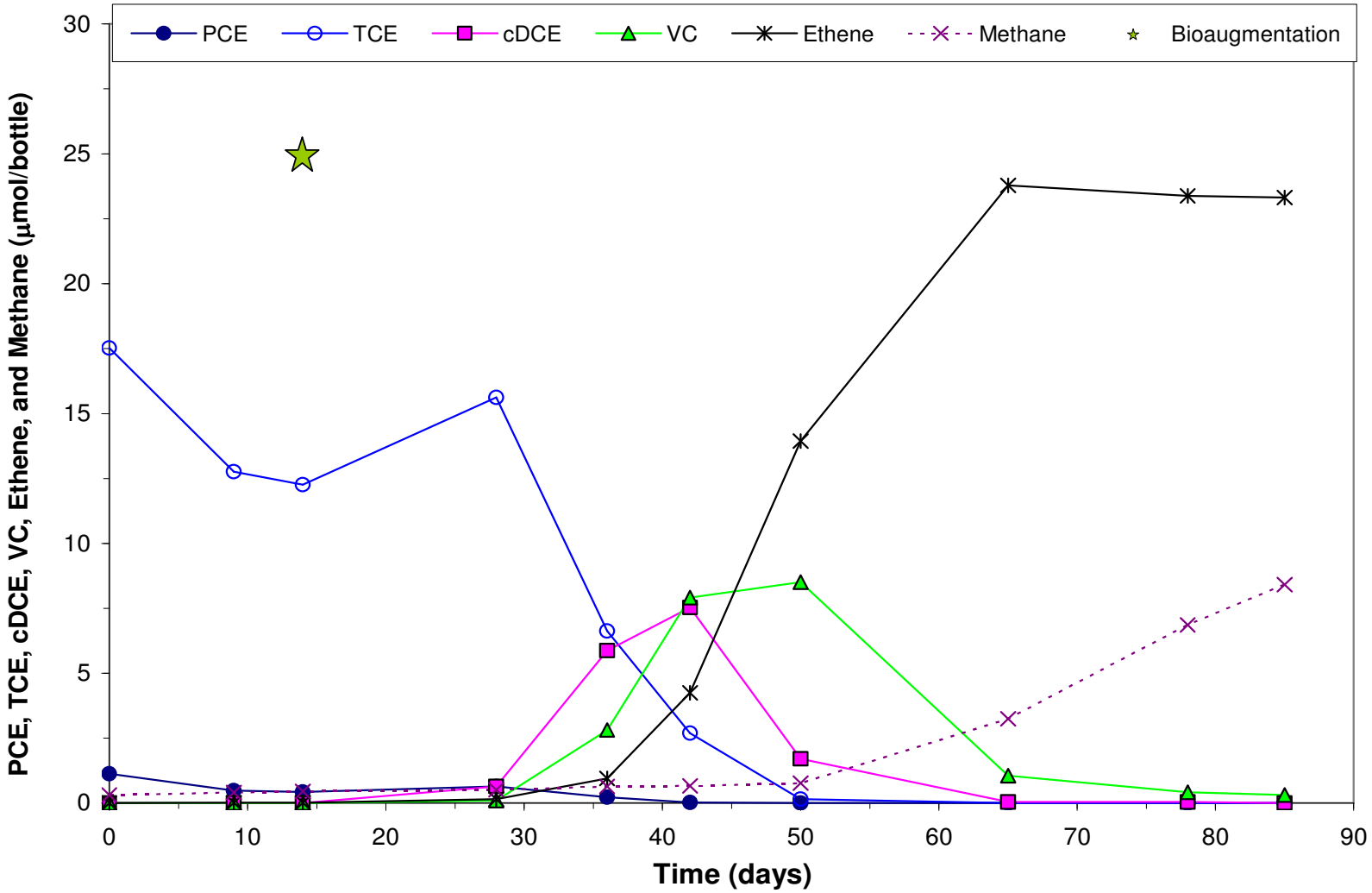

Figure 4.125 Results for one of the EOS® 598 + bioaugmentation-amended bottles (bio + EOS® 598-3).

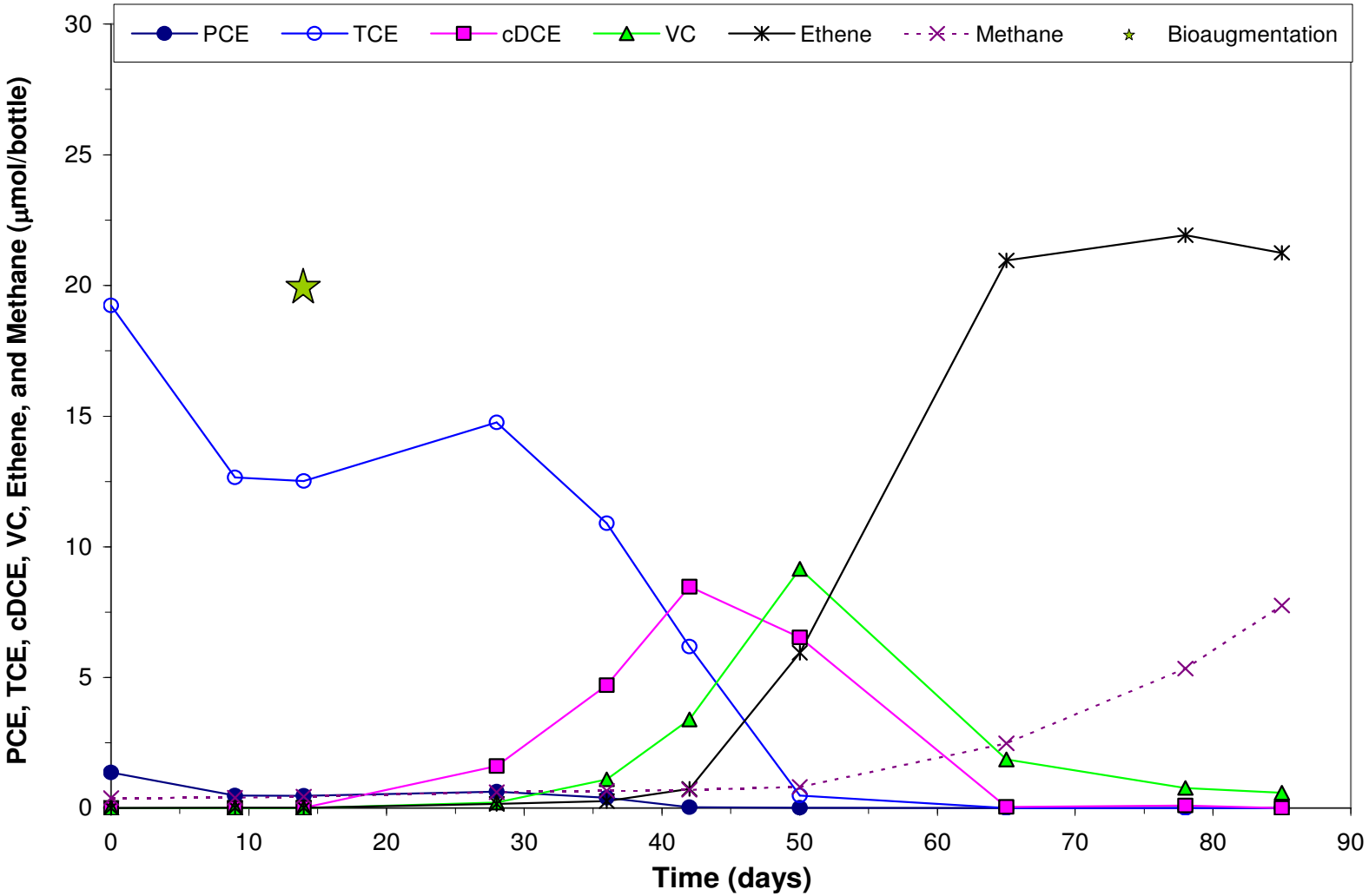

Figure 4.126 Results for one of the EOS® 598B42 + bioaugmentation-amended bottles (bio + EOS® 598B42-1).

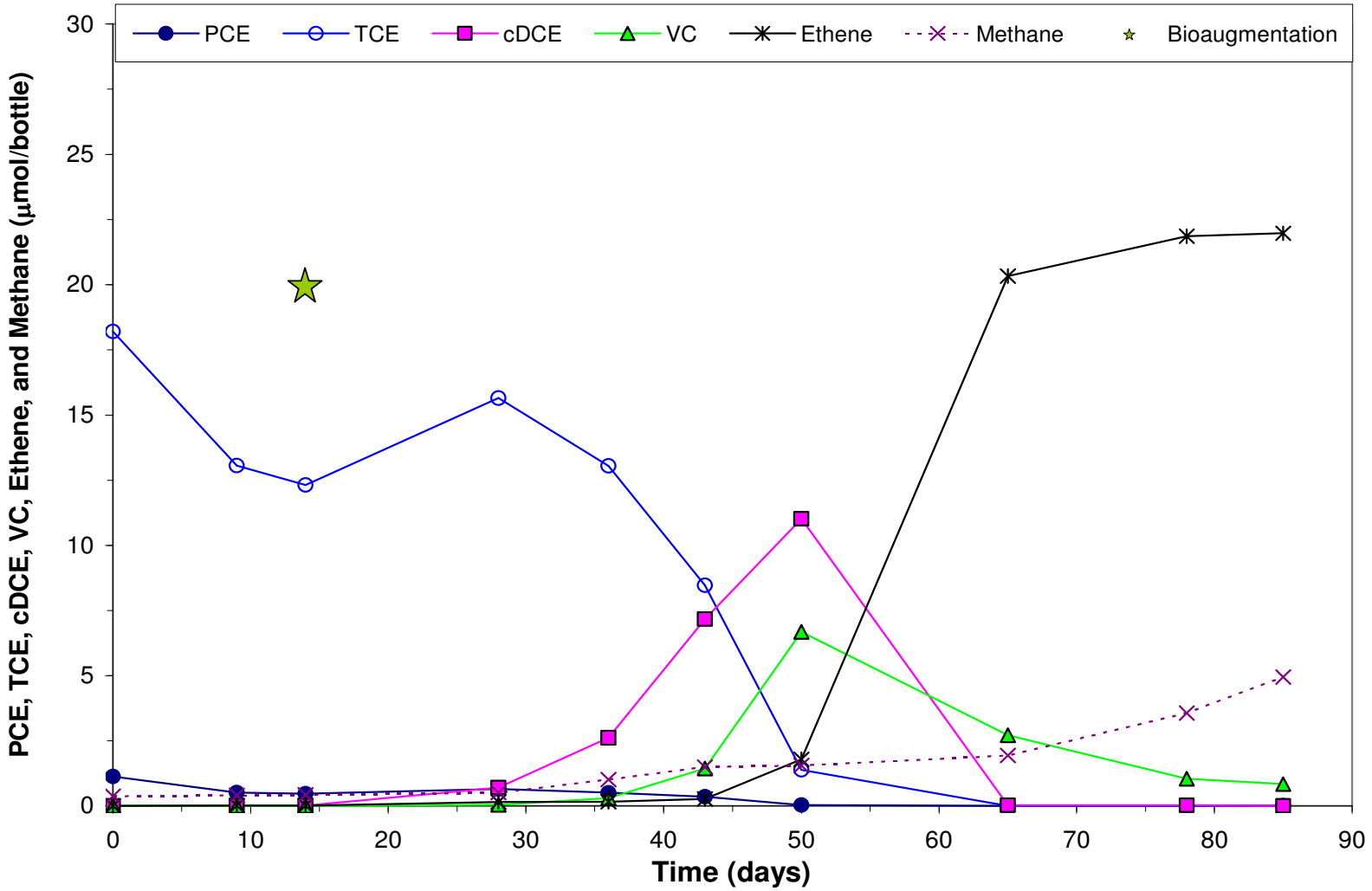

Figure 4.127 Results for one of the EOS® 598B42 + bioaugmentation-amended bottles (bio + EOS® 598B42-2).

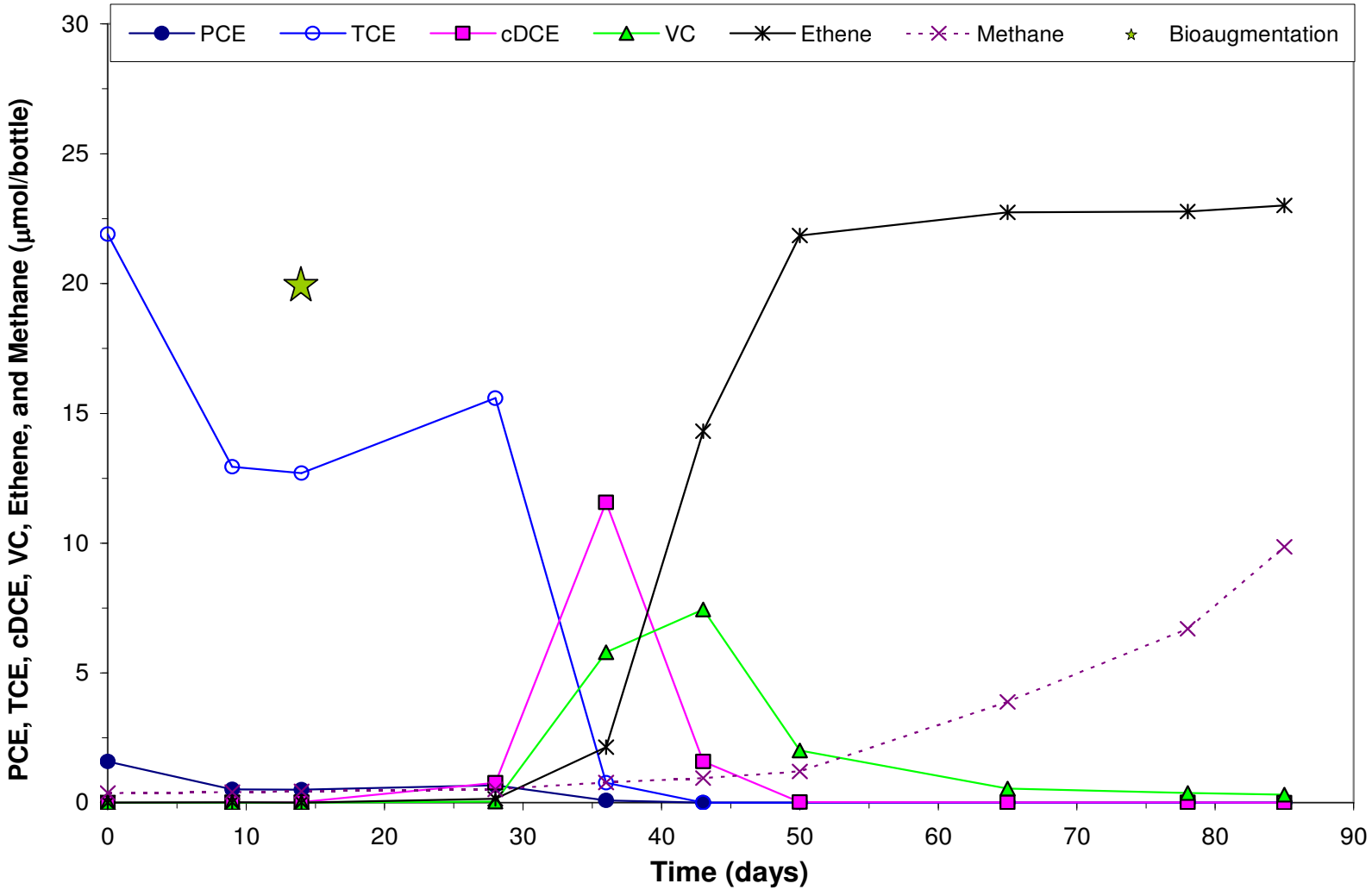

Figure 4.128 Results for one of the EOS® 598B42 + bioaugmentation-amended bottles (bio + EOS® 598B42-3).

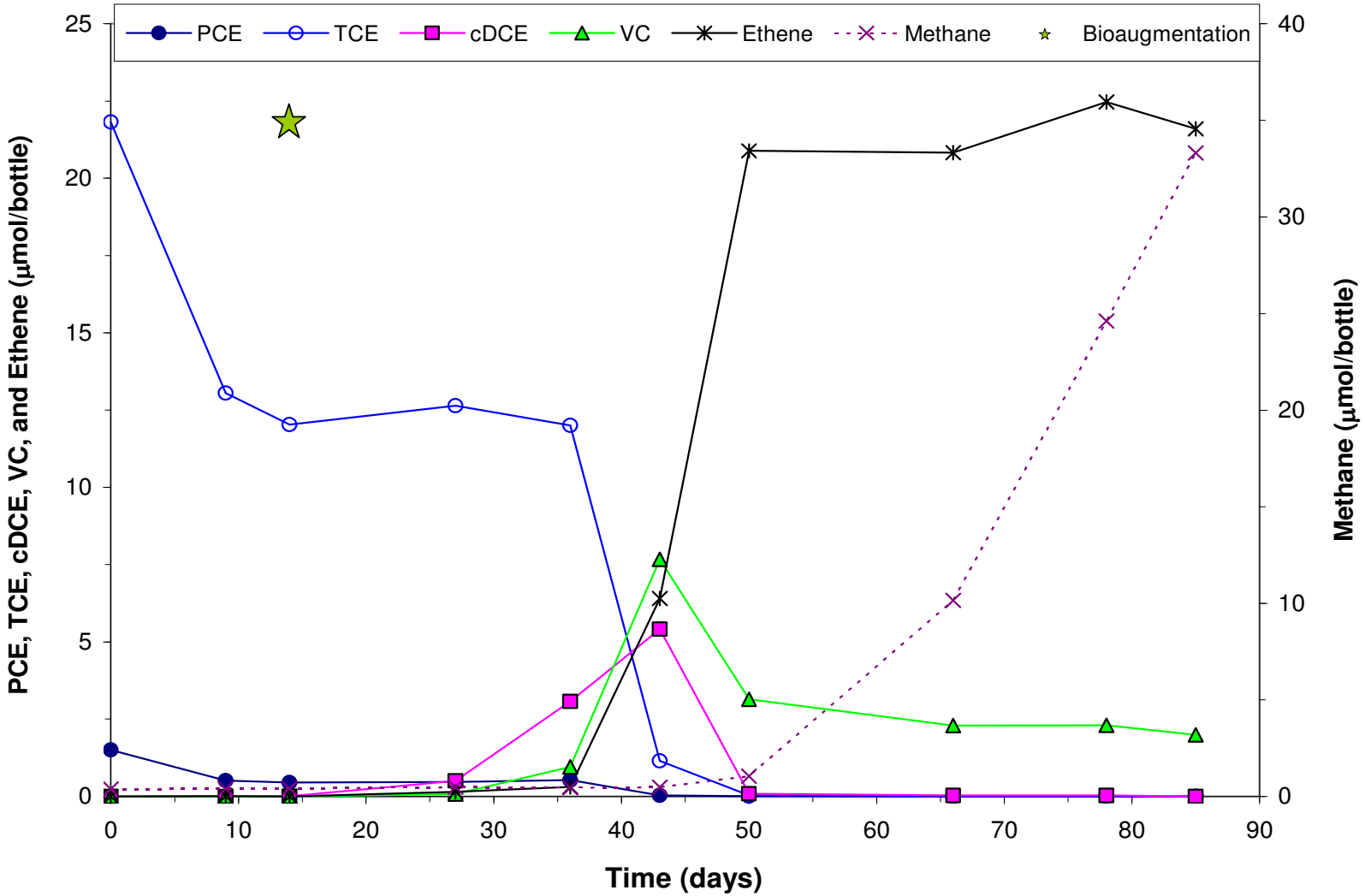

Figure 4.129 Results for one of the EOS® 598B42 + EOS® Vitamin B_{12} solution + bioaugmentation-amended bottles (bio + EOS® 598B42 + B_{12}-1).

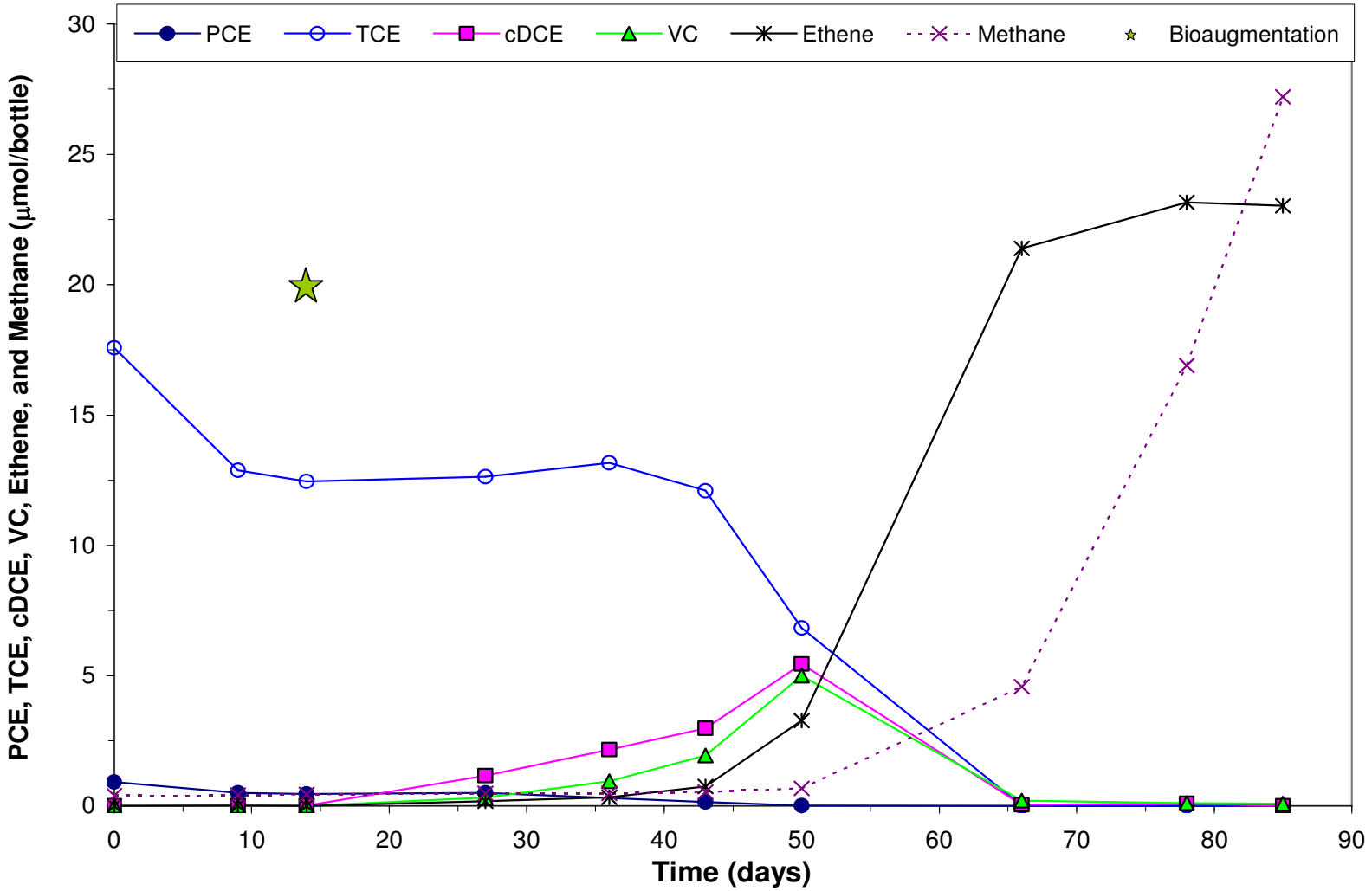

Figure 4.130 Results for one of the EOS® 598B42 + EOS® Vitamin B_{12} solution + bioaugmentation-amended bottles (bio + EOS® 598B42 + B_{12}-2).

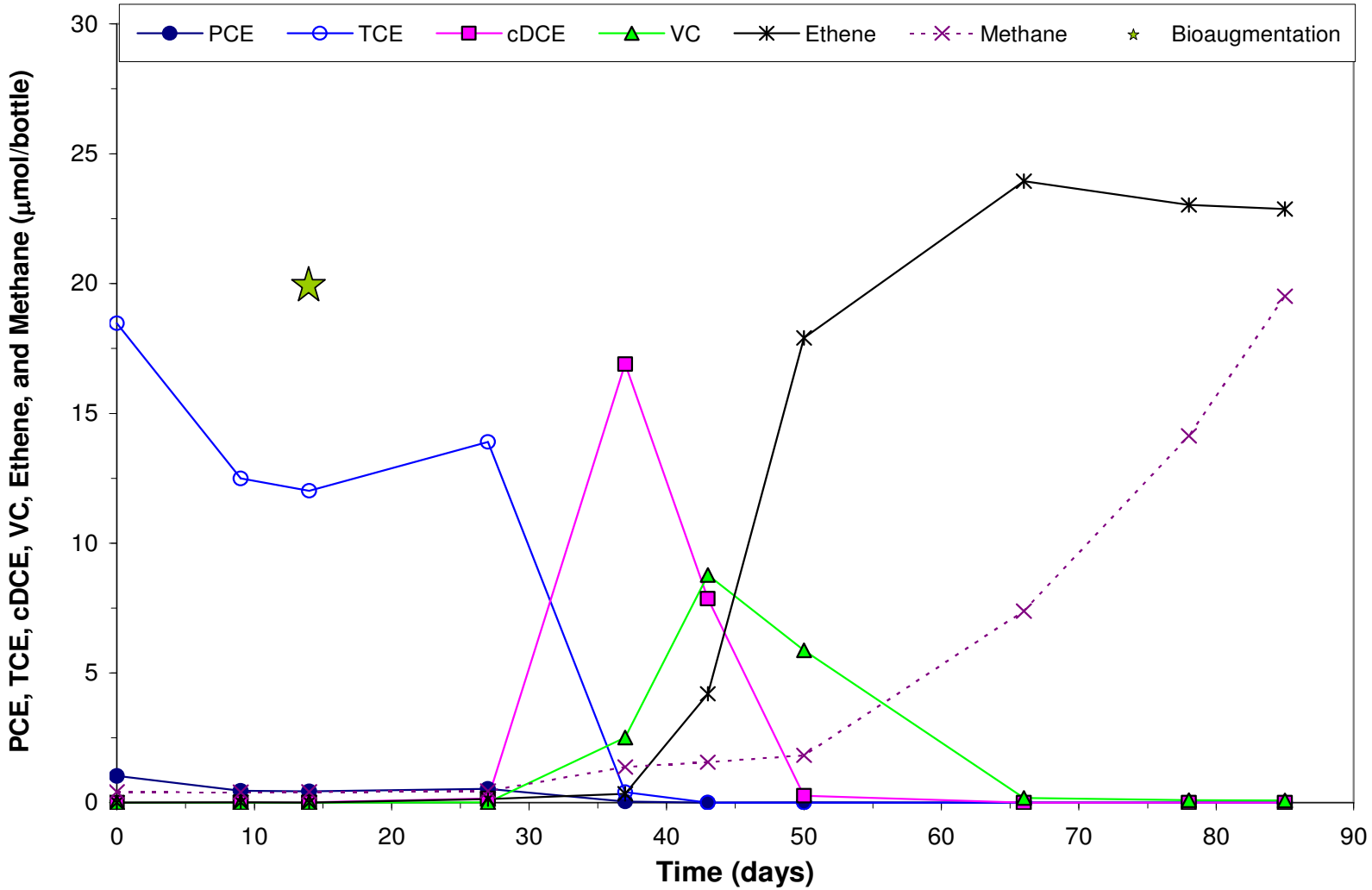

Figure 4.131 Results for one of the EOS® 598B42 + EOS® Vitamin B_{12} solution + bioaugmentation-amended bottles (bio + EOS® 598B42 + B_{12}-3).

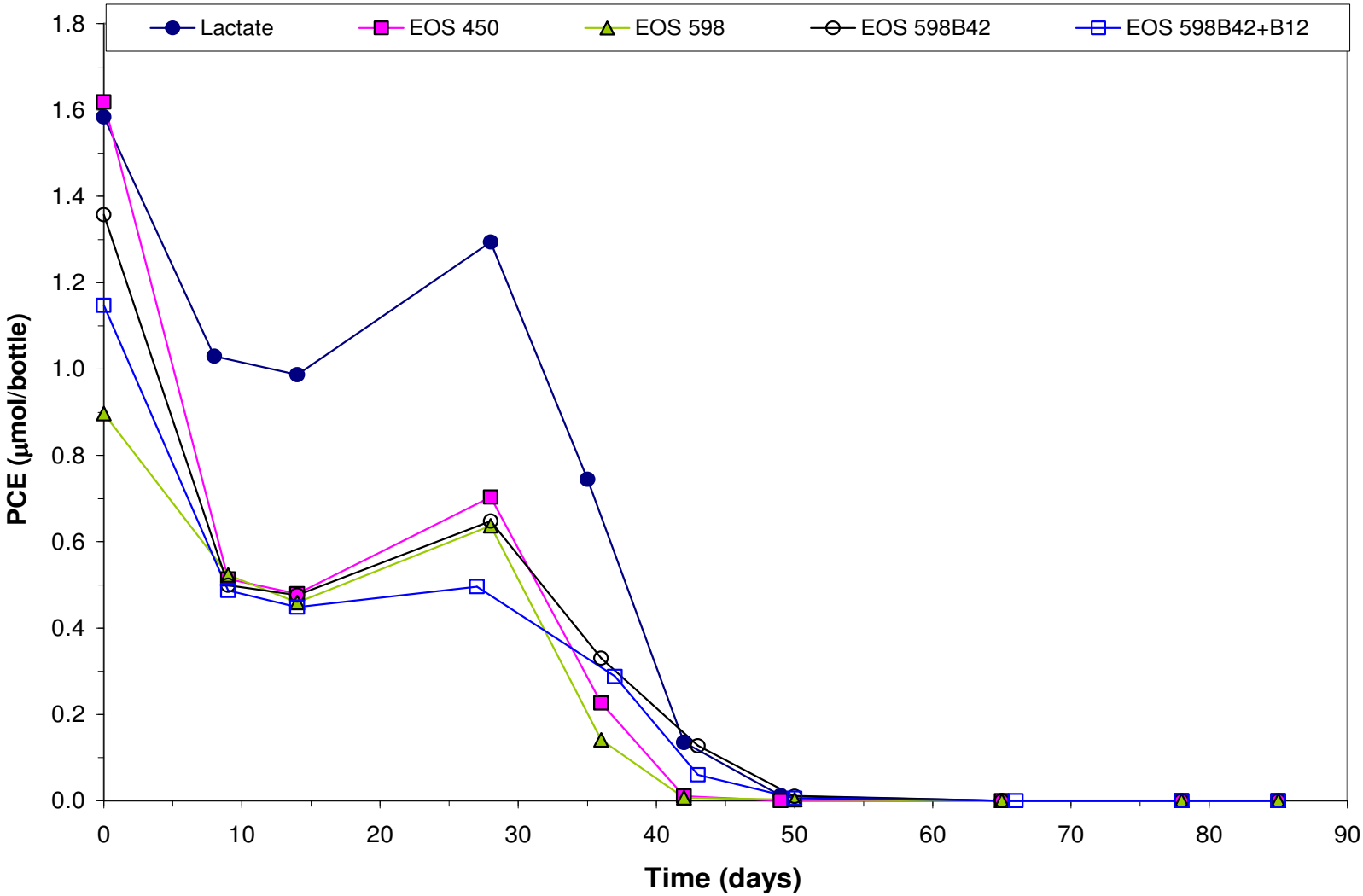

Figure 4.132 Comparison of lactate and EOS® as electron donors on PCE reductive dechlorination (averages of triplicates).

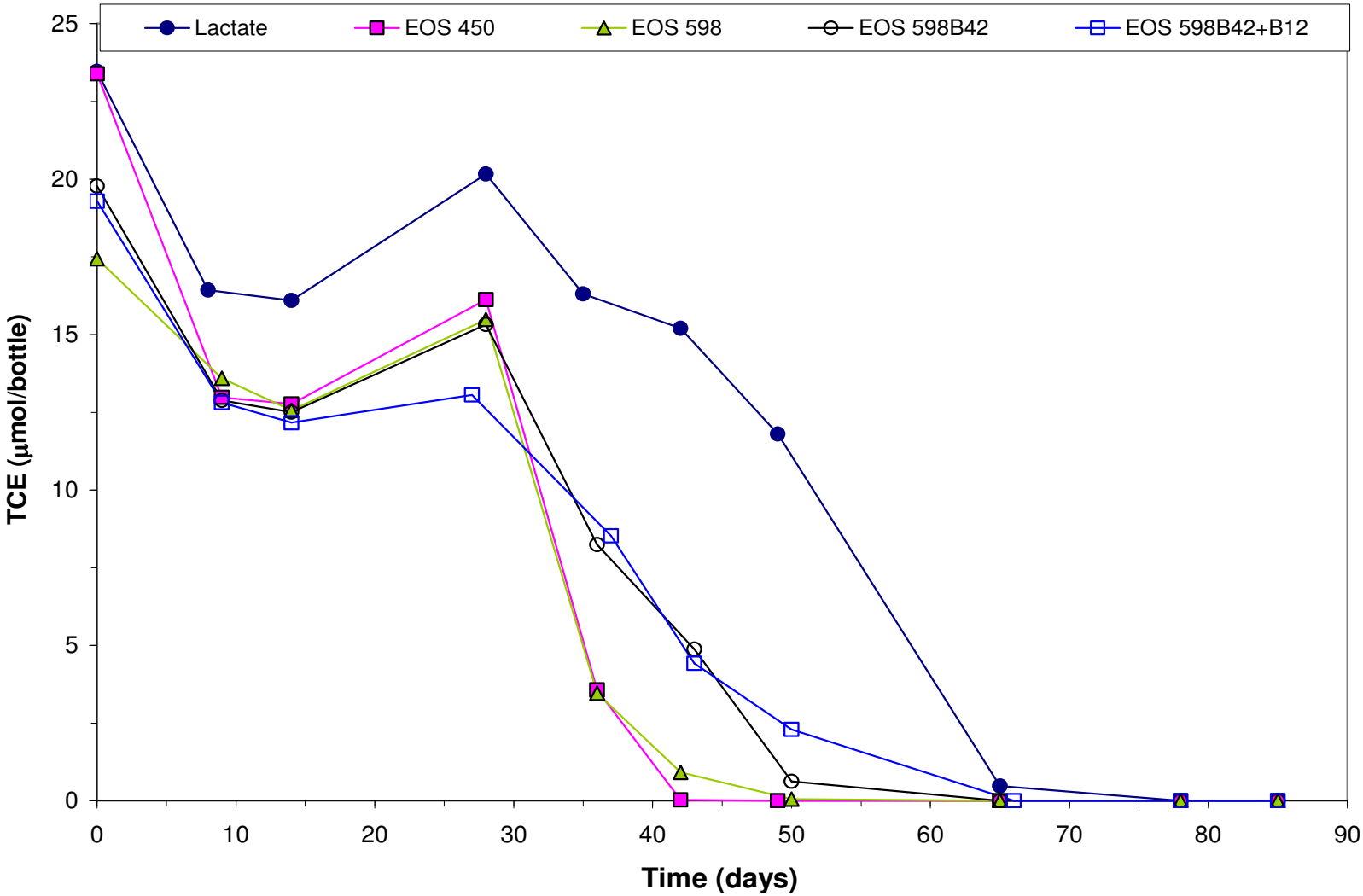

Figure 4.133 Comparison of lactate and EOS® as electron donors on TCE reductive dechlorination (averages of triplicates).

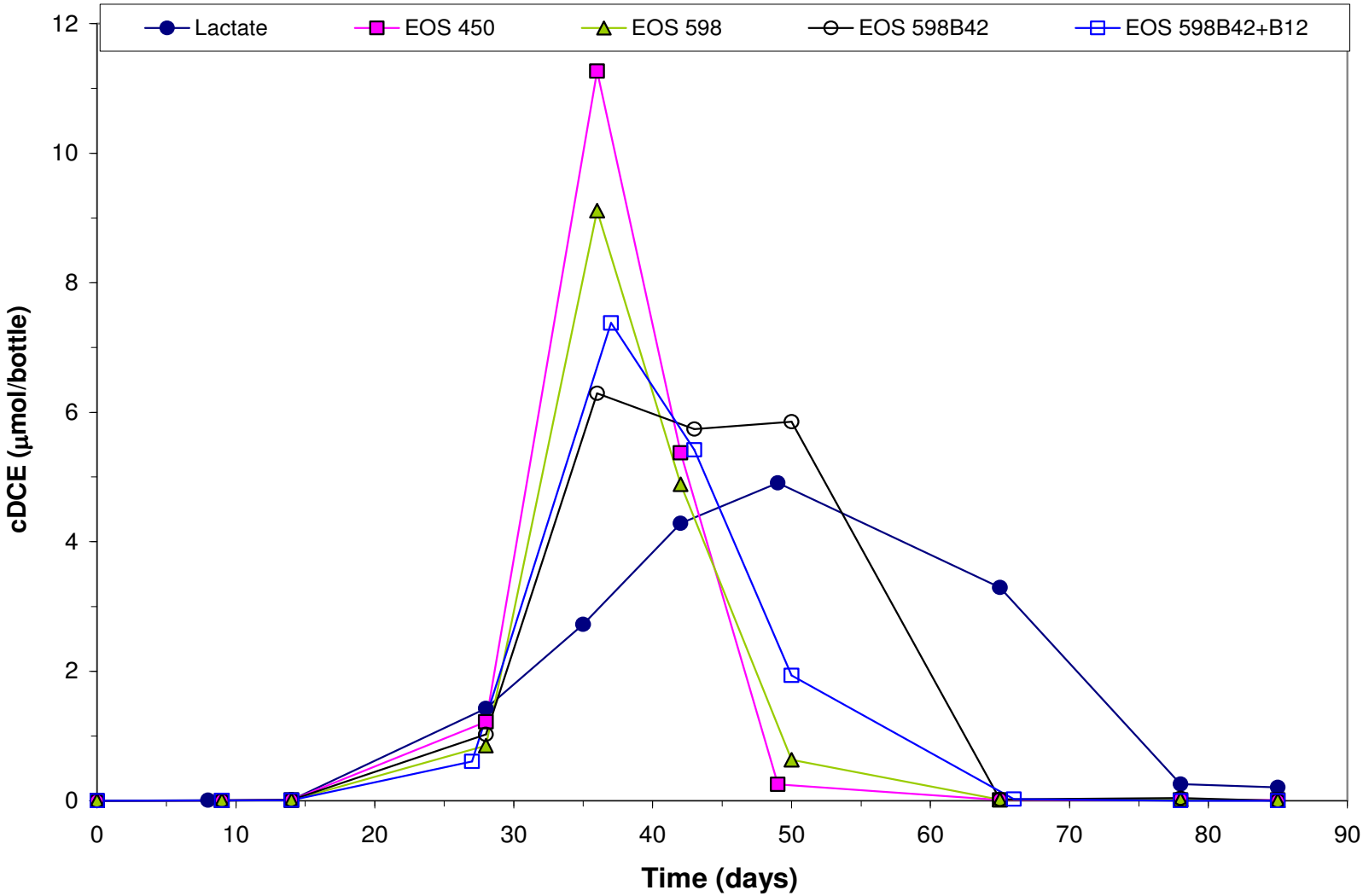

Figure 4.134 Comparison of lactate and EOS® as electron donors on cDCE reductive dechlorination (averages of triplicates).

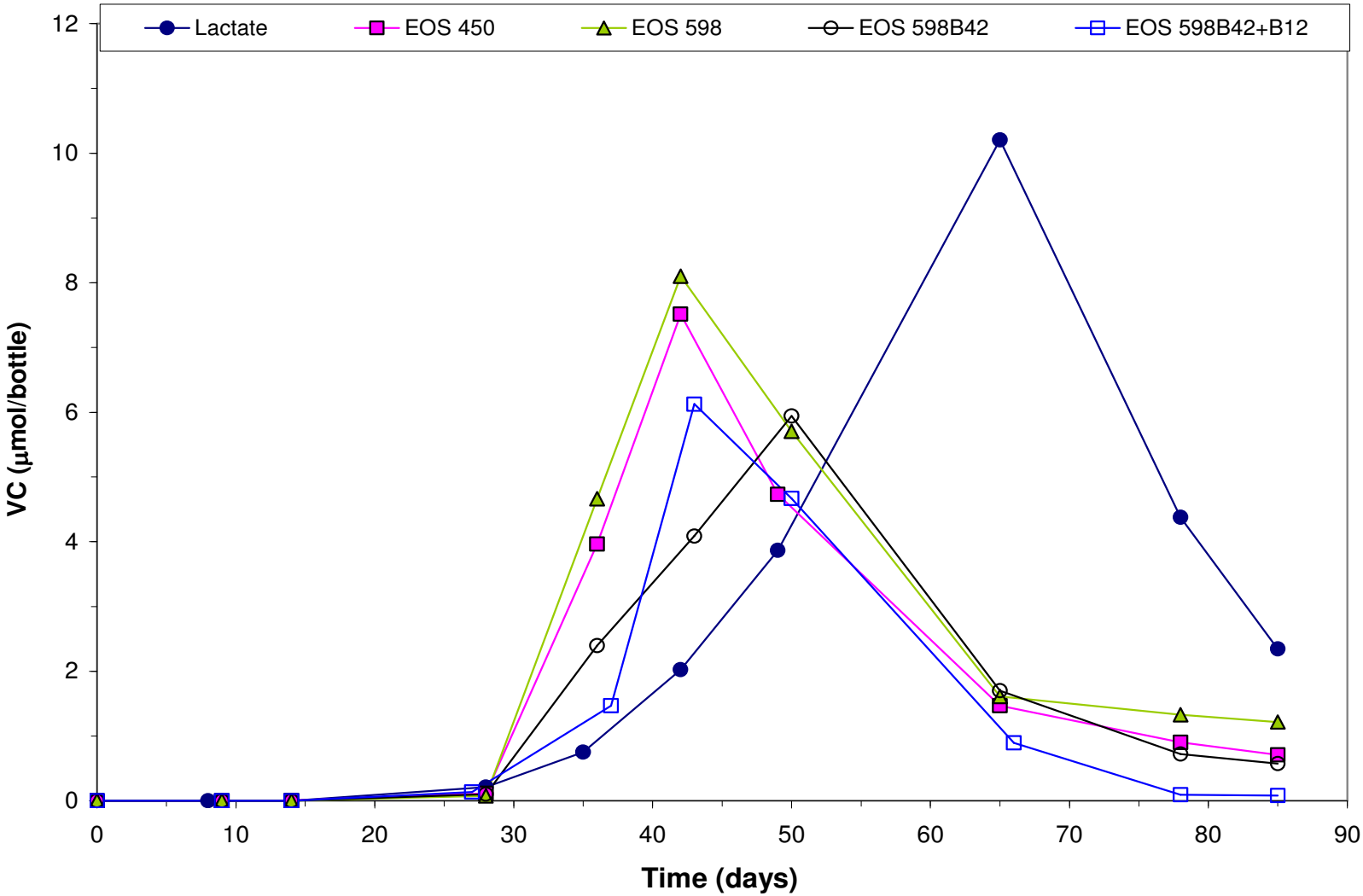

Figure 4.135 Comparison of lactate and EOS® as electron donors on VC reductive dechlorination (averages of triplicates).

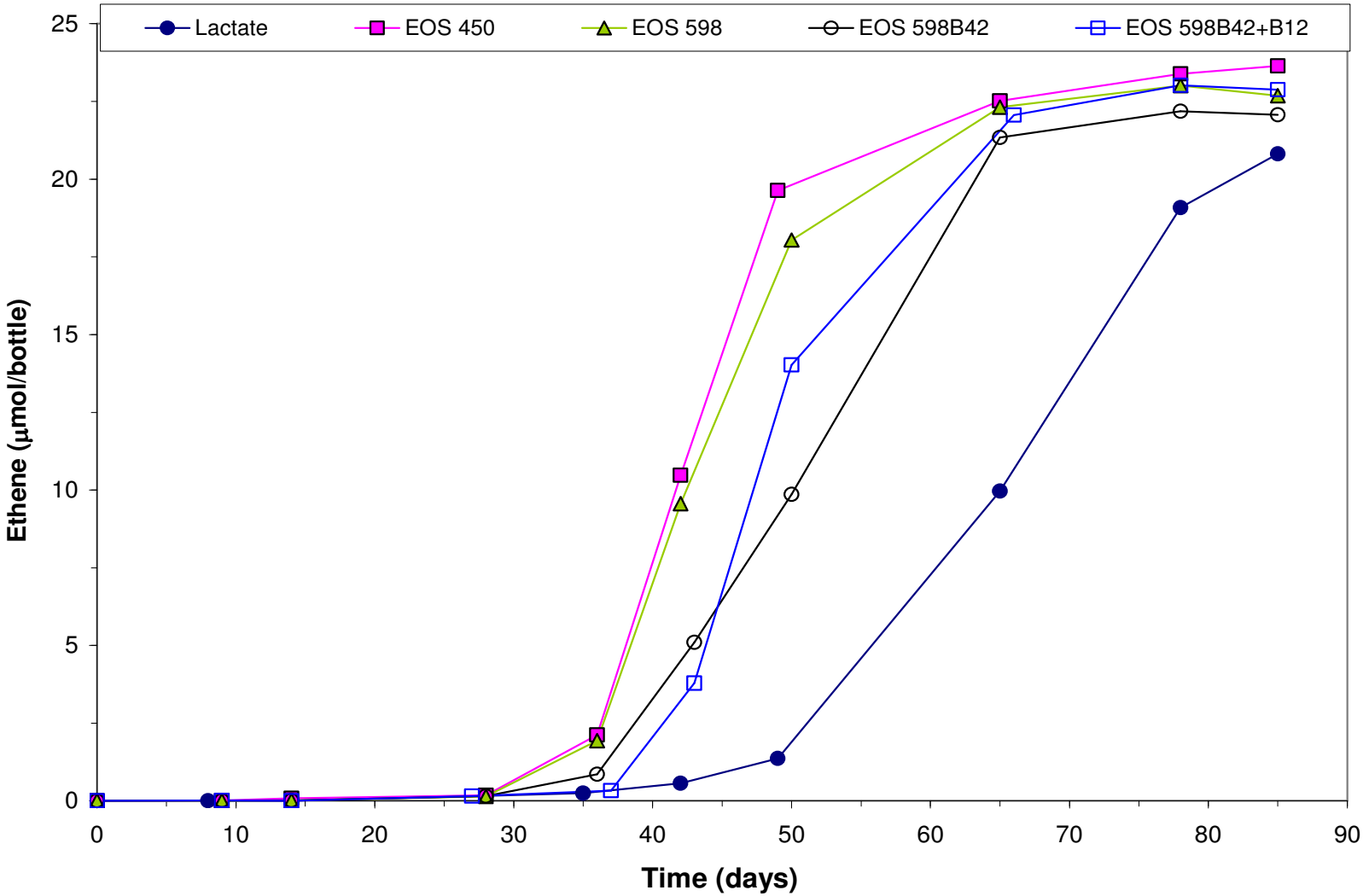

Figure 4.136 Comparison of lactate and EOS® as electron donors on ethene production (averages of triplicates).

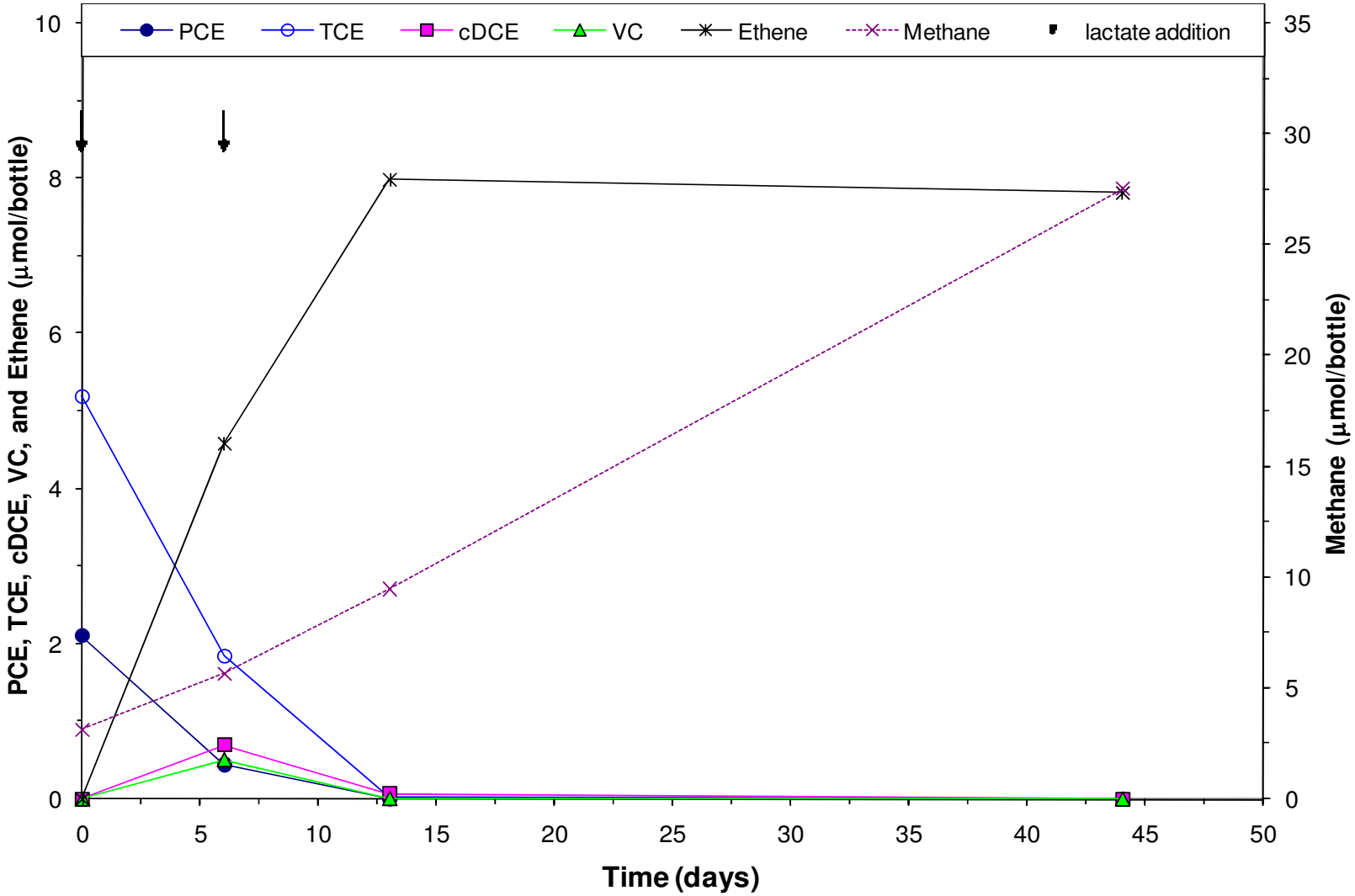

Figure 4.137 Results for one of the unexposed oxygen tolerance bottles (no exposure-1).

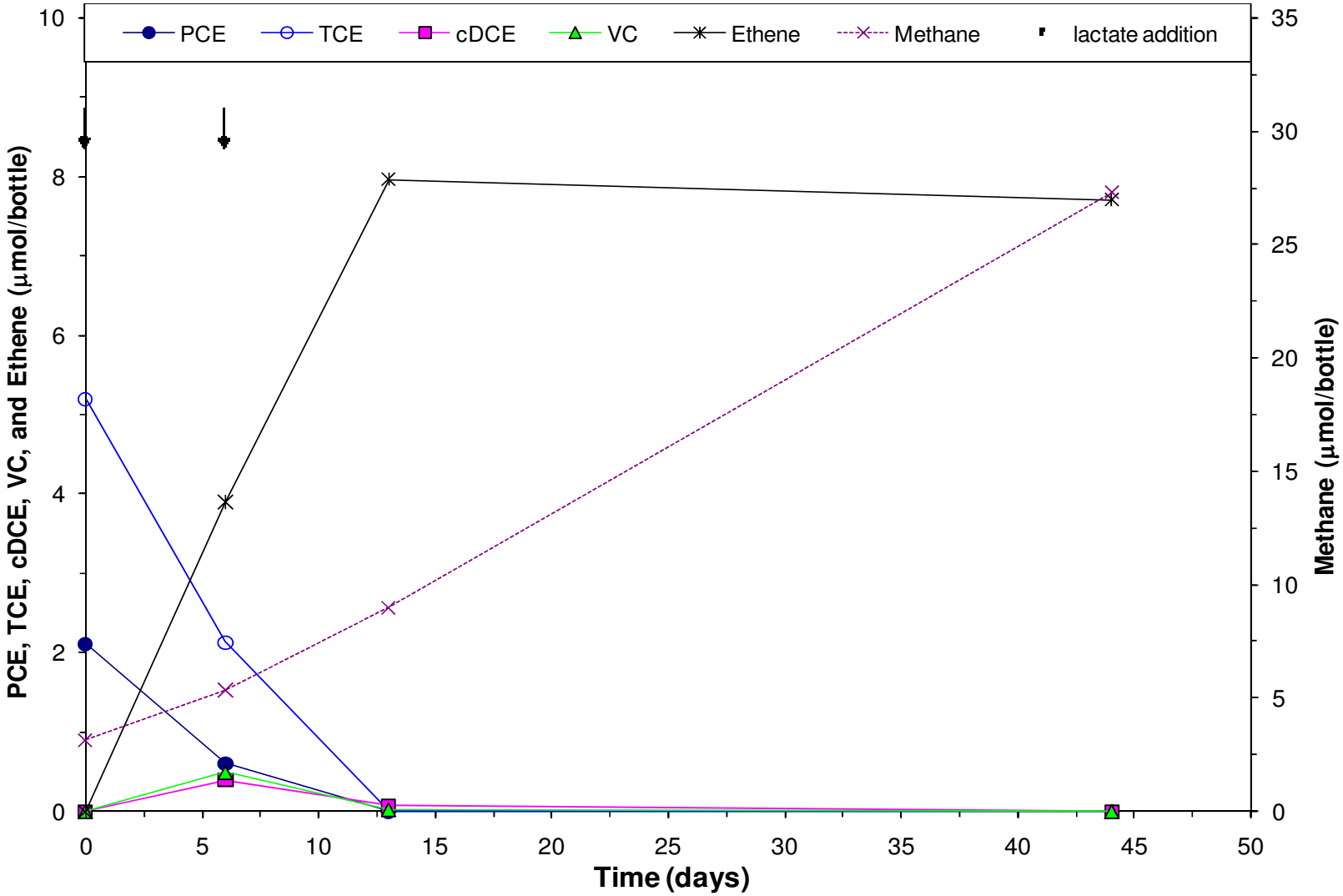

Figure 4.138 Results for one of the unexposed oxygen tolerance bottles (no exposure-2).

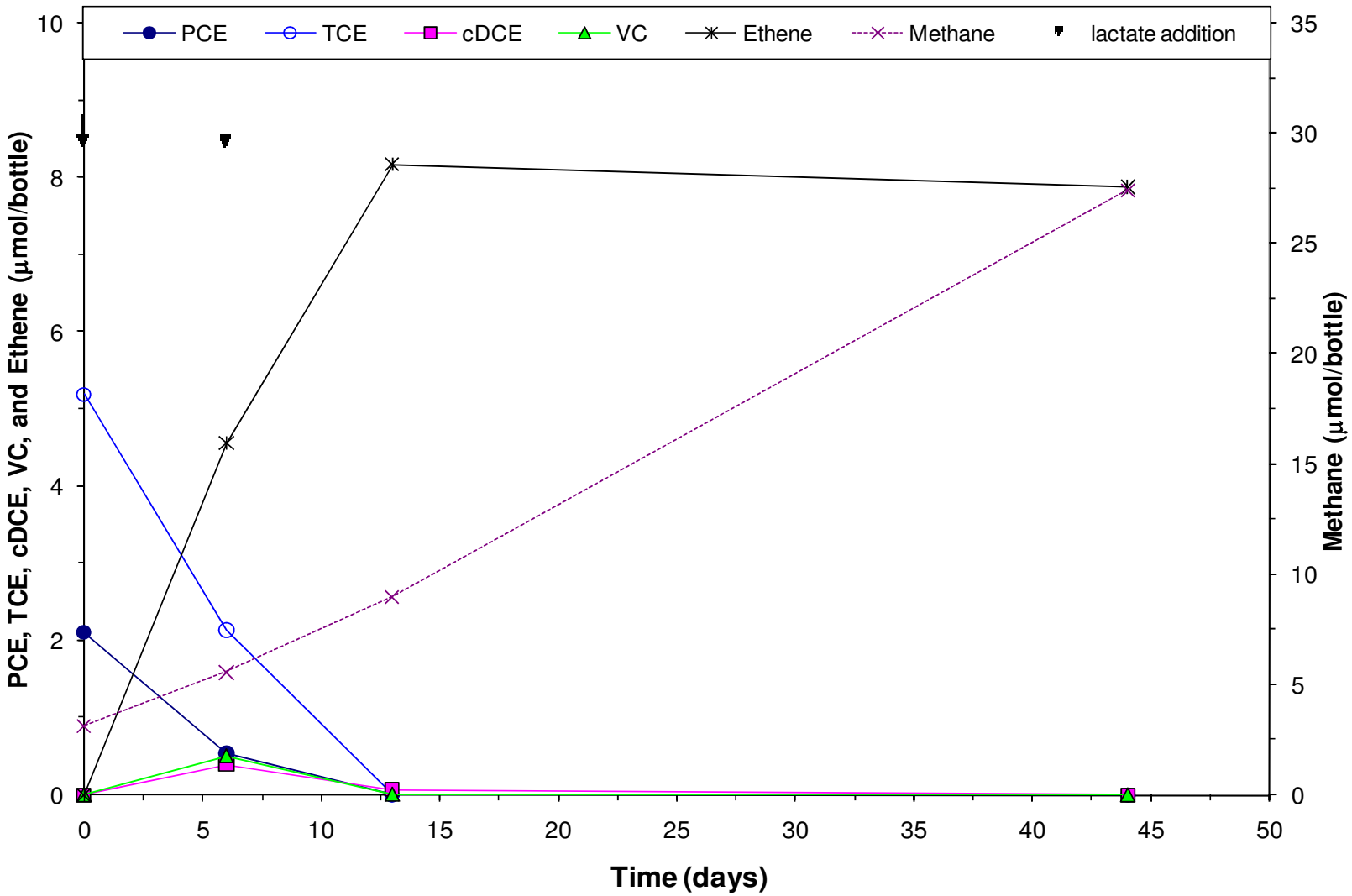

Figure 4.139 Results for one of the unexposed oxygen tolerance bottles (no exposure-3).

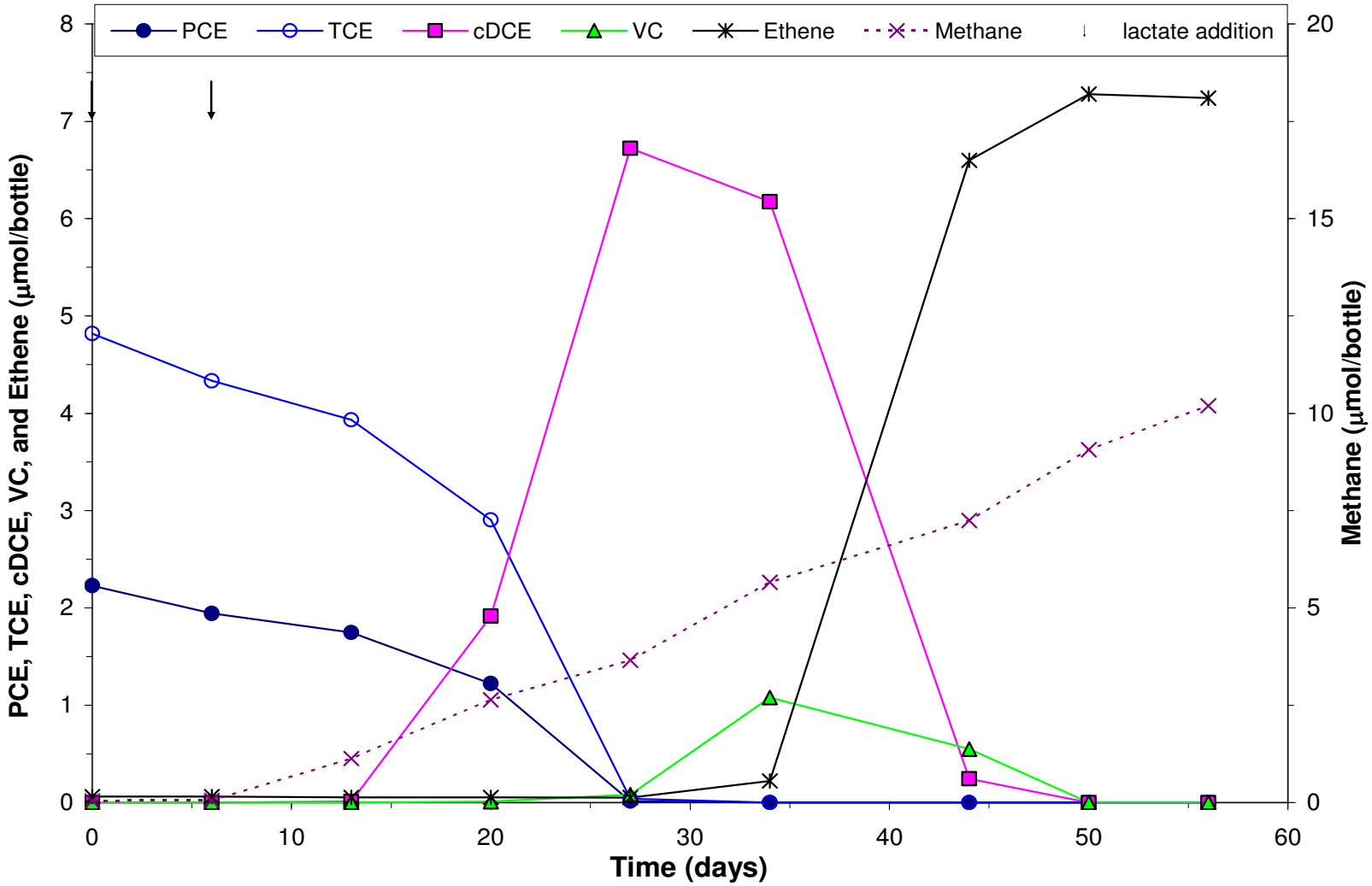

Figure 4.140 Results for one of the 24-hour exposed oxygen tolerance bottles (24 hr exposure-1).

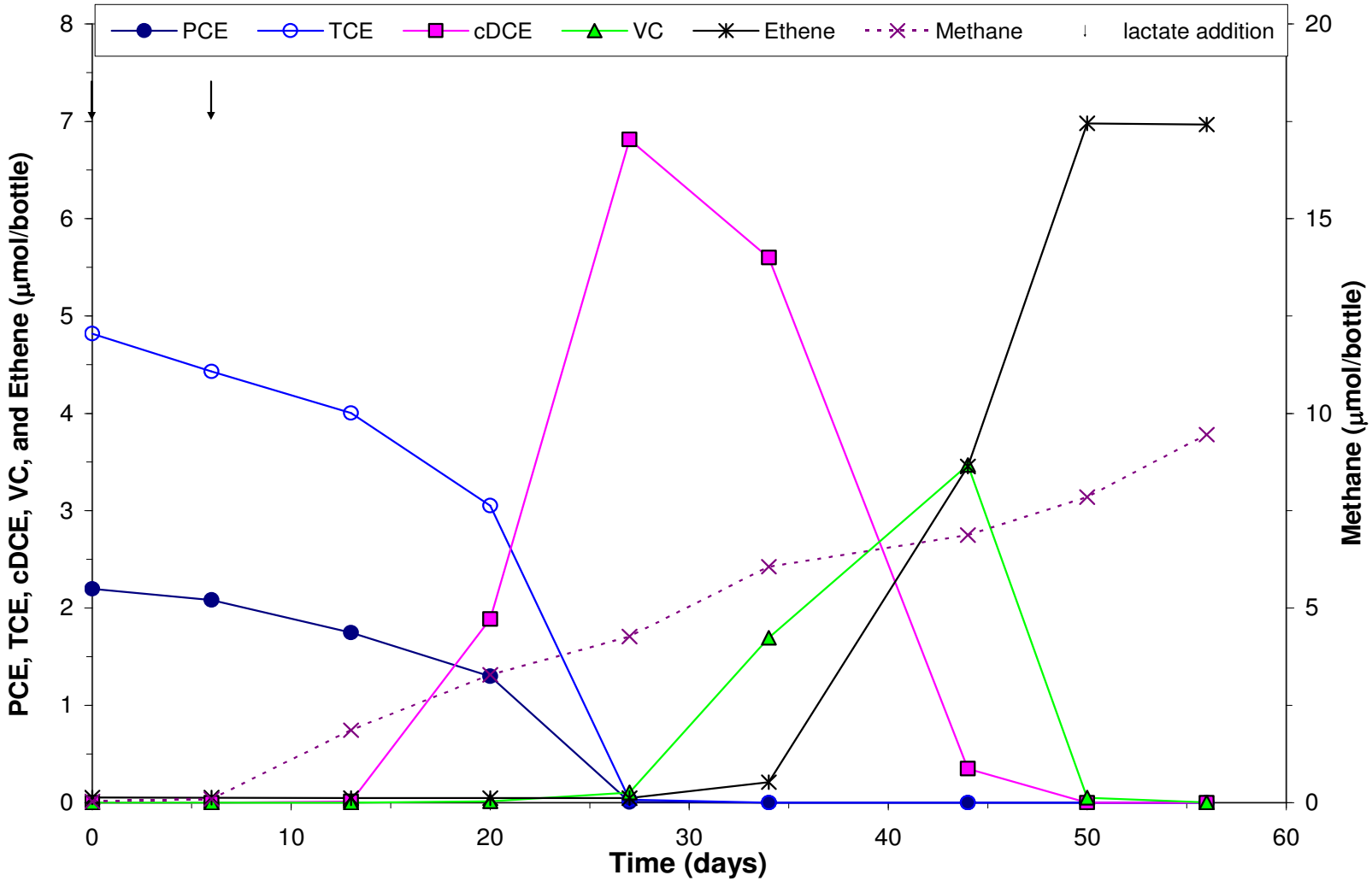

Figure 4.141 Results for one of the 24-hour exposed oxygen tolerance bottles (24 hr exposure-2).

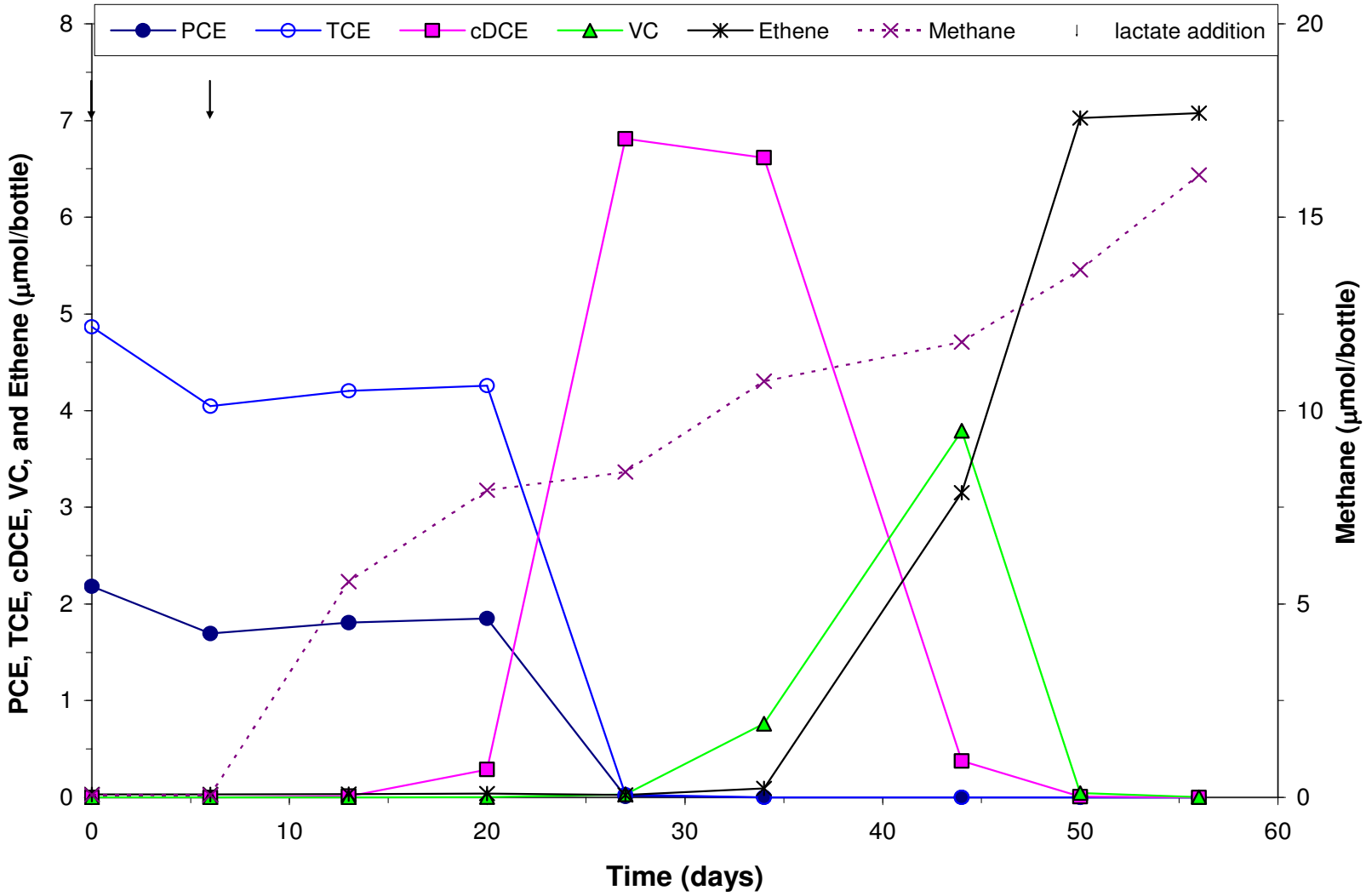

Figure 4.142 Results for one of the 24-hour exposed oxygen tolerance bottles (24 hr exposure-3).

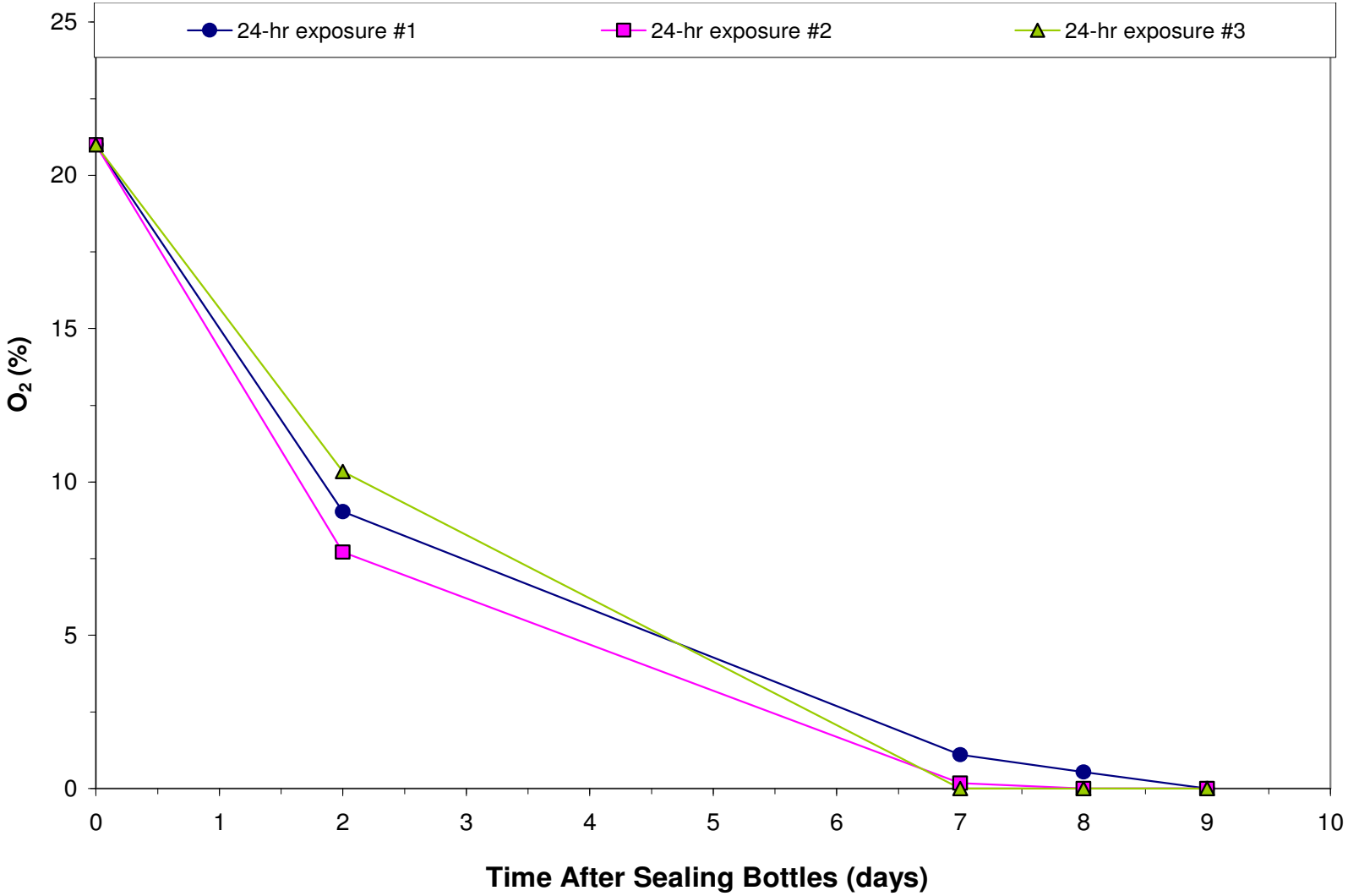

Figure 4.143 Results for percent oxygen in headspace of 24-hour exposed bottles in oxygen tolerance experiment.

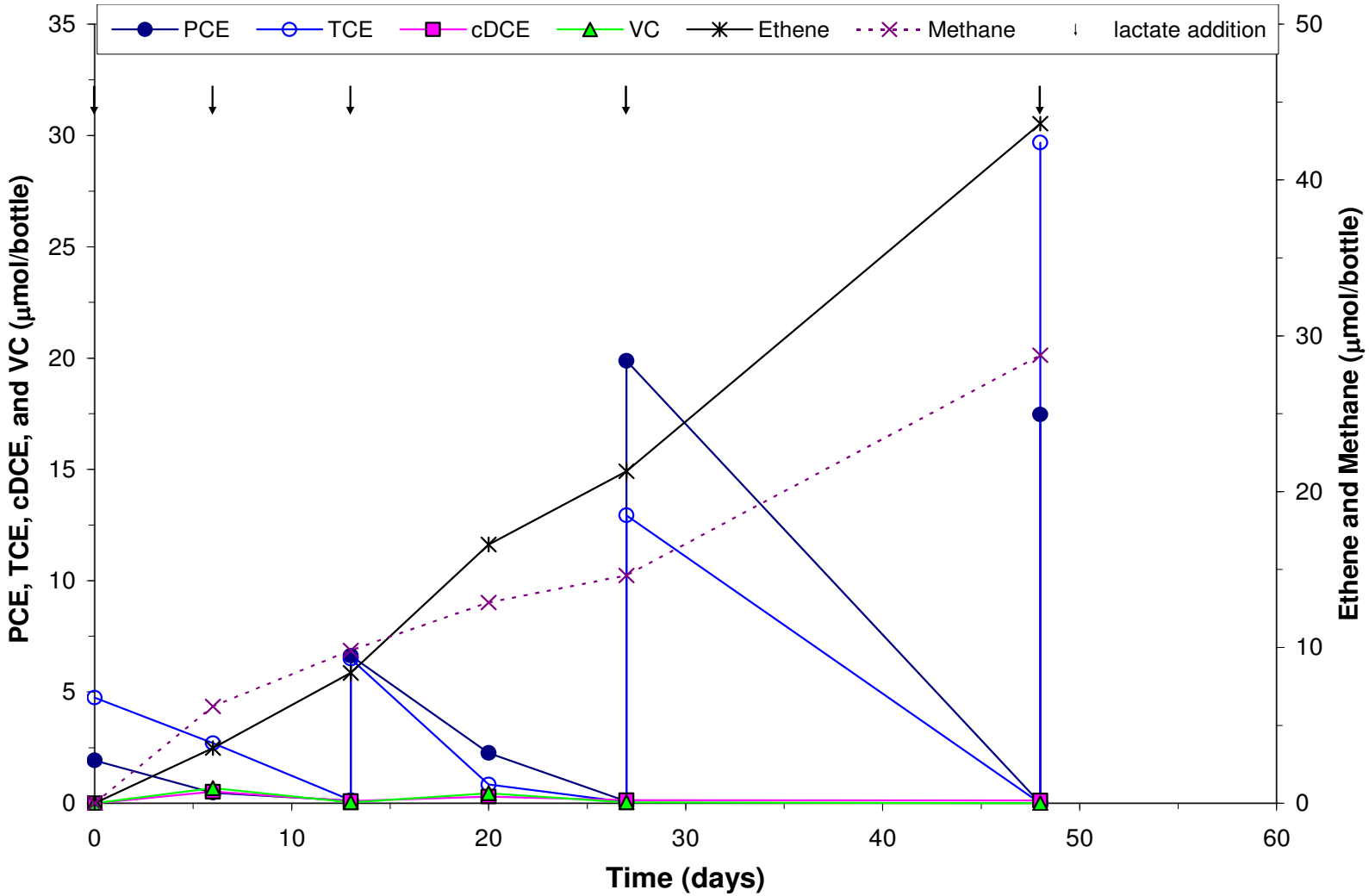

Figure 4.144 Results for one of the pH 7.0±0.25 bottles (pH 7.0±0.25-1). Bottle was broken and discarded on day 48, therefore, no data is shown after day 48.

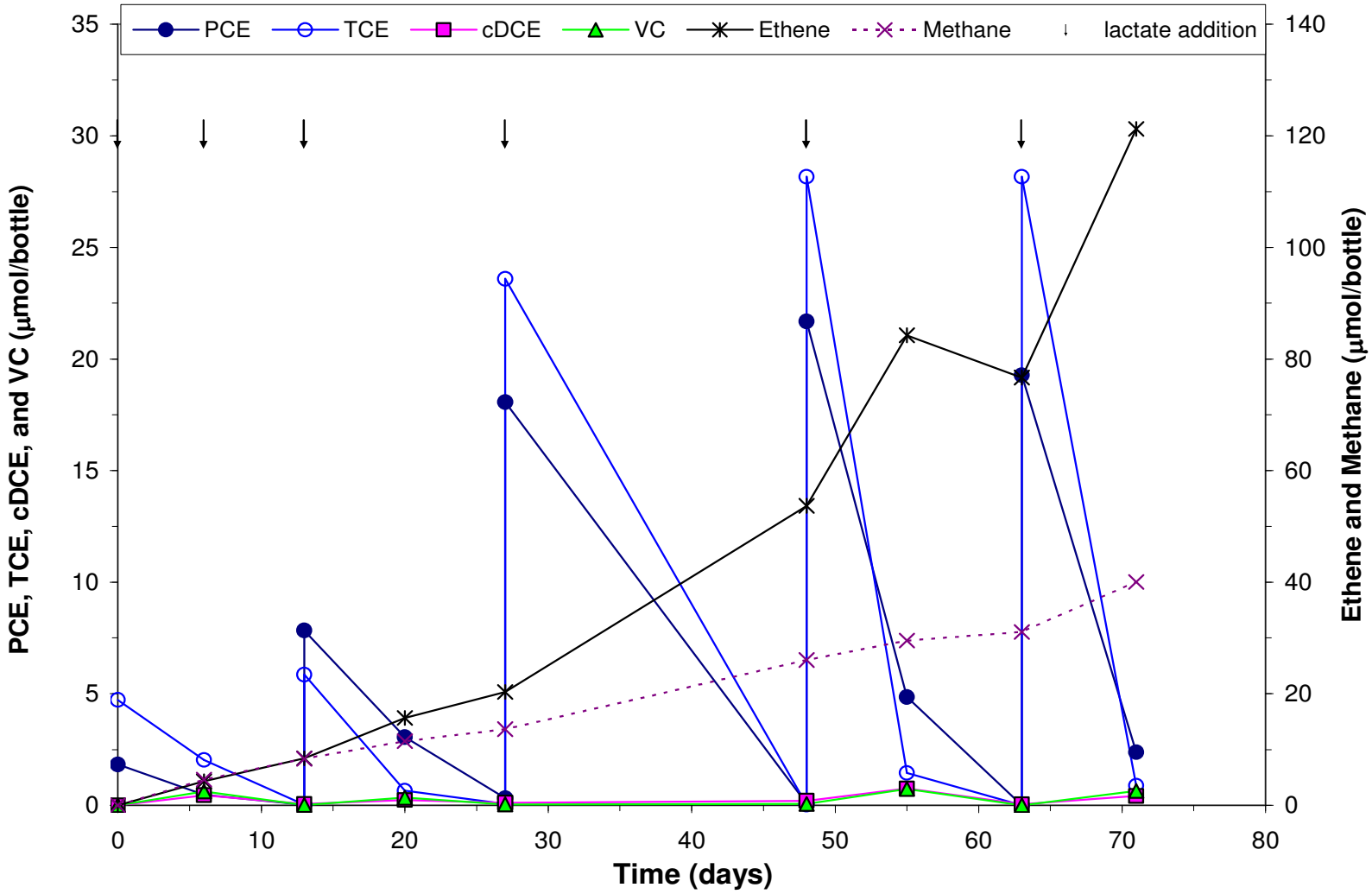

Figure 4.145 Results for one of the pH 7.0±0.25 bottles (pH 7.0±0.25-2).

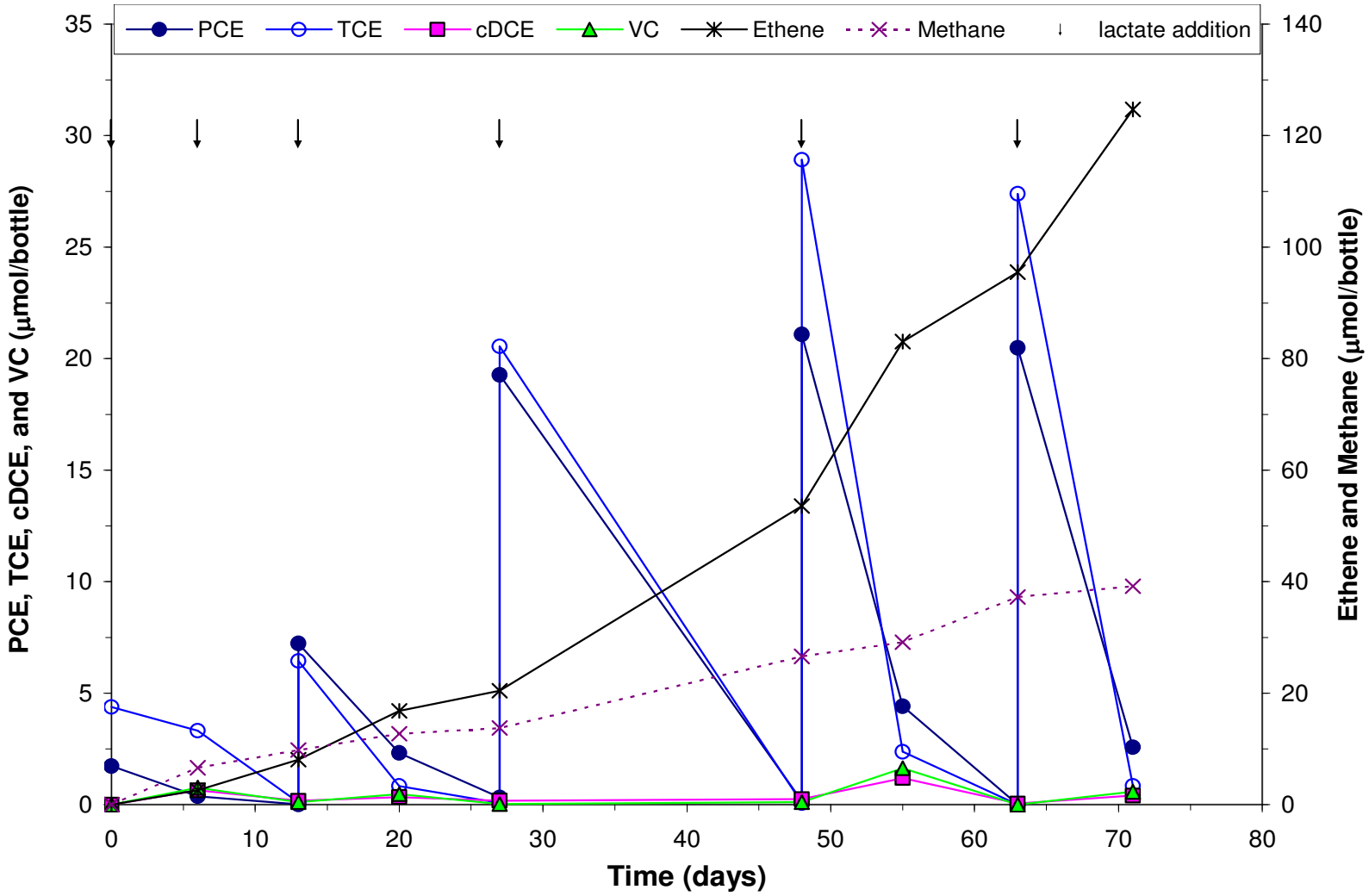

Figure 4.146 Results for one of the pH 7.0±0.25 bottles (pH 7.0±0.25-3).

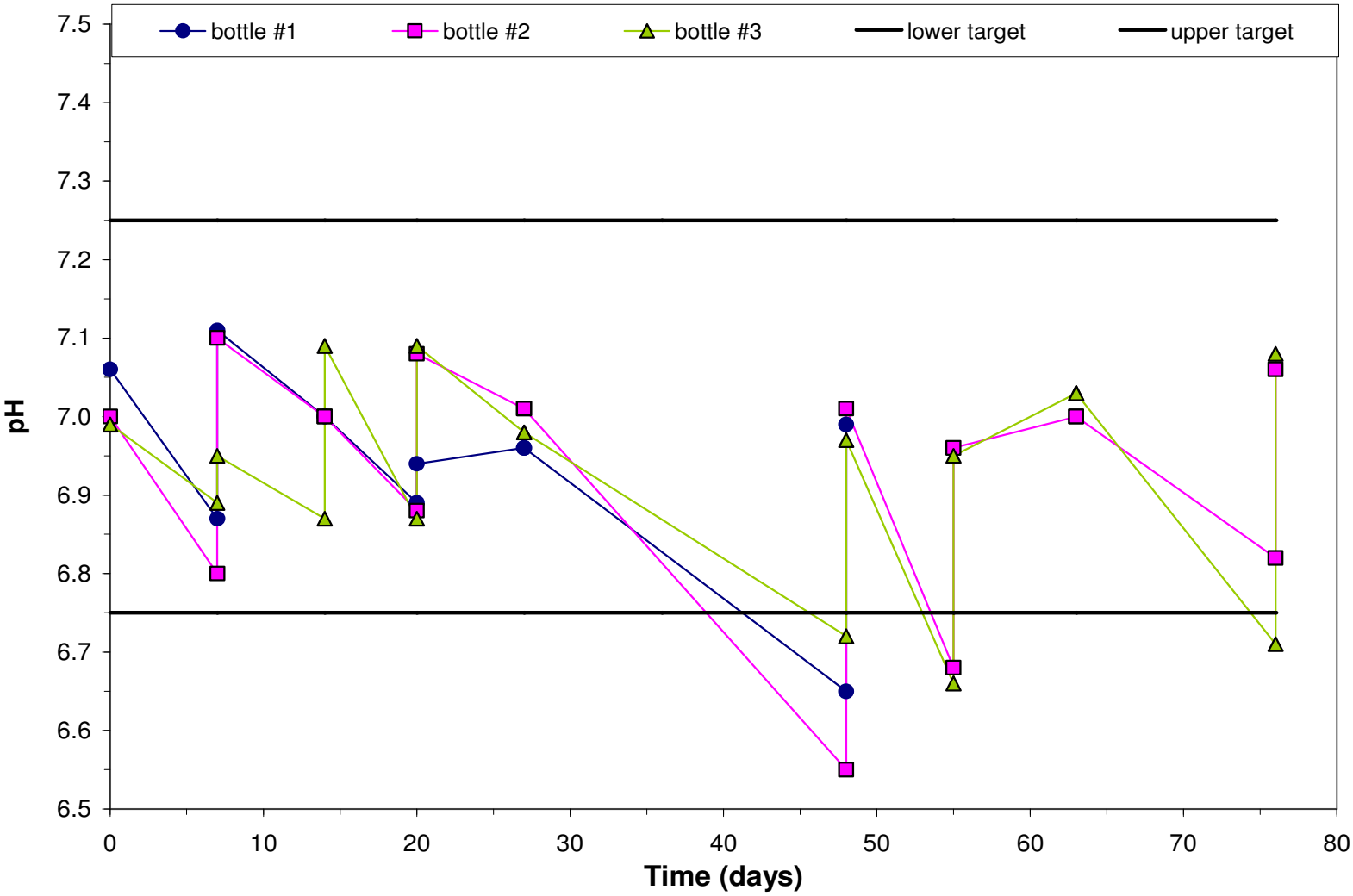

Figure 4.147 Results for pH adjustment of the pH 7.0±0.25 bottles.

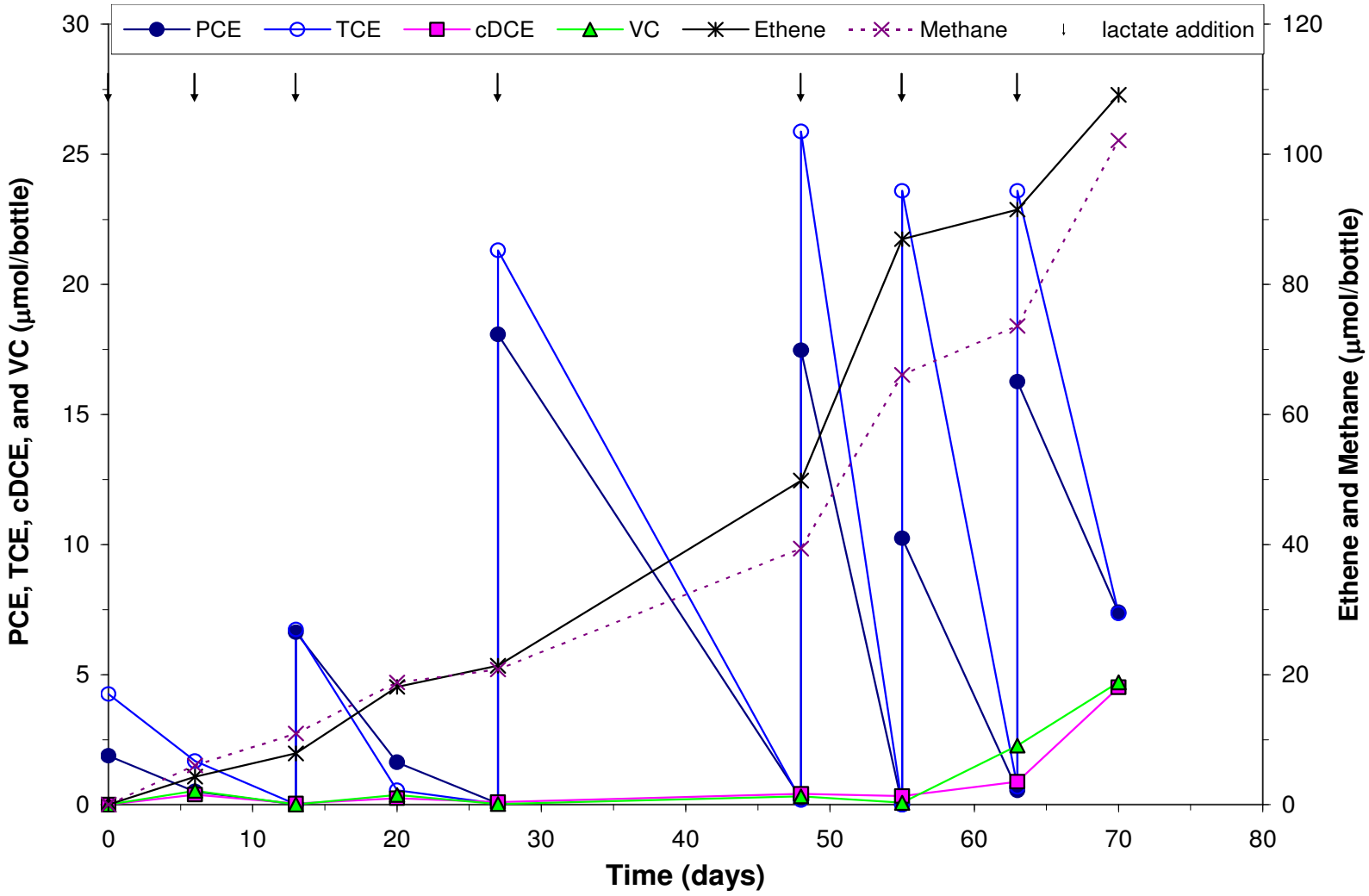

Figure 4.148 Results for one of the pH 7.0 with no pH adjustment bottles (pH 7.0 no adjustment-1).

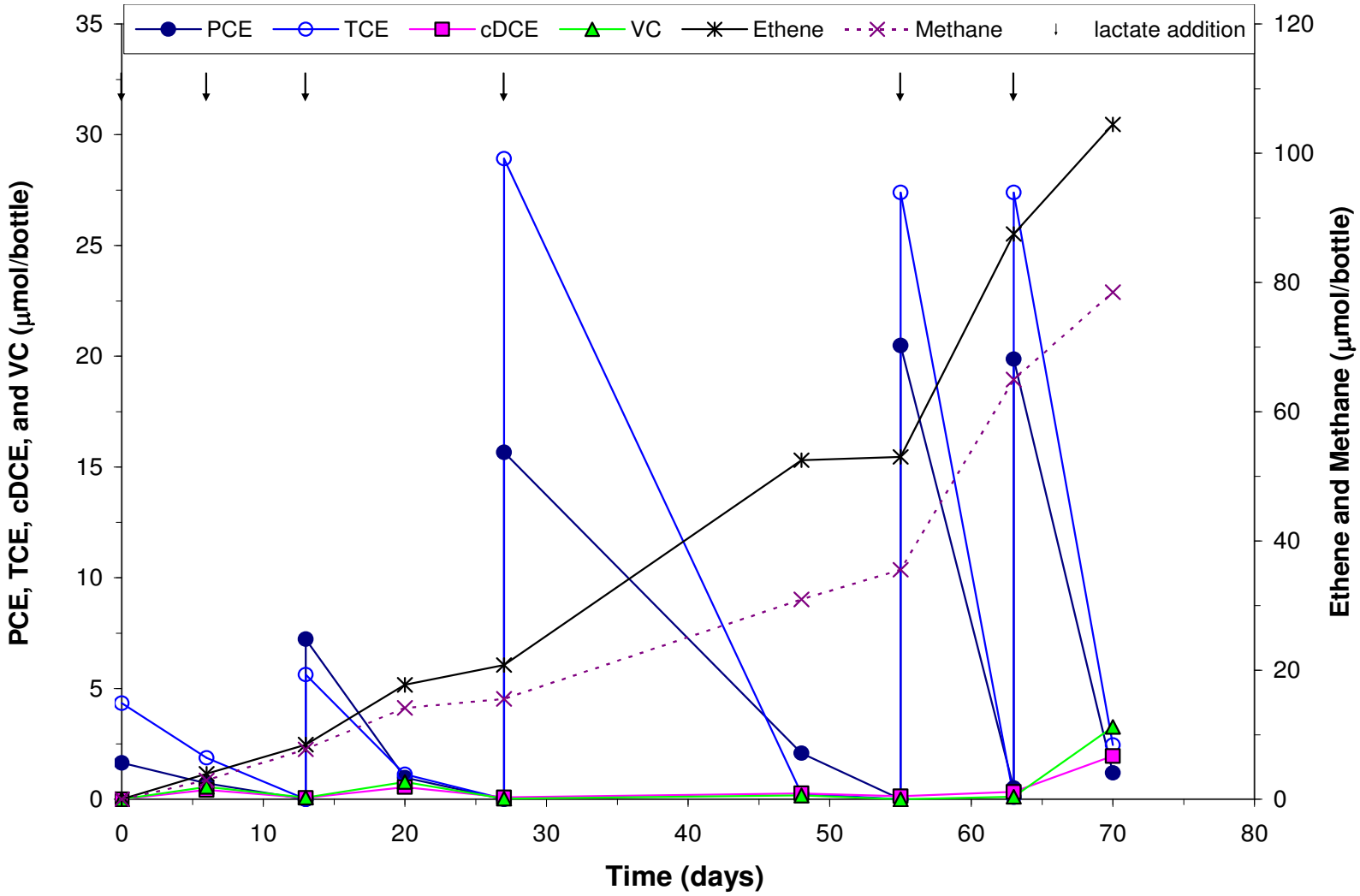

Figure 4.149 Results for one of the pH 7.0 with no pH adjustment bottles (pH 7.0 no adjustment-2).

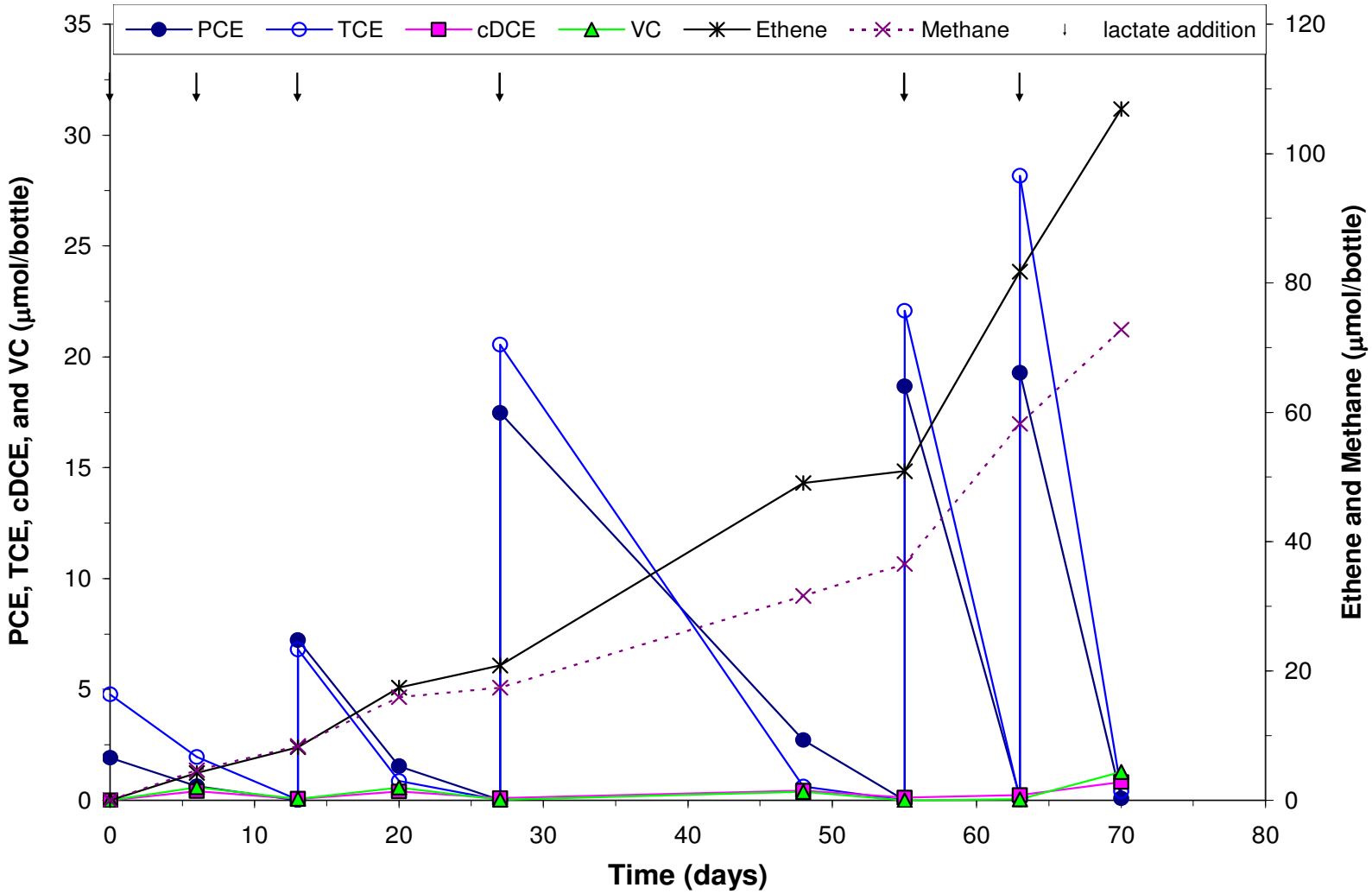

Figure 4.150 Results for one of the pH 7.0 with no pH adjustment bottles (pH 7.0 no adjustment-3).

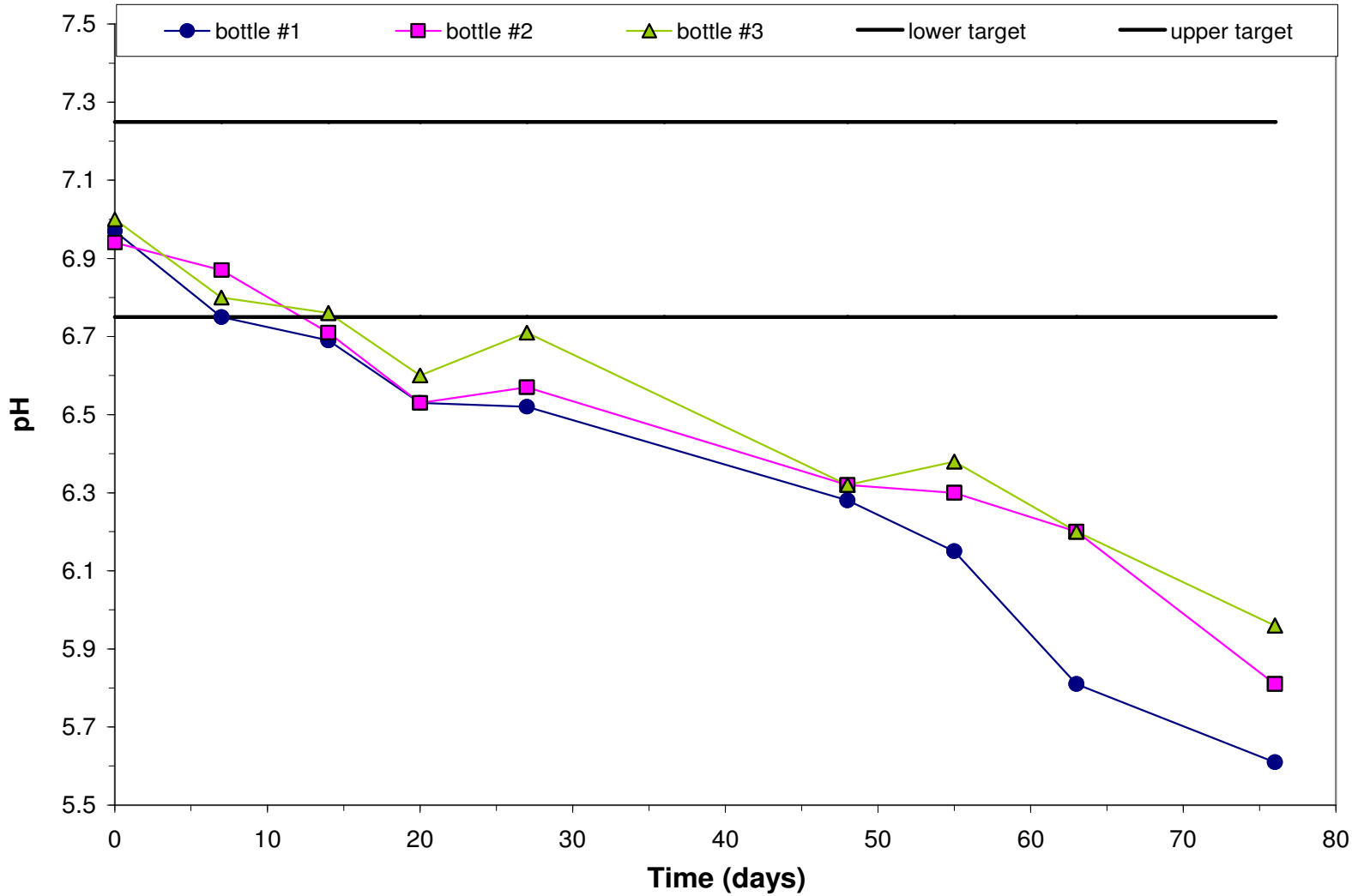

Figure 4.151 Results for pH adjustment of the pH 7.0 with no pH adjustment bottles.

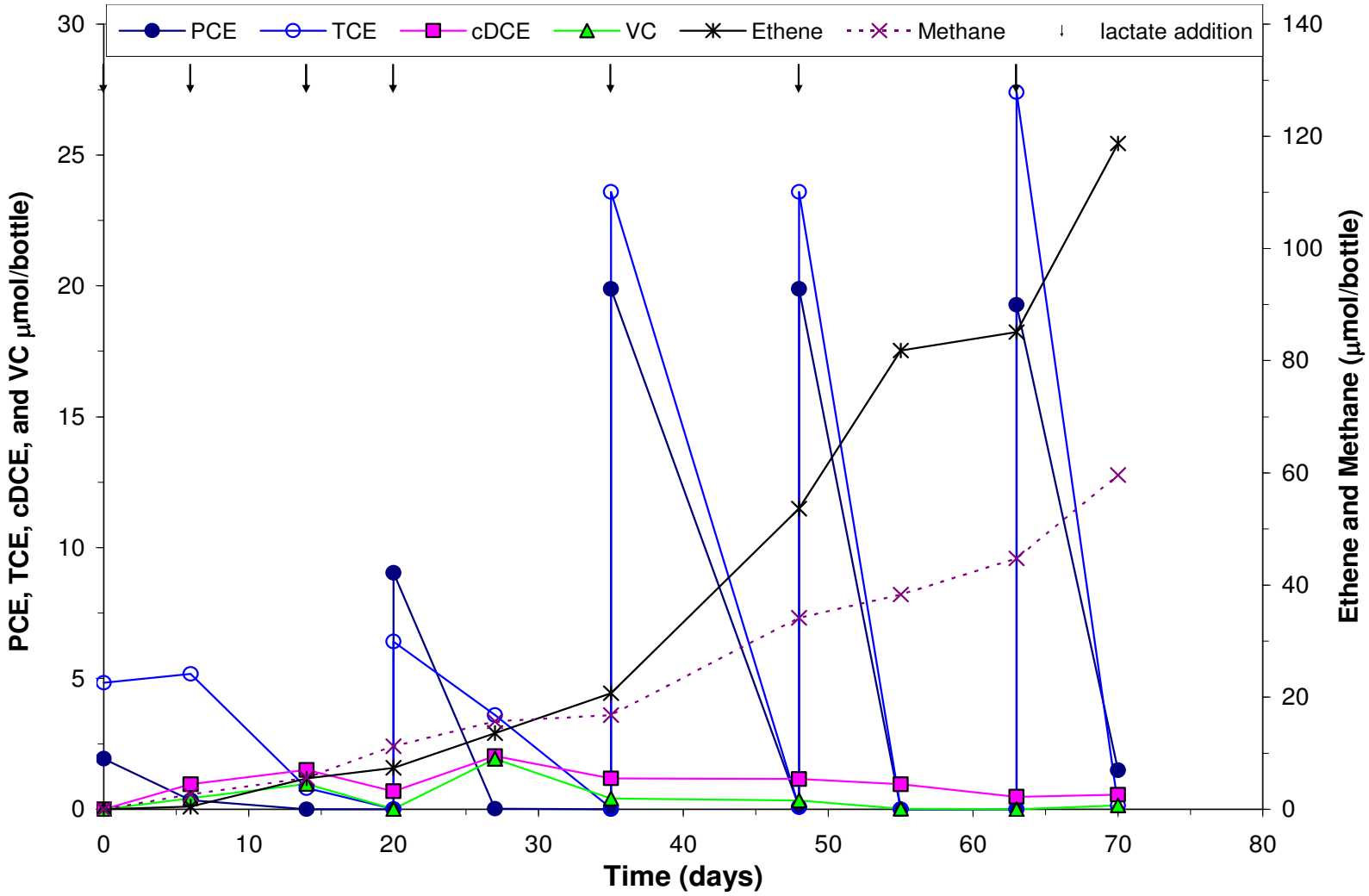

Figure 4.152 Results for one of the pH 6.5±0.25 bottles (pH 6.5±0.25-1).

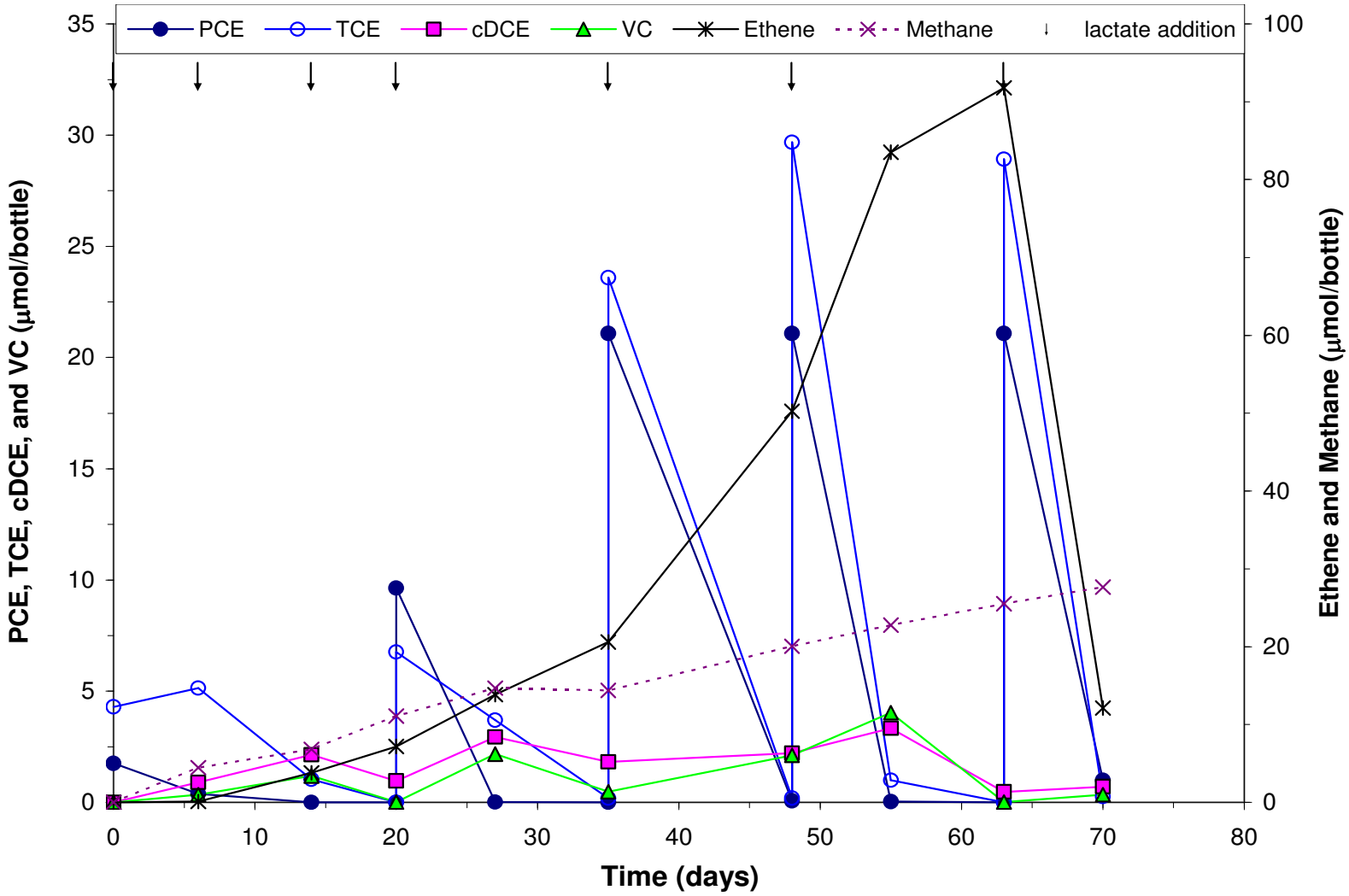

Figure 4.153 Results for one of the pH 6.5±0.25 bottles (pH 6.5±0.25-2).

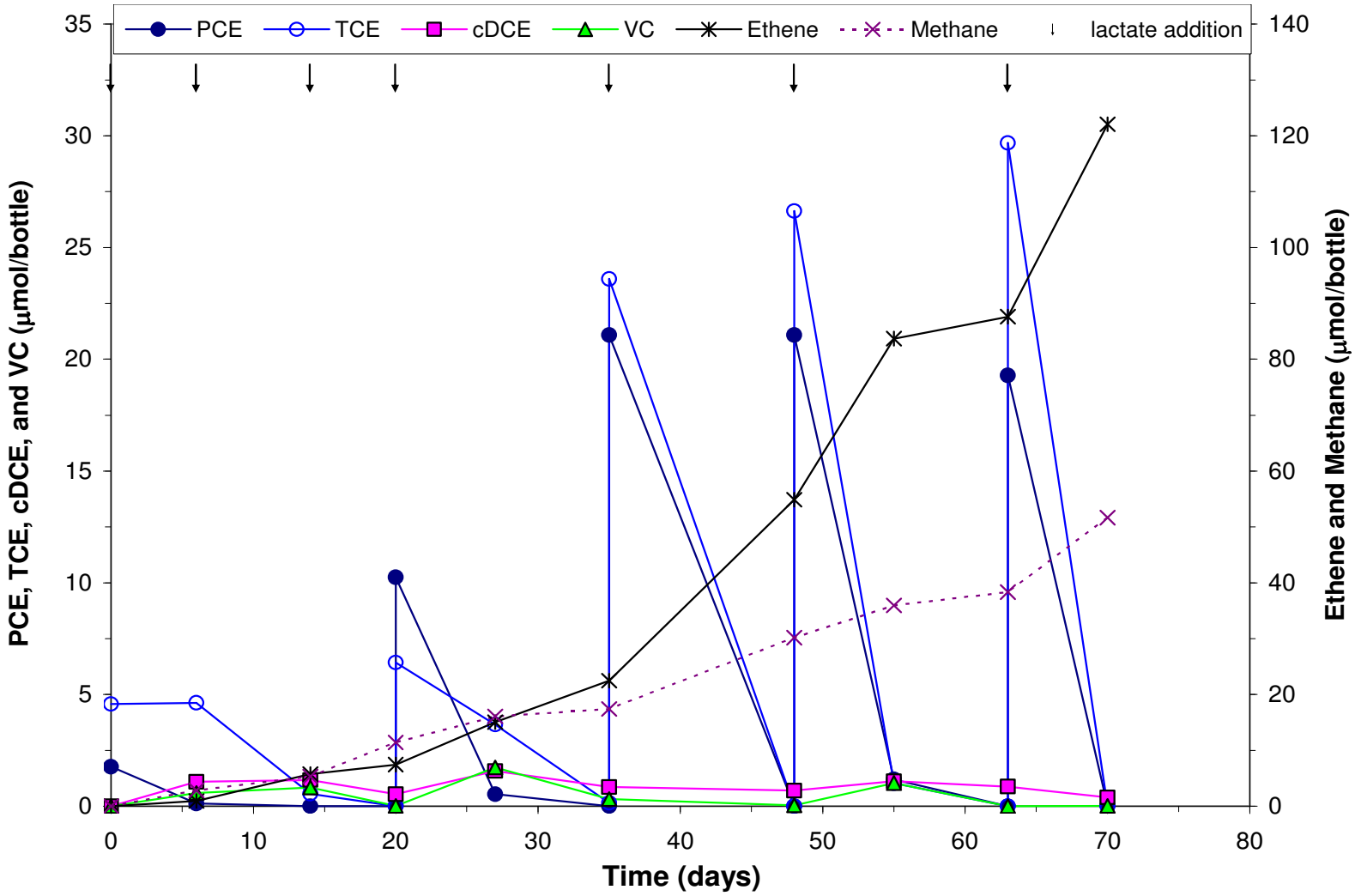

Figure 4.154 Results for one of the pH 6.5±0.25 bottles (pH 6.5±0.25-3).

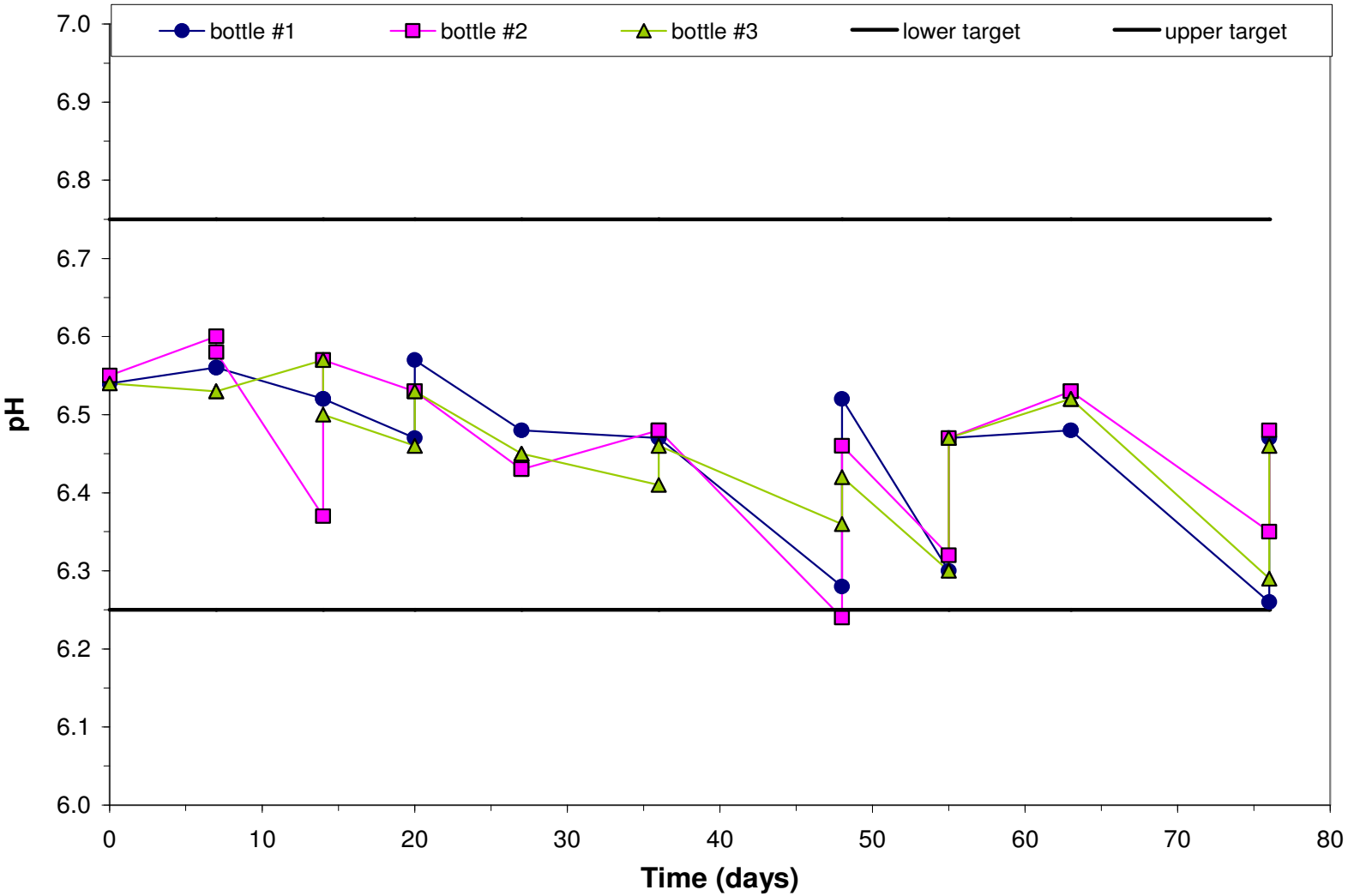

Figure 4.155 Results for pH adjustment of the pH 6.5±0.25 bottles.

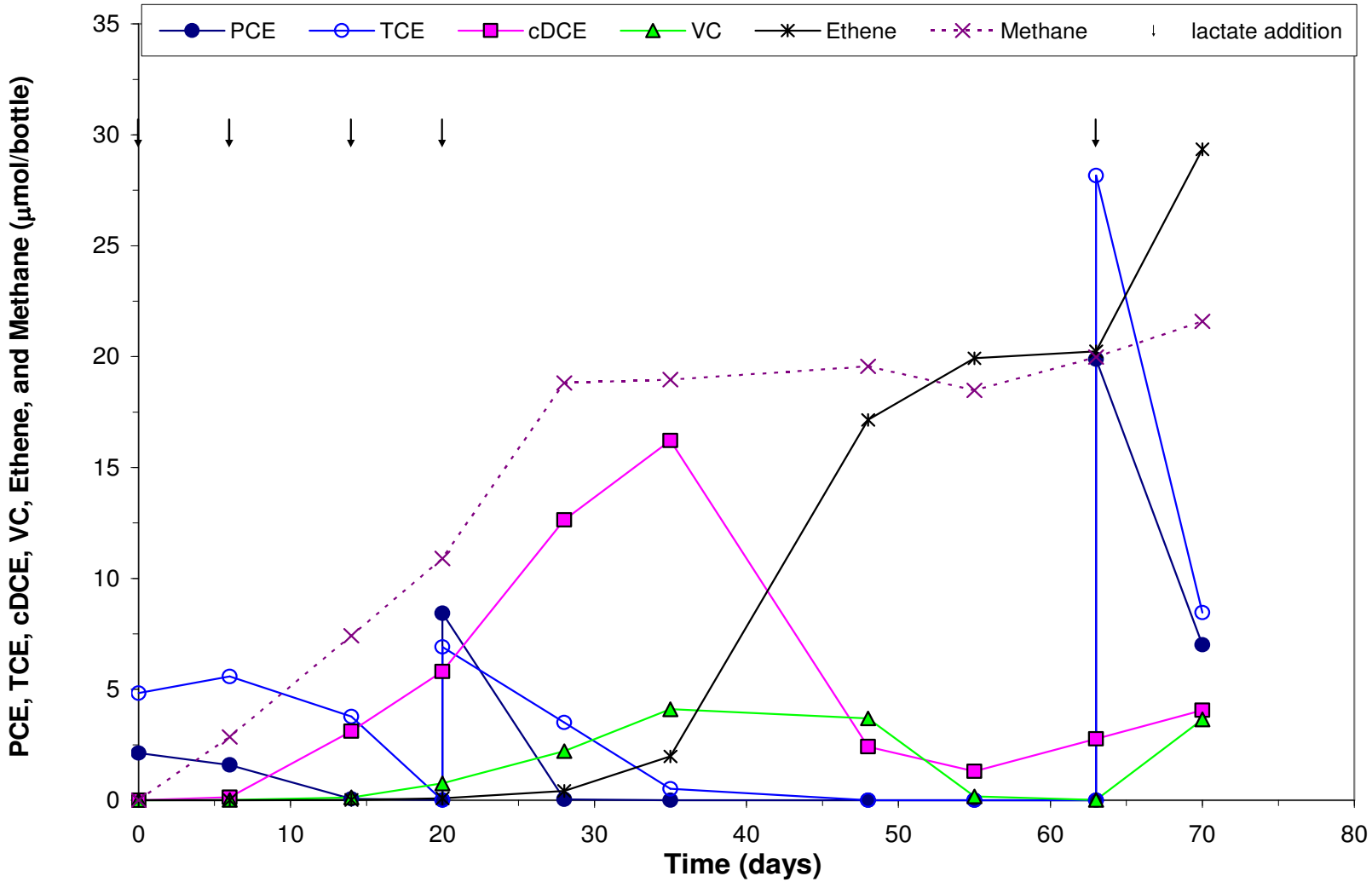

Figure 4.156 Results for one of the pH 6.0±0.25 bottles (pH 6.0±0.25-1).

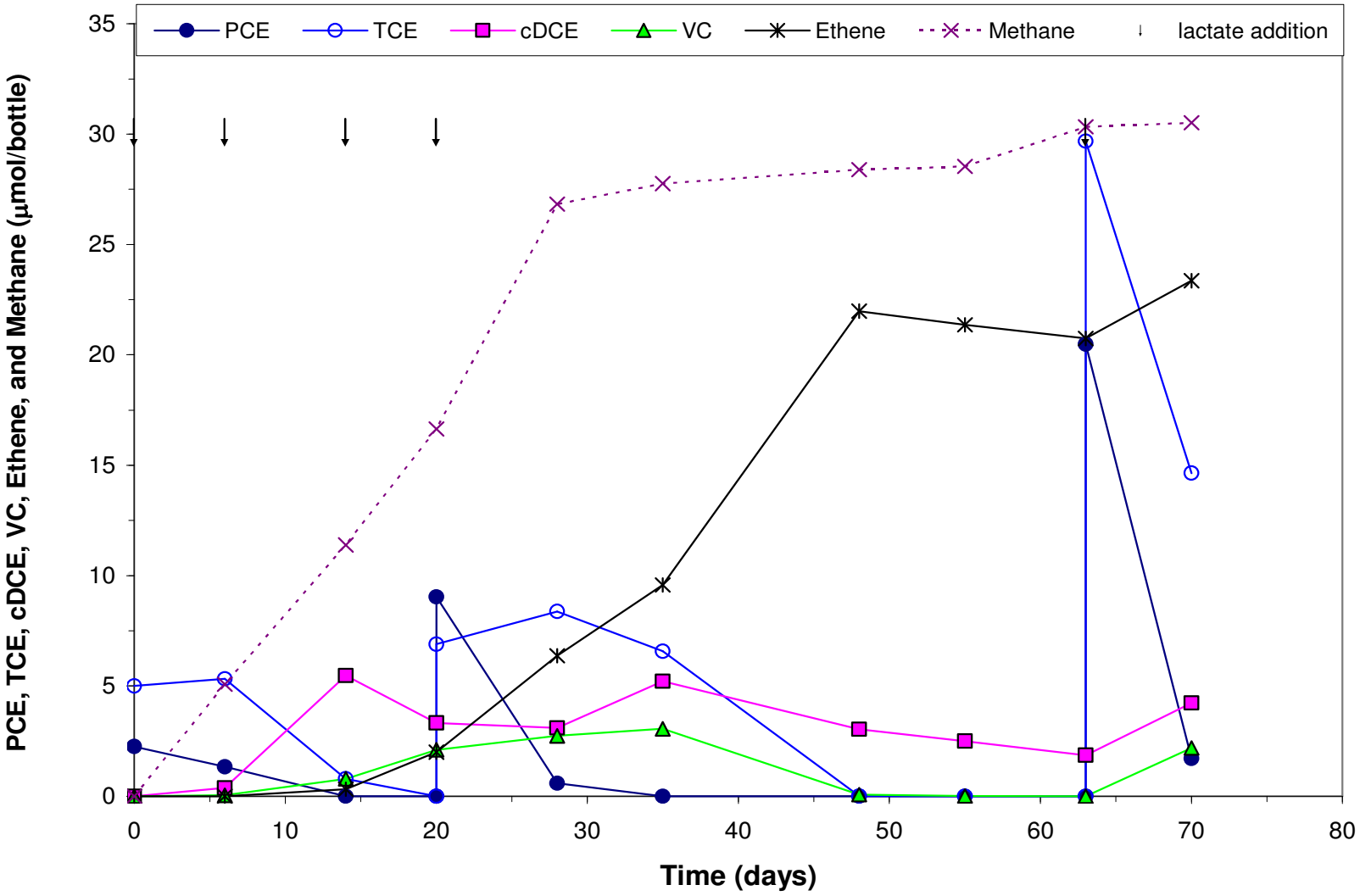

Figure 4.157 Results for one of the pH 6.0±0.25 bottles (pH 6.0±0.25-2).

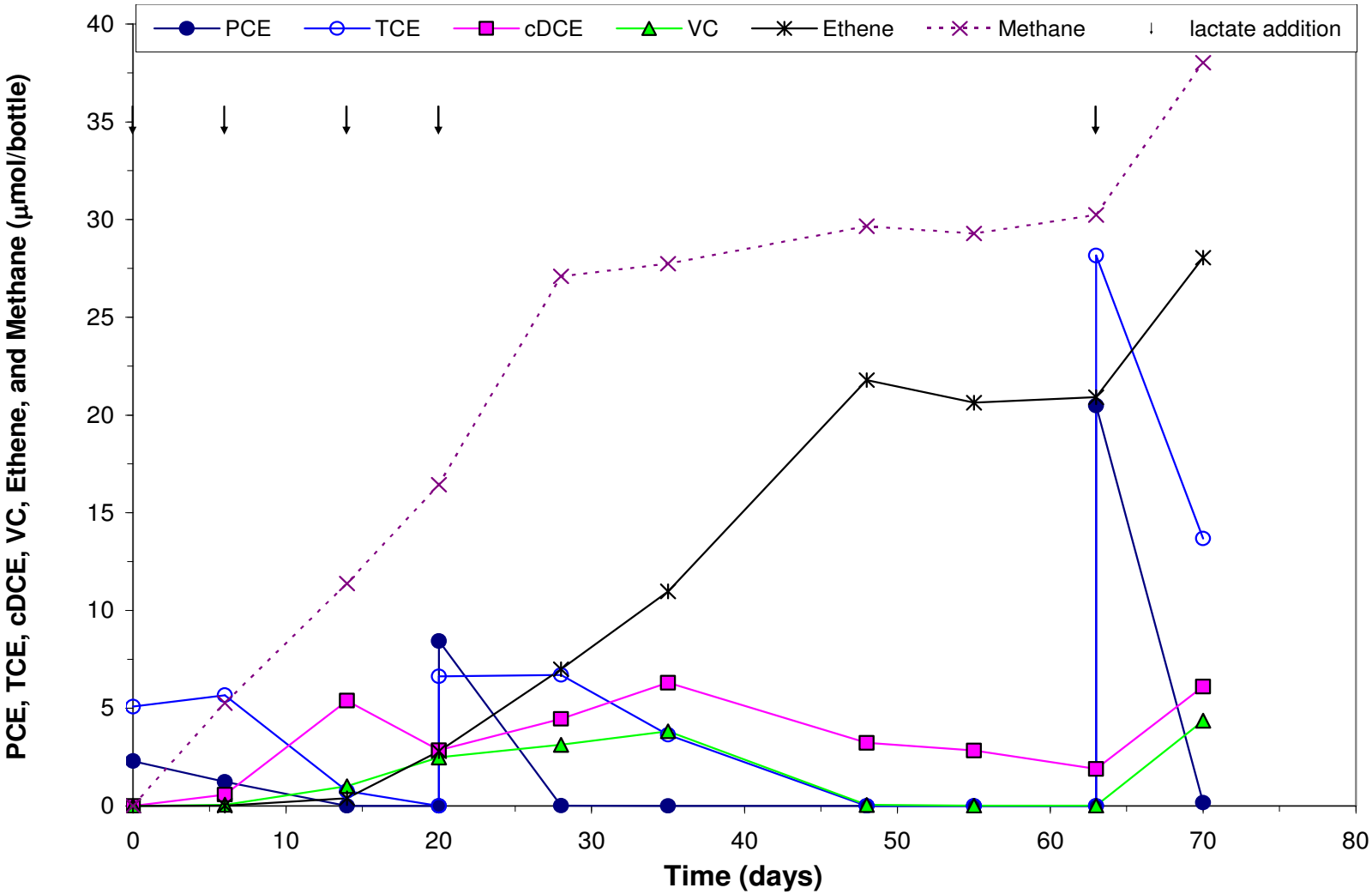

Figure 4.158 Results for one of the pH 6.0±0.25 bottles (pH 6.0±0.25-3).

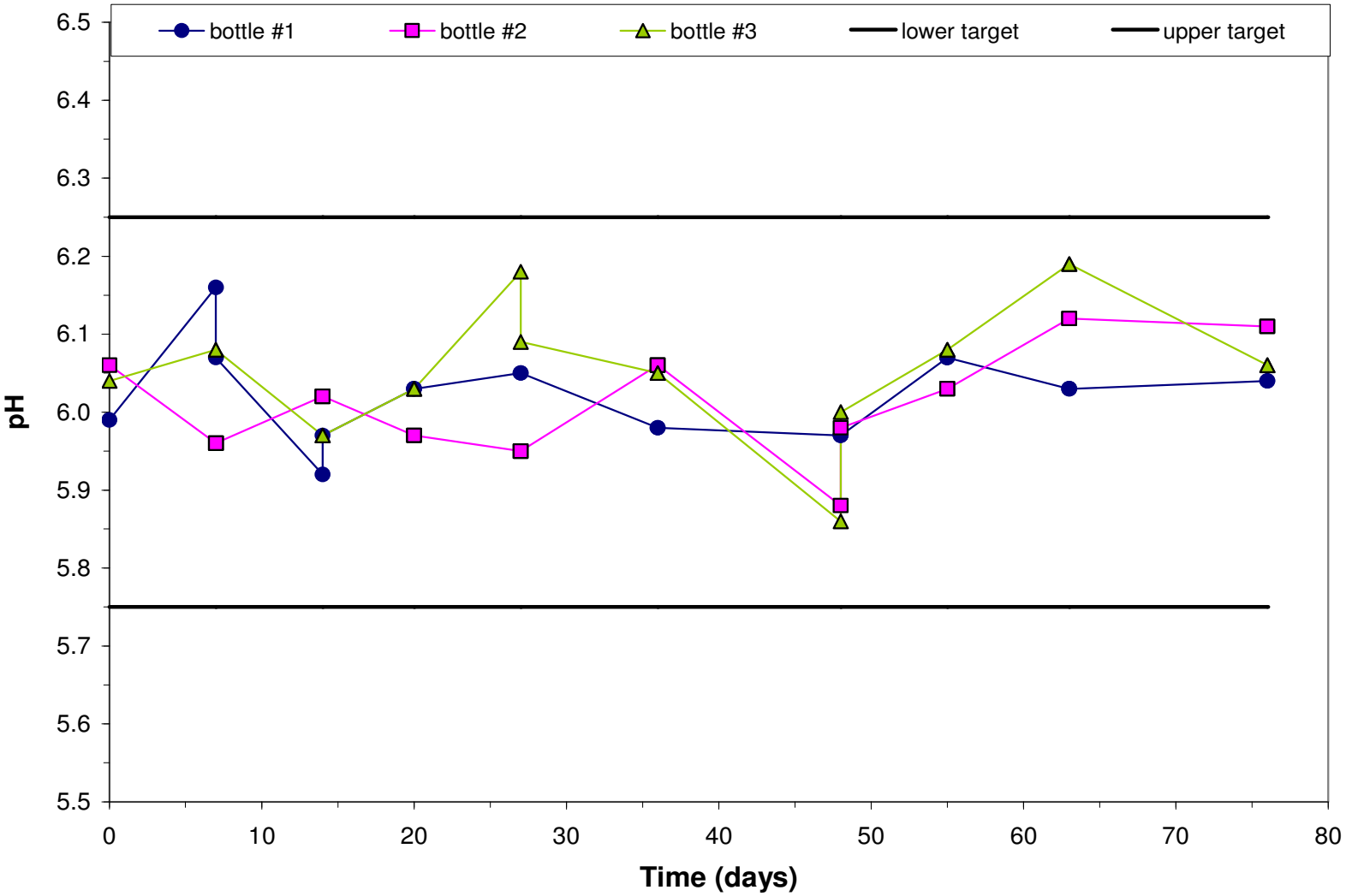

Figure 4.159 Results for pH adjustment of the pH 6.0±0.25 bottles.

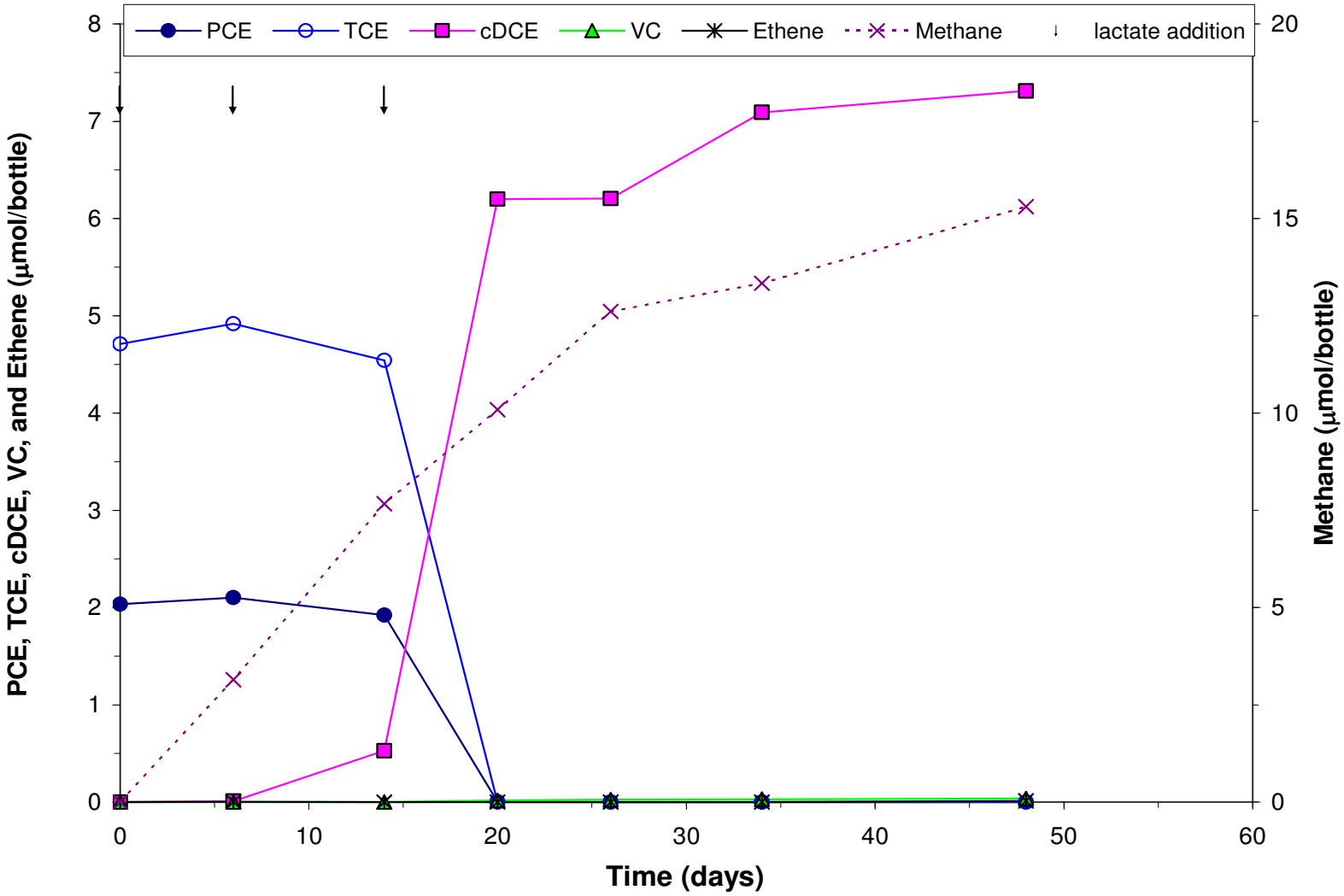

Figure 4.160 Results for one of the pH 5.5±0.25 bottles (pH 5.5±0.25-1). Bottle was broken and discarded on day 48, therefore, no data is shown after day 48.

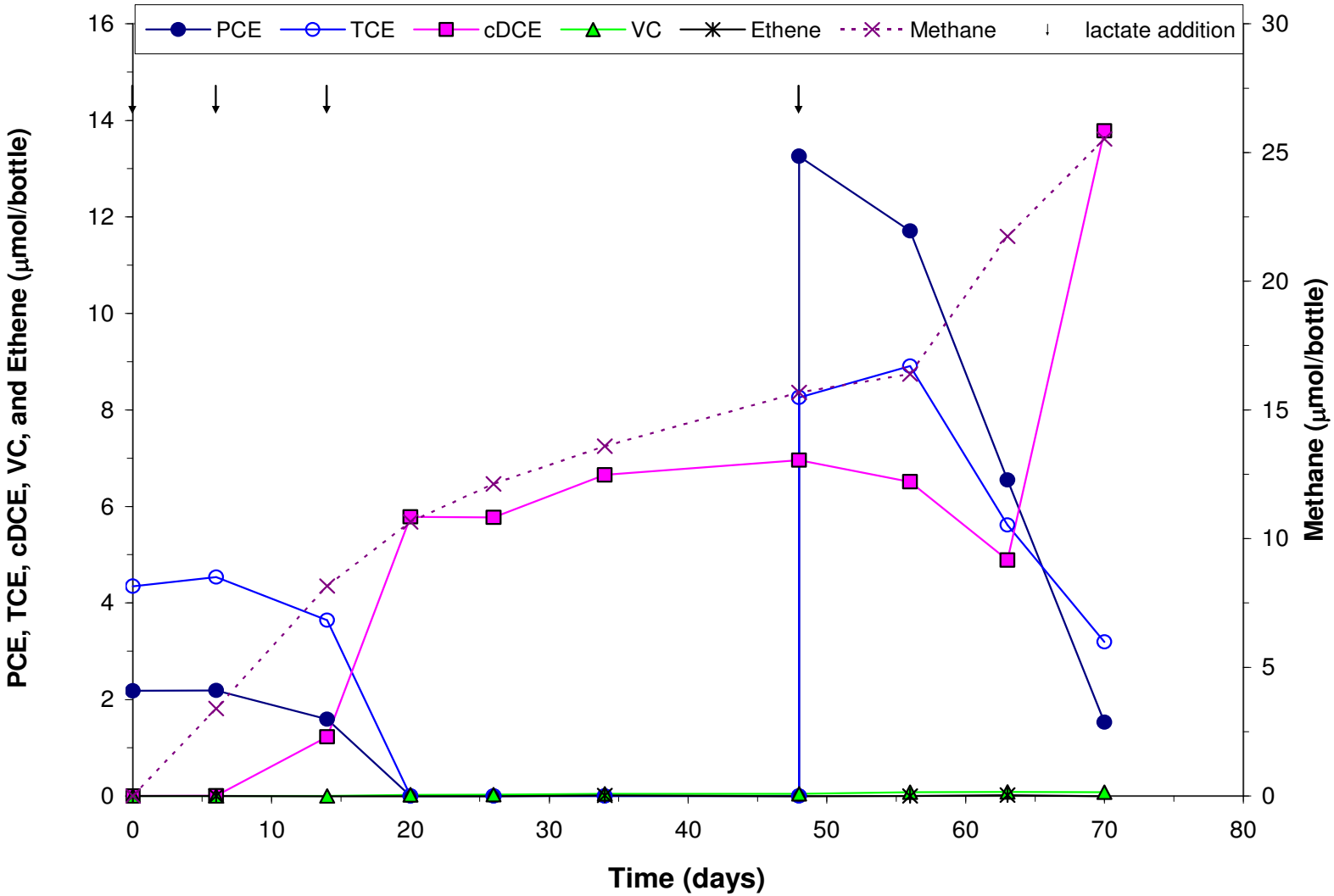

Figure 4.161 Results for one of the pH 5.5±0.25 bottles (pH 5.5±0.25-2).

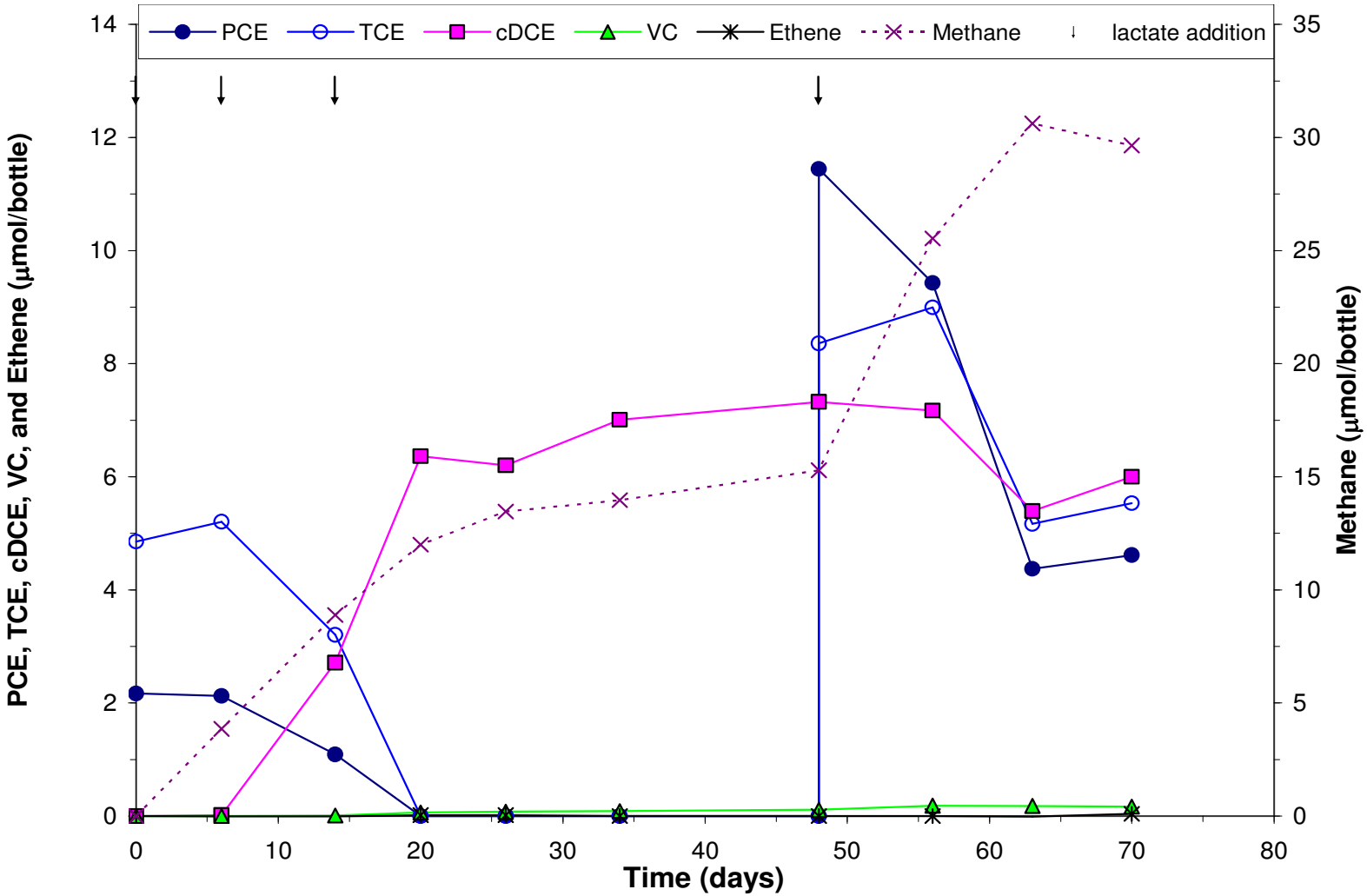

Figure 4.162 Results for one of the pH 5.5±0.25 bottles (pH 5.5±0.25-3).

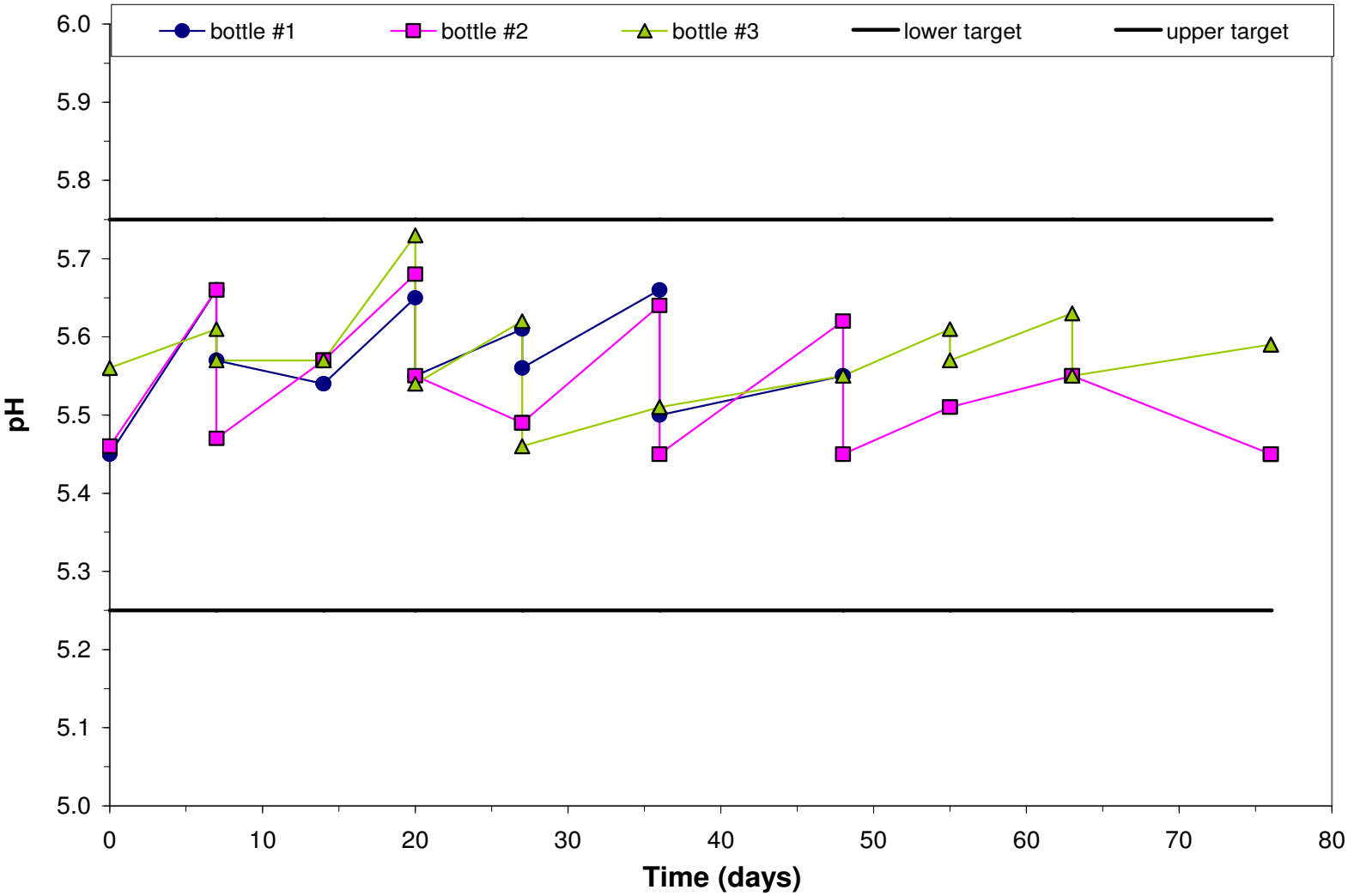

Figure 4.163 Results for pH adjustment of the pH 5.5±0.25 bottles.

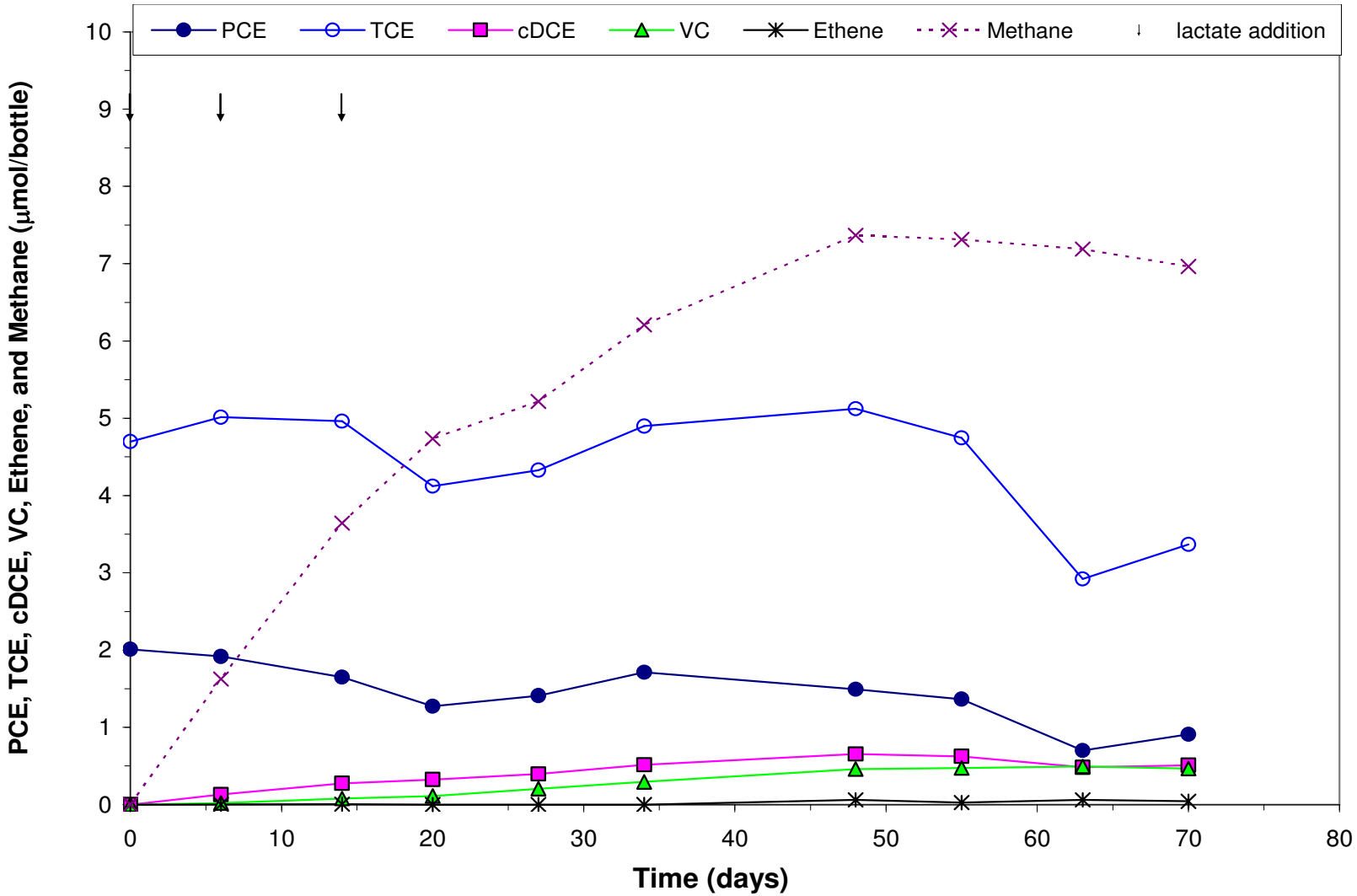

Figure 4.164 Results for one of the pH 8.5±0.25 bottles (pH 8.5±0.25-1).

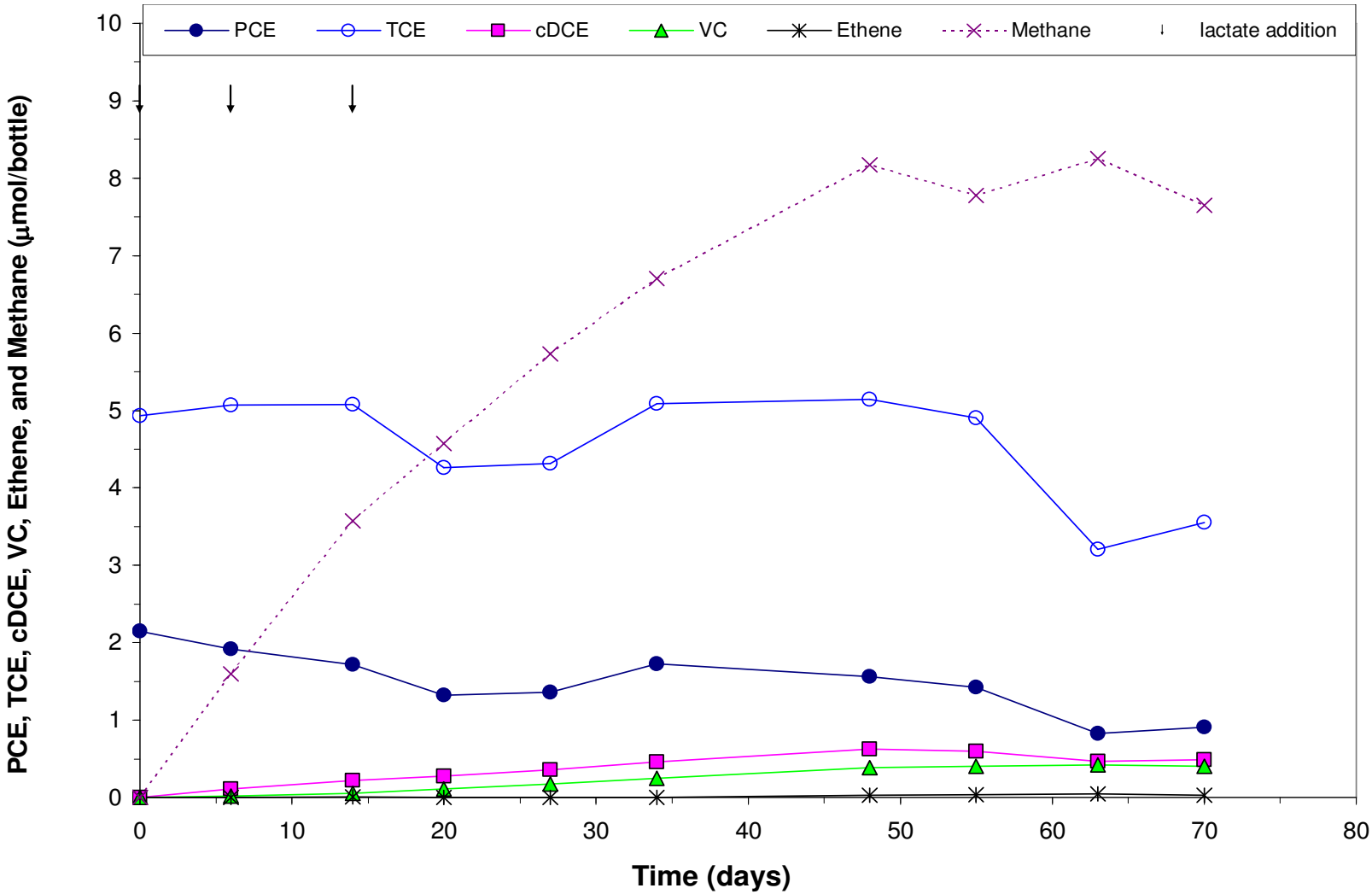

Figure 4.165 Results for one of the pH 8.5±0.25 bottles (pH 8.5±0.25-2).

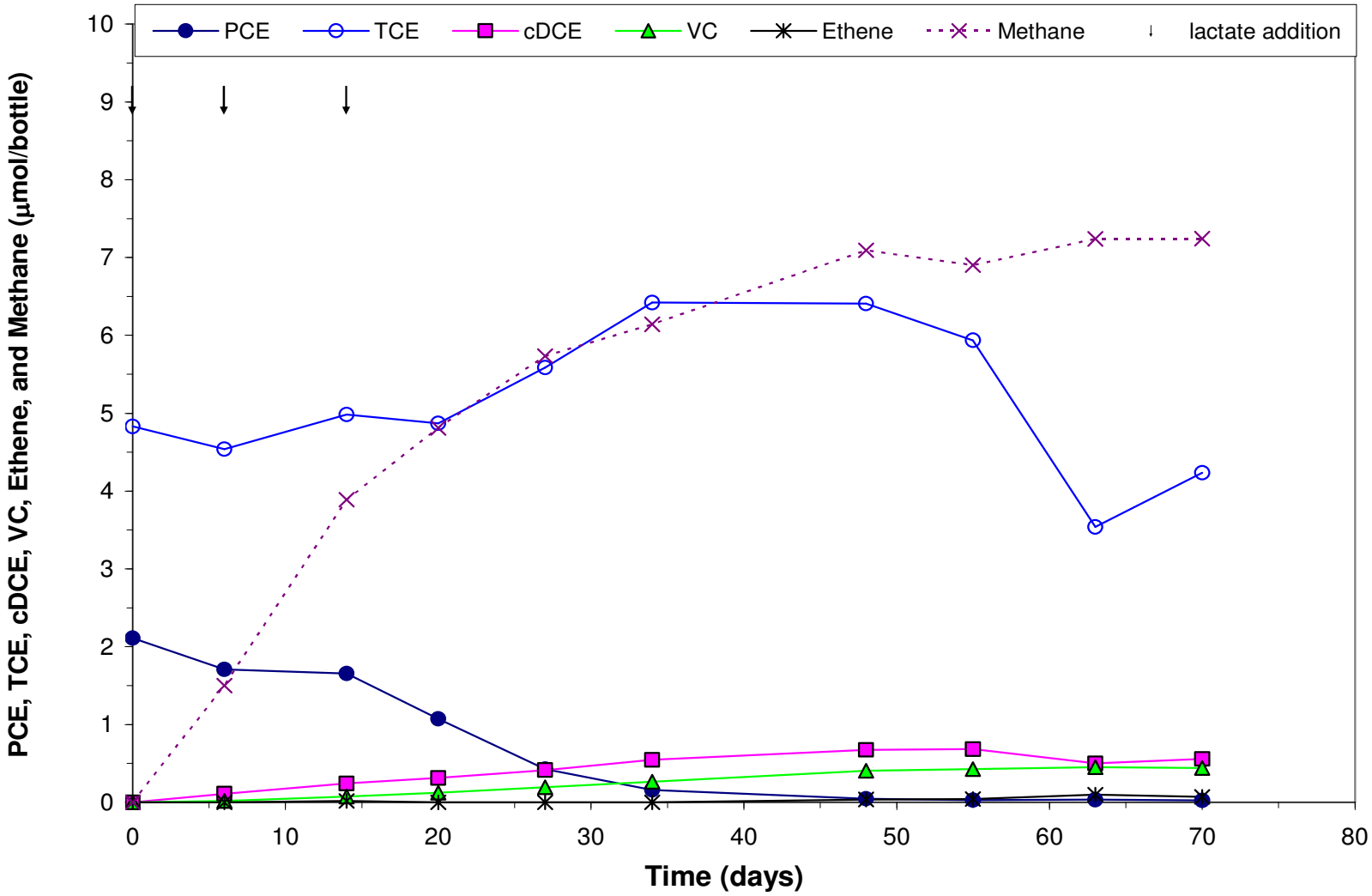

Figure 4.166 Results for one of the pH 8.5±0.25 bottles (pH 8.5±0.25-3).

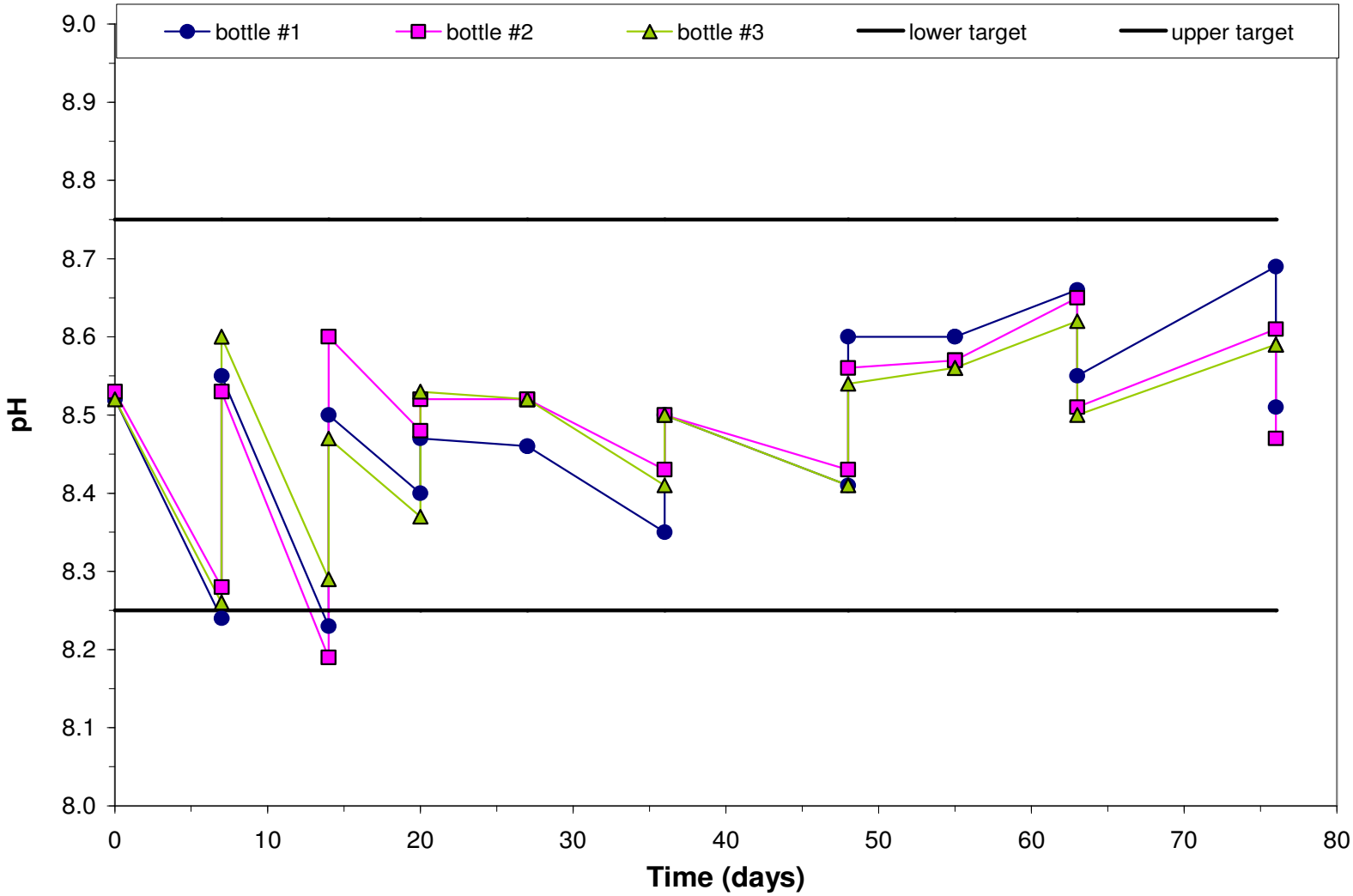

Figure 4.167 Results for pH adjustment of the pH 8.5±0.25 bottles.

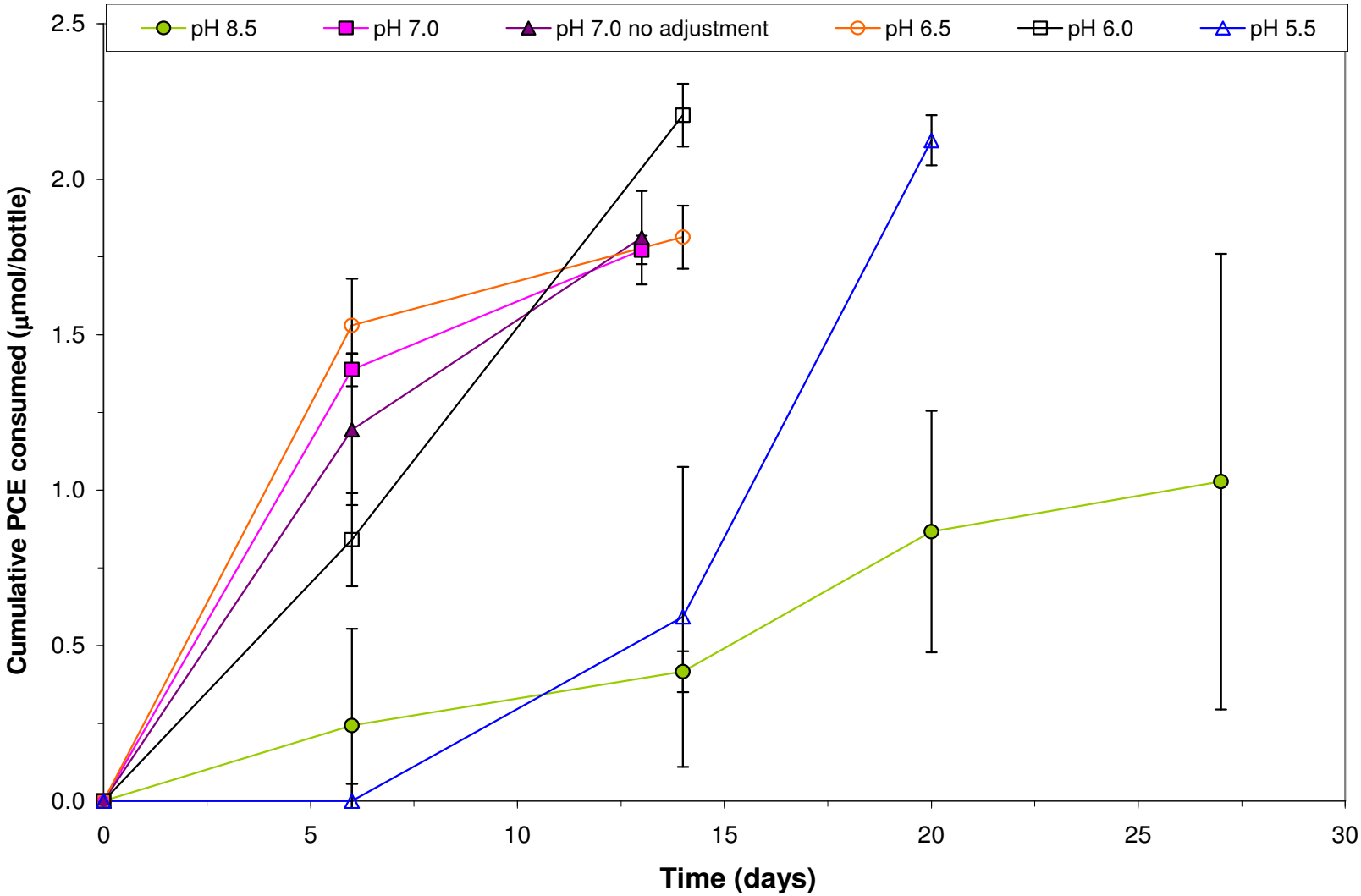

Figure 4.168 Comparison of pH effect on PCE consumption based on the first PCE and TCE addition at all pH levels (averages of triplicates). Error bars represent ± one standard deviation.

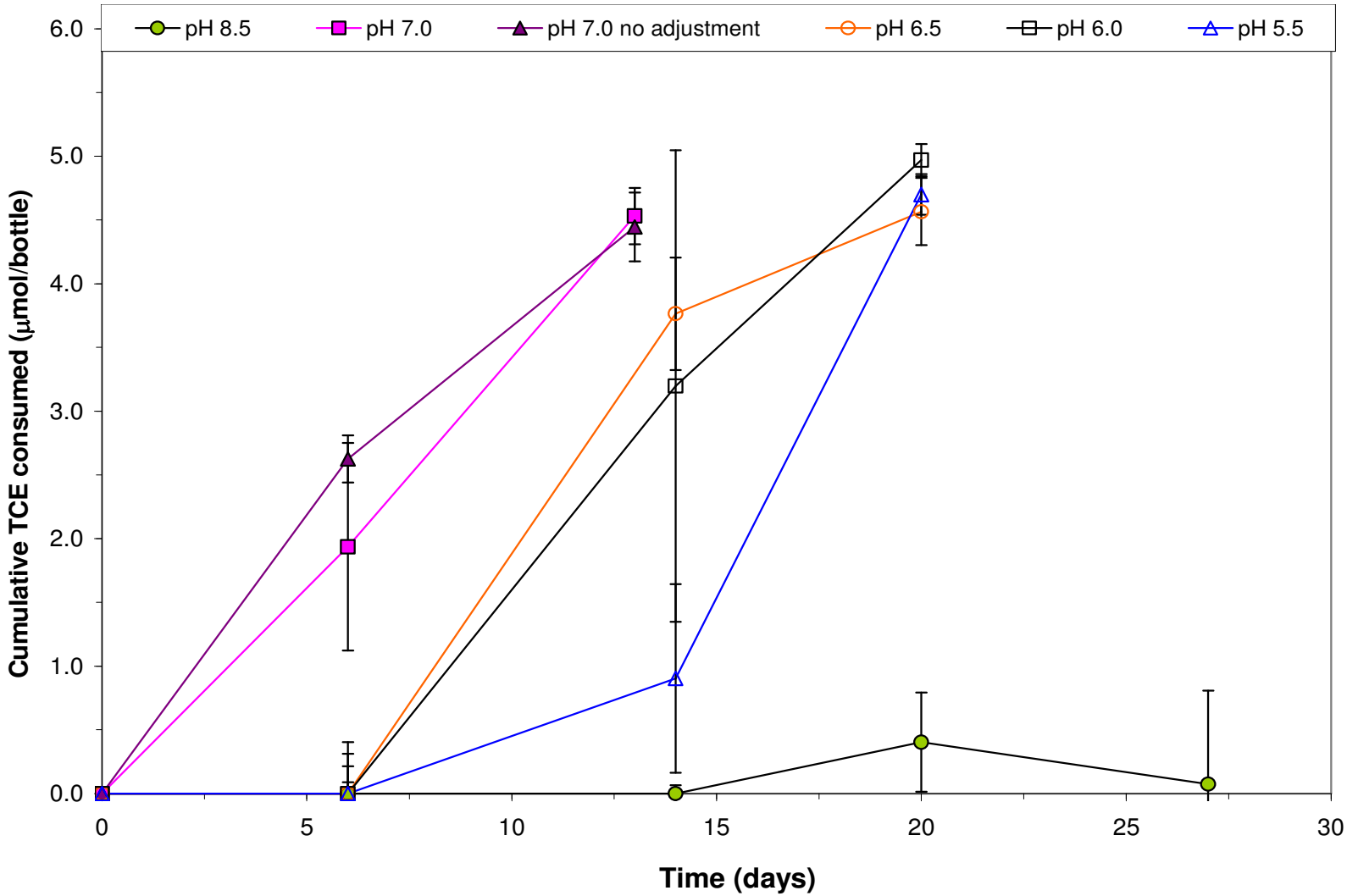

Figure 4.169 Comparison of pH effect on TCE consumption based on the first PCE and TCE addition at all pH levels (averages of triplicates). Error bars represent ± one standard deviation.

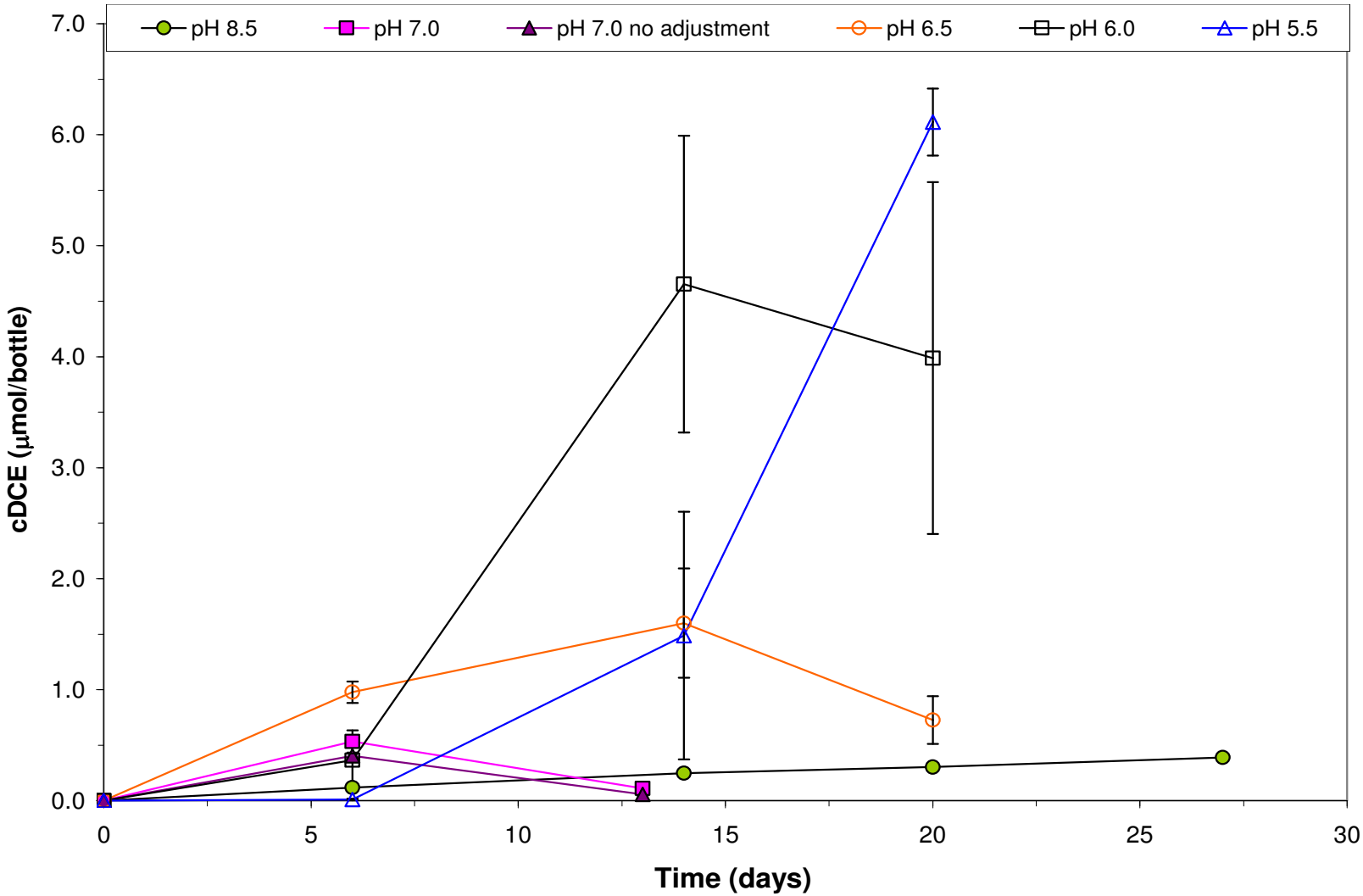

Figure 4.170 Comparison of pH effect on cDCE dechlorination based on the first PCE and TCE addition at all pH levels (averages of triplicates). Error bars represent ± one standard deviation.

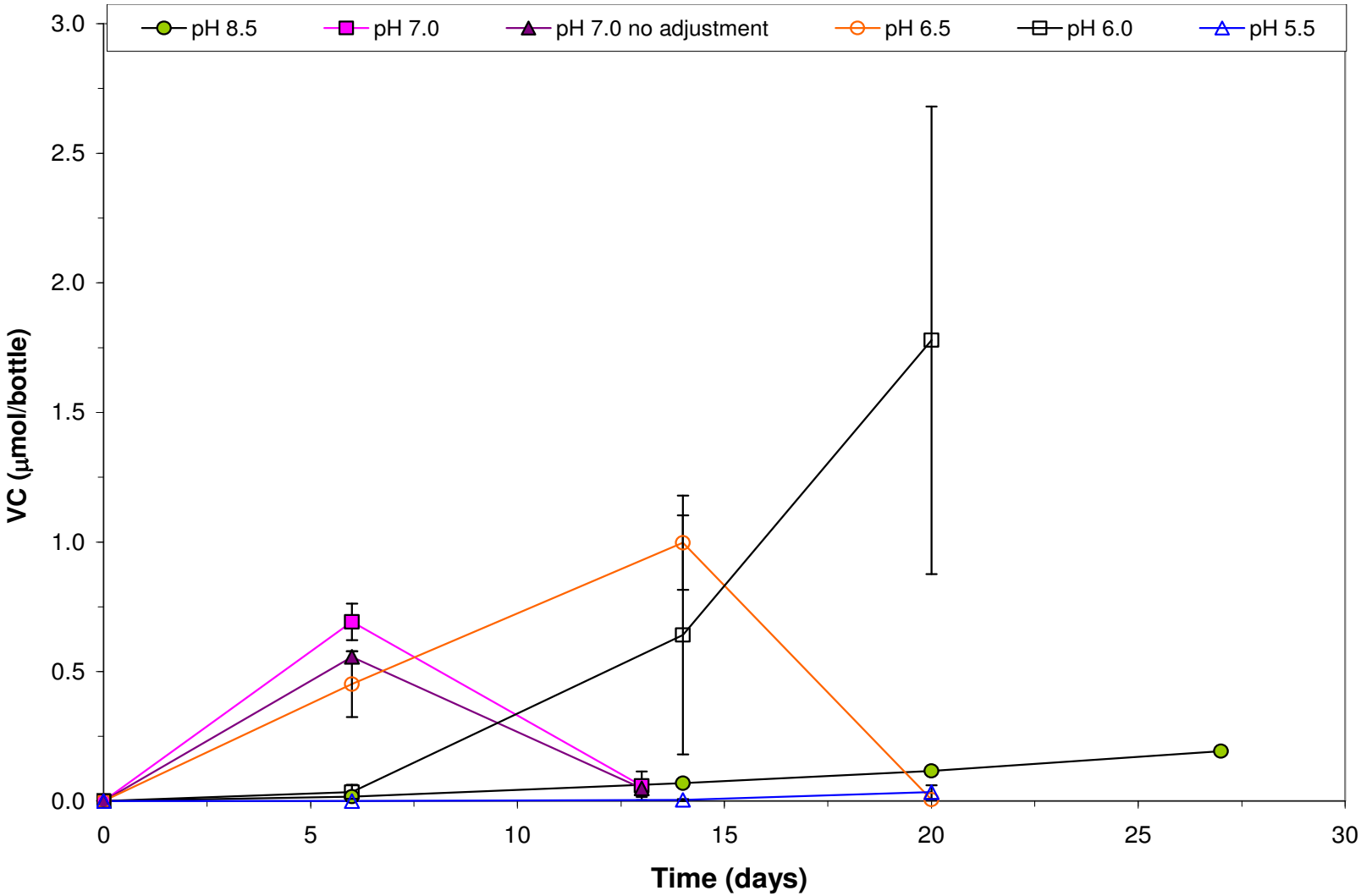

Figure 4.171 Comparison of pH effect on VC dechlorination based on the first PCE and TCE addition at all pH levels (averages of triplicates). Error bars represent ± one standard deviation.

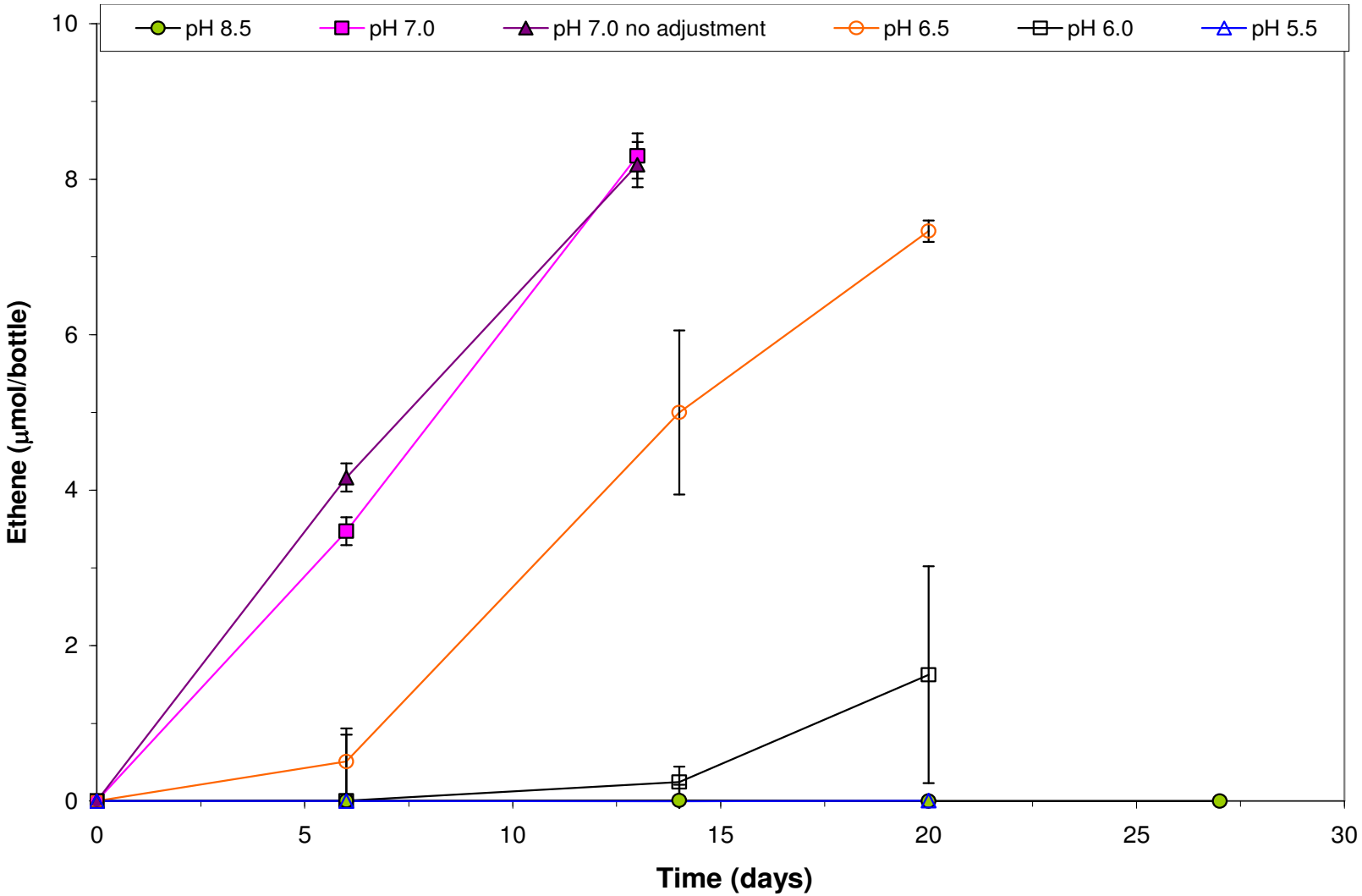

Figure 4.172 Comparison of pH effect on ethene production based on the first PCE and TCE addition at all pH levels (averages of triplicates). Error bars represent ± one standard deviation.

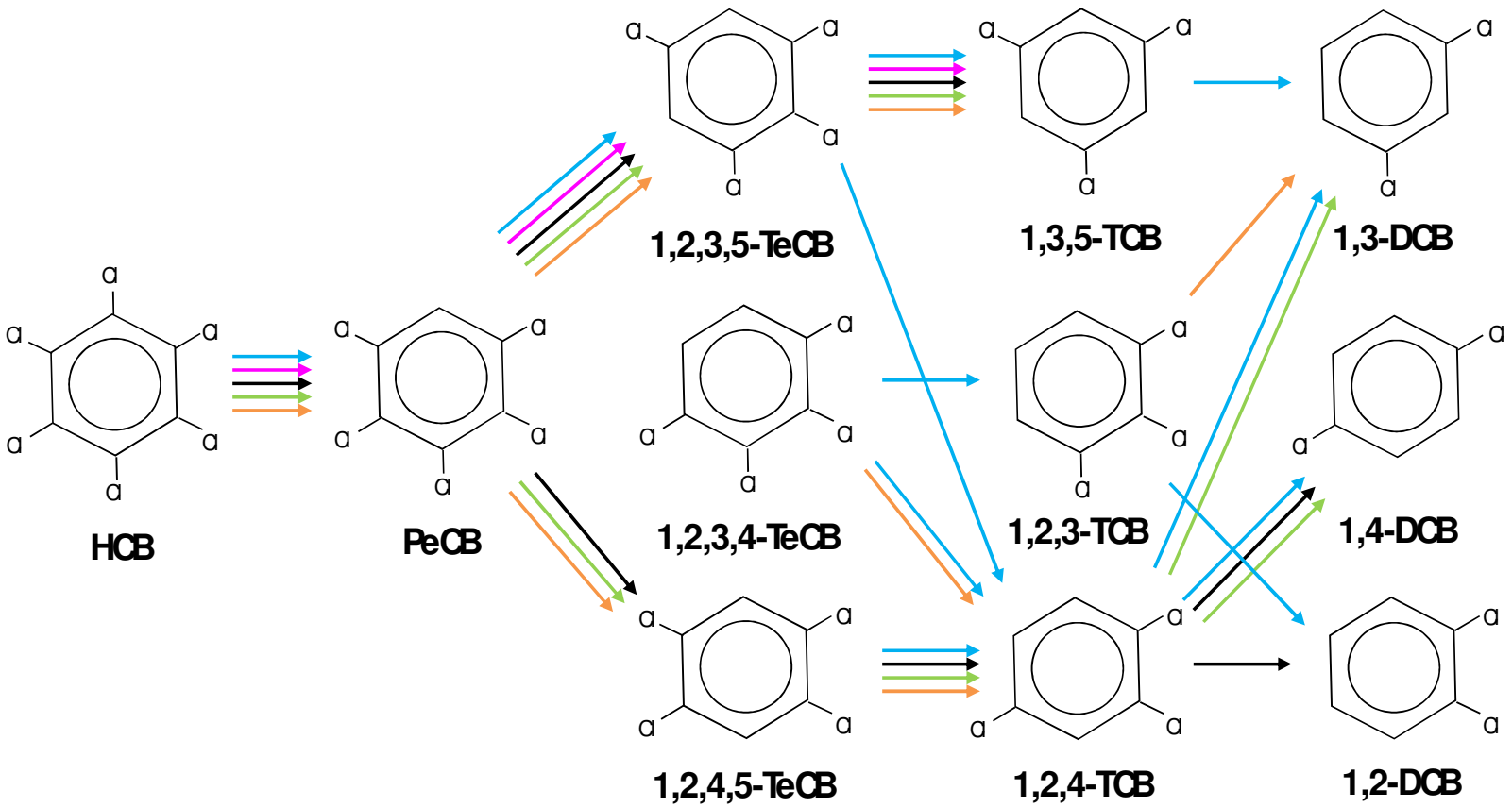

Figure 5.1 Reported pathways for reductive dechlorination of chlorinated benzenes. Pathway reported for SRS enrichment culture is indicated by black arrows. Pathways reported in literature are denoted by colored arrows: green = ref (28), blue = ref (19), pink = ref (49), and orange = ref (5).

APPENDICES

Appendix A

Maps of the PBRP (from Mark Amidon, SRNL)

Figure A.1 PCE concentration in the P-Area chlorinated ethene plume.

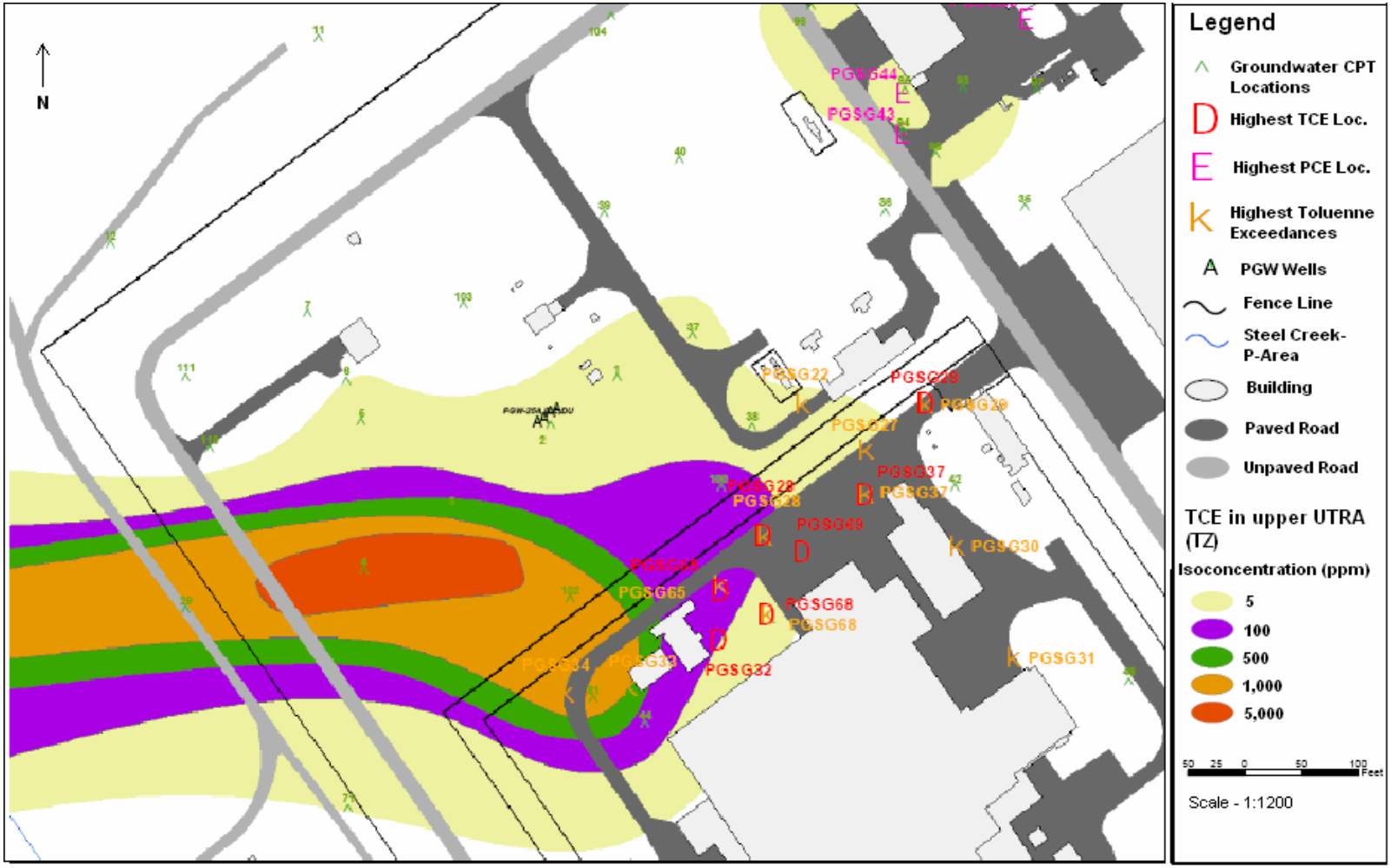

Figure A.2 TCE concentration in the P-Area chlorinated ethene plume.

Appendix B

Minimal Media Preparation

Reagents and stock solutions needed for media:

- Phosphate buffer
  In a 100 mL volumetric flask add 5.25 g K_2HPO_4. Fill to 100 mL with DDI water.

- Salt solution
  In a 100 mL volumetric flask add:
  5.35 g NH_4Cl
  0.46976 g $CaCl_2 \cdot 2H_2O$
  0.17787 g $FeCl_2 \cdot H_2O$
  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 $MnCl_2 \cdot 4H_2O$
  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 $Al_2(SO_4)_3 \cdot 16H_2O$
  1 mL concentrated HCl.
  Fill to 100 mL with DDI water.

- Magnesium sulfate solution
  In a 100 mL volumetric flask add 6.25 g $MgSO_4 \cdot 7H_2O$. Fill to 100 mL with DDI water.

- Bicarbonate solution
  In a 500 mL volumetric flask add 8.0 g $NaHCO_3$. Fill to 500 mL with DDI water.

- Redox solution
  In a 10 mL volumetric flask add 0.01 g resazurin. Fill to 10 mL with DDI water.

- Yeast extract solution
  In a 100 mL volumetric flask add 0.5 g yeast extract. Fill to 100 mL with DDI water.

- Ferrous sulfide

For 1 L of media, weigh into separate glass vials:

0.24 g of $Na_2S \cdot 9H_2O$

0.1448 g $FeCl_2 \cdot H_2O$

Media Preparation

1) 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
905 mL DDI water

2) Autoclave the above solution and allow to cool.

3) Add:

50 mL filter sterilized bicarbonate solution
10 mL filter sterilized yeast extract

4) Transfer the bottle to the glove box along with the vials of sodium sulfide and ferrous chloride and 10 mL of sterile DDI water. When the O_2 reaches zero, add the 0.24 g of $Na_2S \cdot 9H_2O$ and rinse the vial with ~5 mL of sterile DDI water. Wait until the media turns from pink to clear.

5) Then add the 0.1448g $FeCl_2 \cdot H_2O$. Rinse the vial with ~5 mL of sterile DDI water.

6) After dispensing the media, remove bottles from the glove box and purge the headspace with oxygen-free gas containing 70%N_2 and 30%CO_2. This will lower the media pH to approximately 7.

Appendix C

Canister and Maintenance Diagram

Figure C.1 Photo of canister #1 modified with valves and Mininert® sampling port.

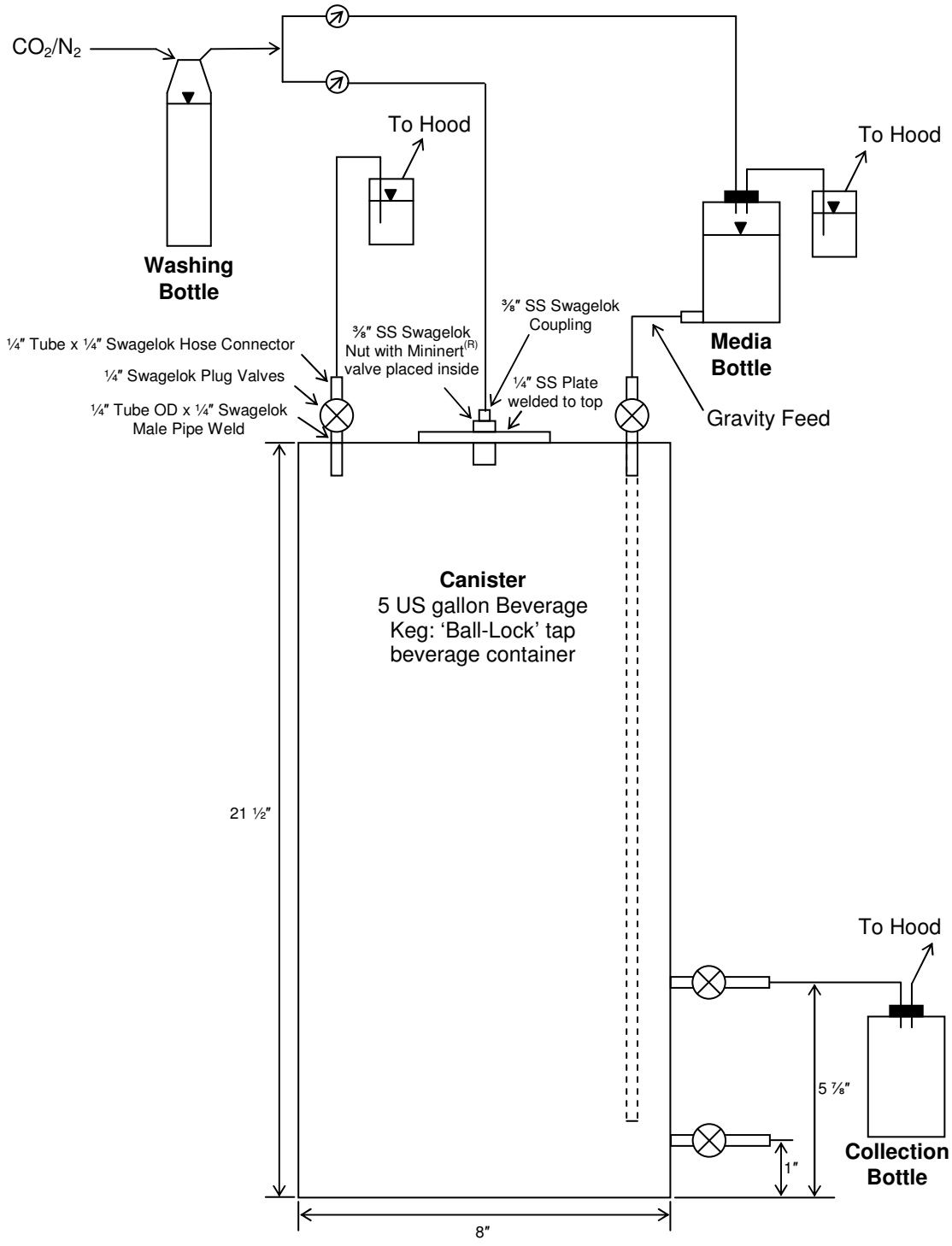

Figure C.2 Diagram of canister maintenance process for wasting liquid and adding media.

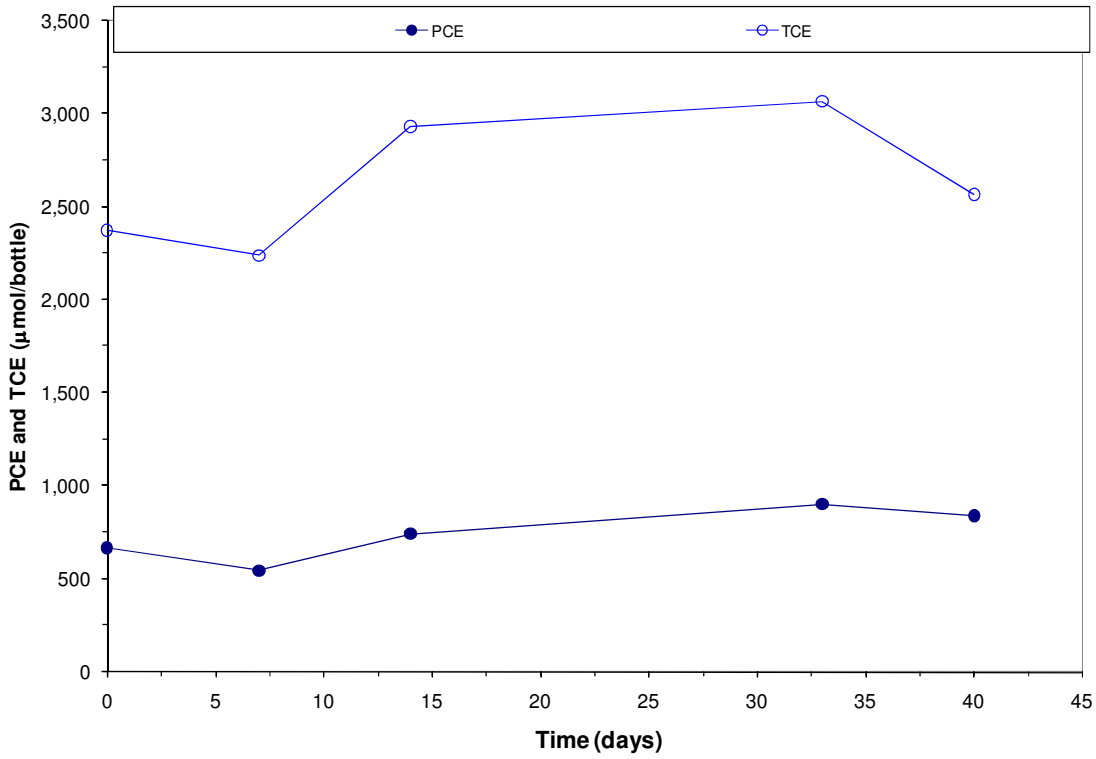

Figure C.3 Leak test for canister #1 (PCE + TCE + DDI water).

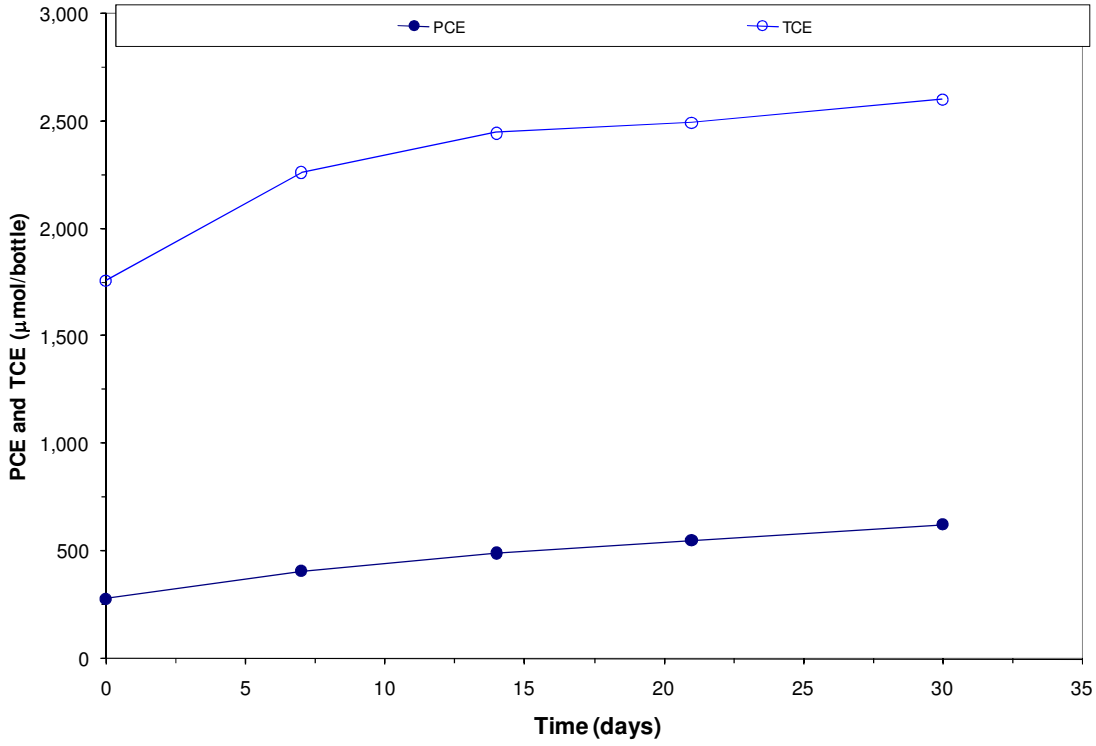

Figure C.4 Leak test for canister #2 (PCE + TCE + DDI water).

Appendix D

Results for Acetone Effect on Reductive Dechlorination of PCE and TCE

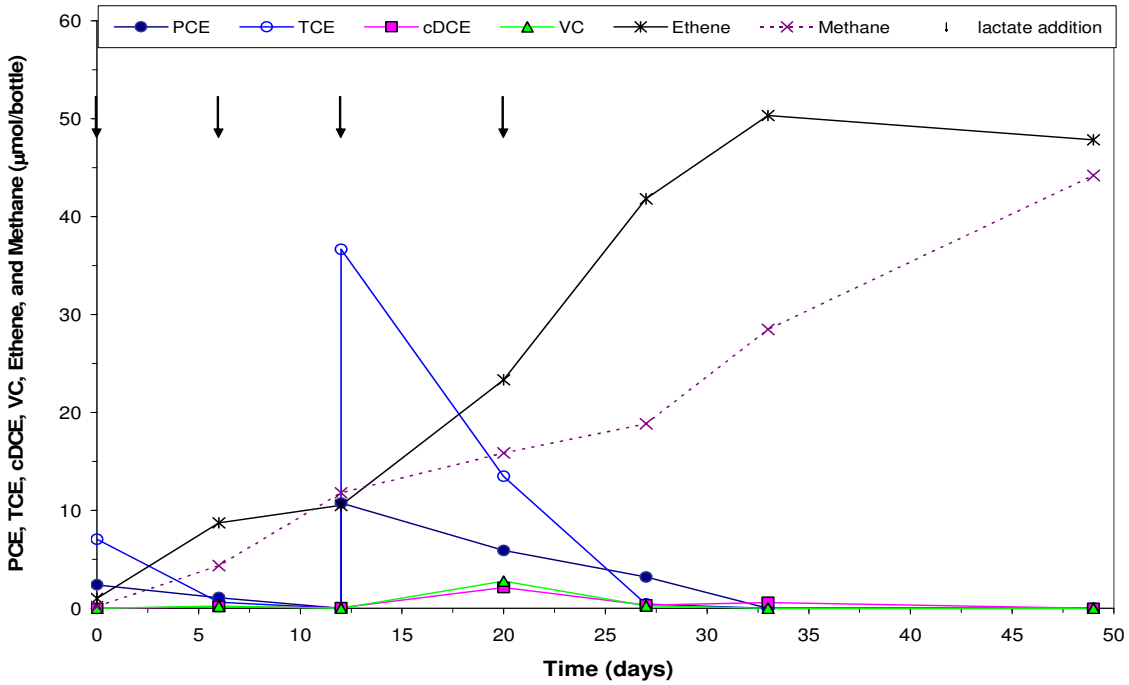

Figure D.1 Results for one of the bottles testing the effect of acetone on PCE and TCE dechlorination (PCE+TCE only-1).

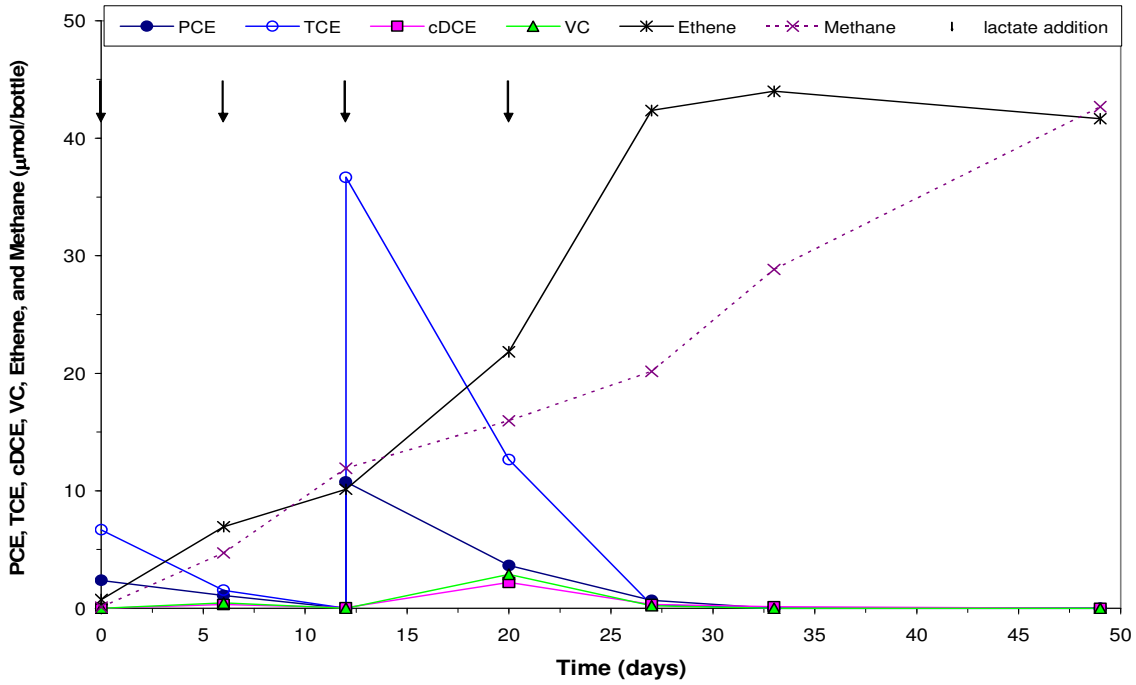

Figure D.2 Results for one of the bottles testing the effect of acetone on PCE and TCE dechlorination (PCE+TCE only-2).

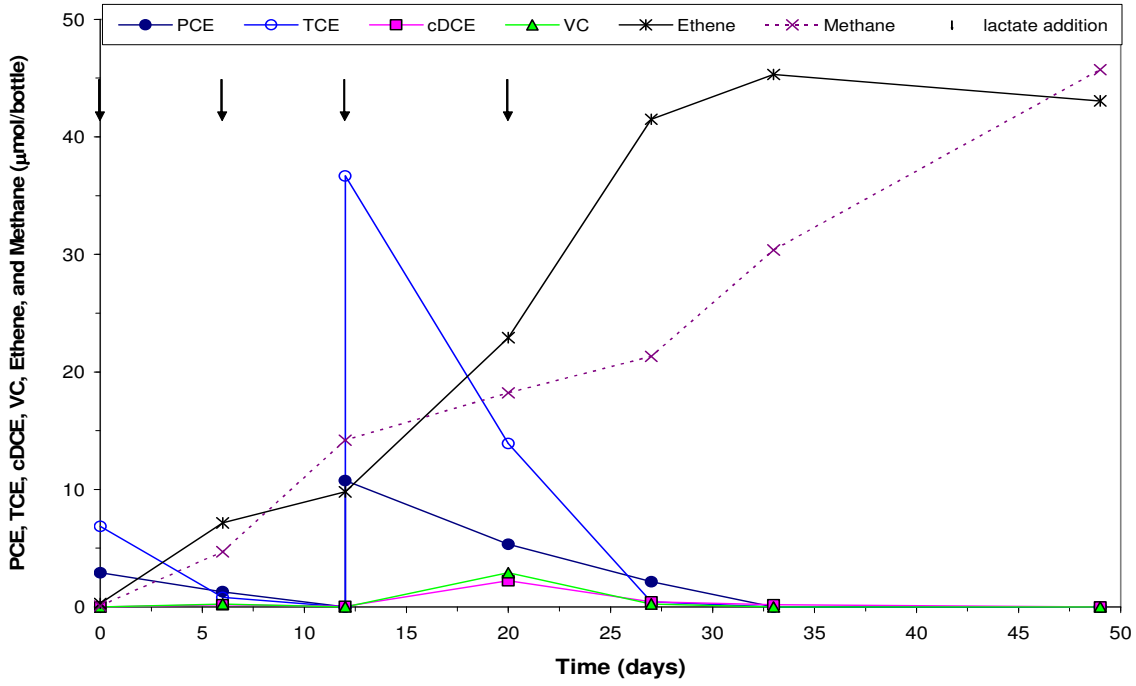

Figure D.3 Results for one of the bottles testing the effect of acetone on PCE and TCE dechlorination (PCE+TCE only-3).

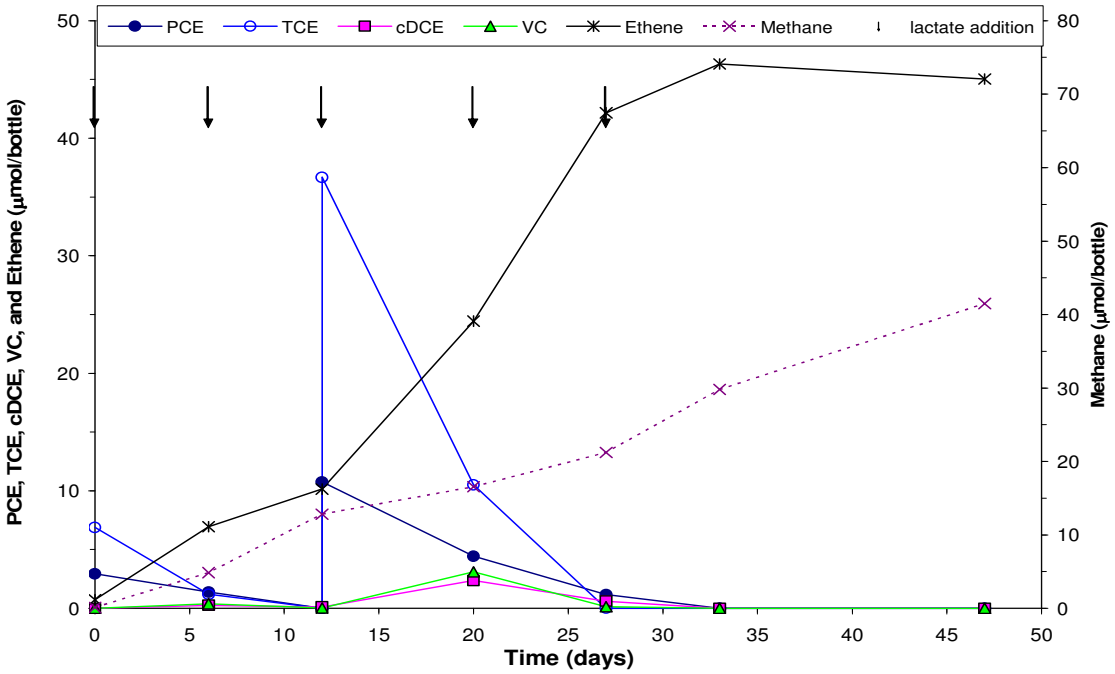

Figure D.4 Results for one of the bottles testing the effect of acetone on PCE and TCE dechlorination (PCE+TCE+acetone-1).

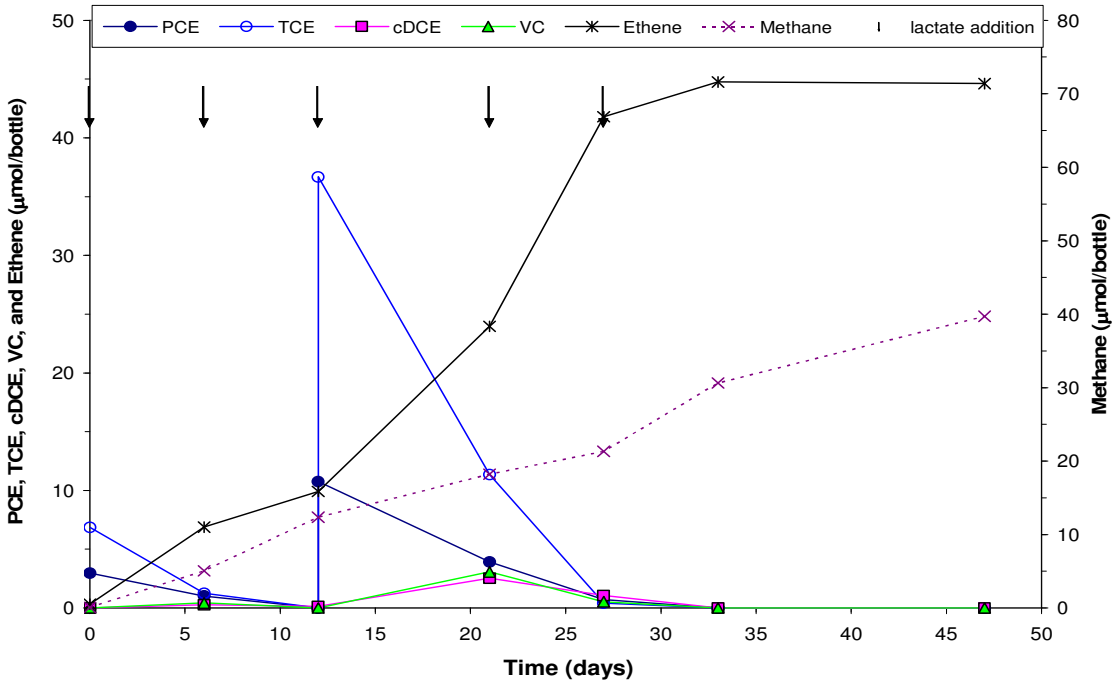

Figure D.5 Results for one of the bottles testing the effect of acetone on PCE and TCE dechlorination (PCE+TCE+acetone-2).

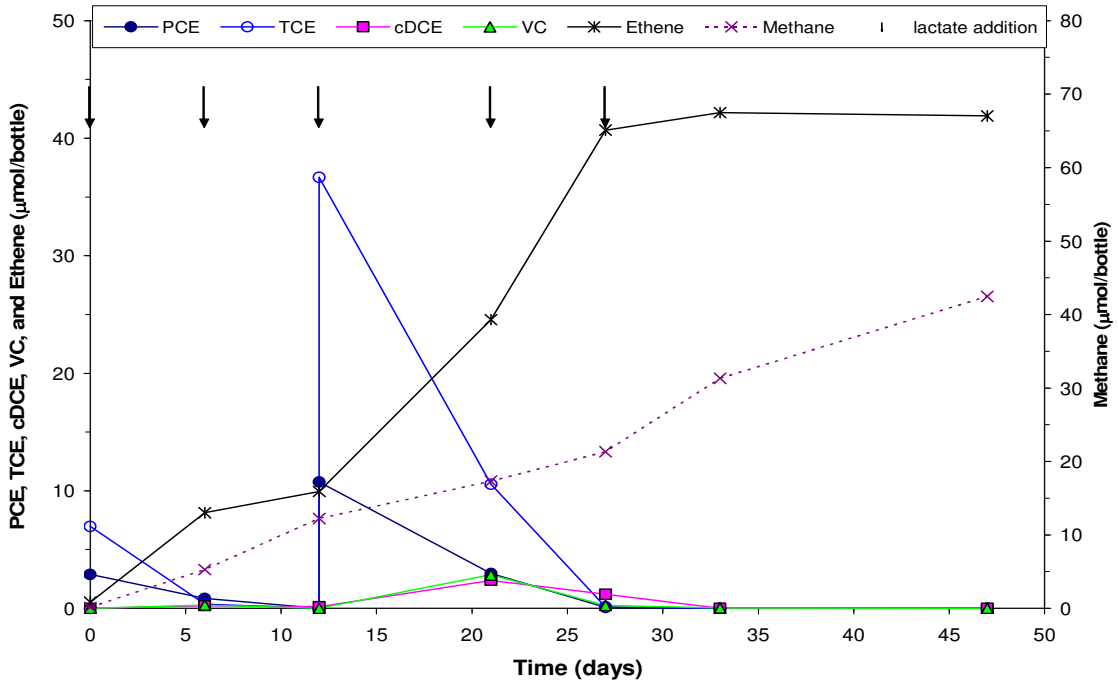

Figure D.6 Results for one of the bottles testing the effect of acetone on PCE and TCE dechlorination (PCE+TCE+acetone-3).

Appendix E

Results for Phase 1 of Oxygen Tolerance Experiment

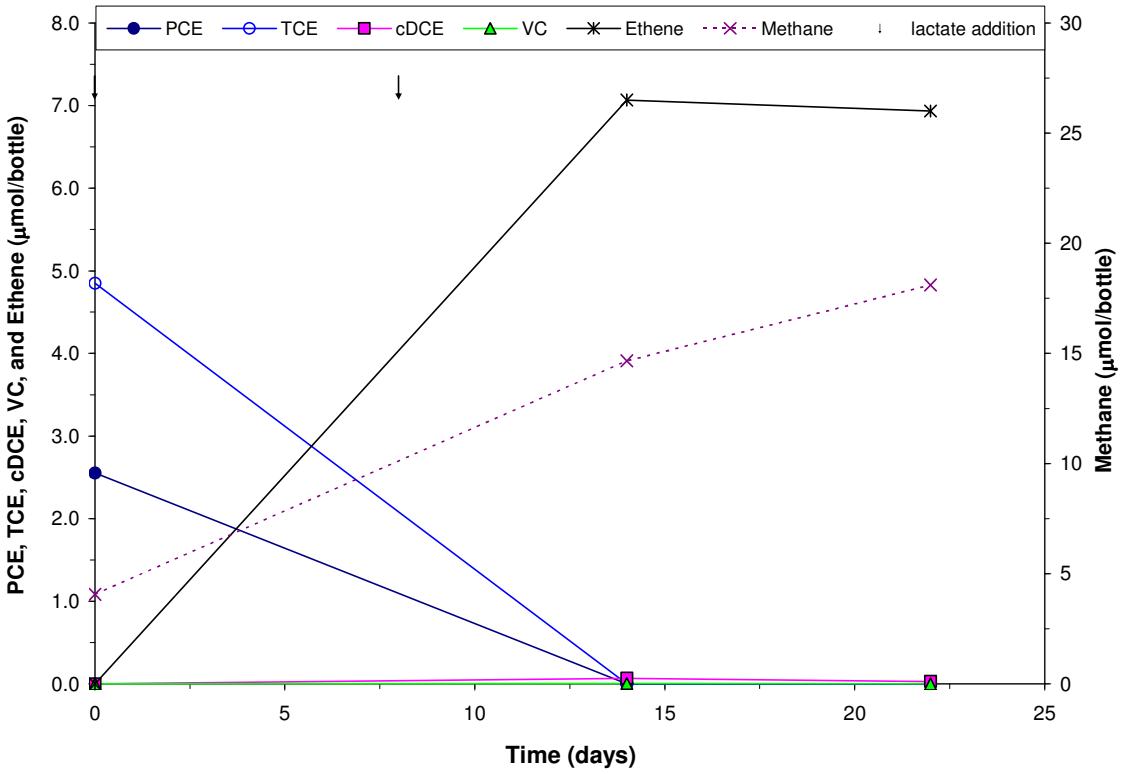

Figure E.1 Results for the single bottle not exposed to air in Phase 1 oxygen tolerance experiment.

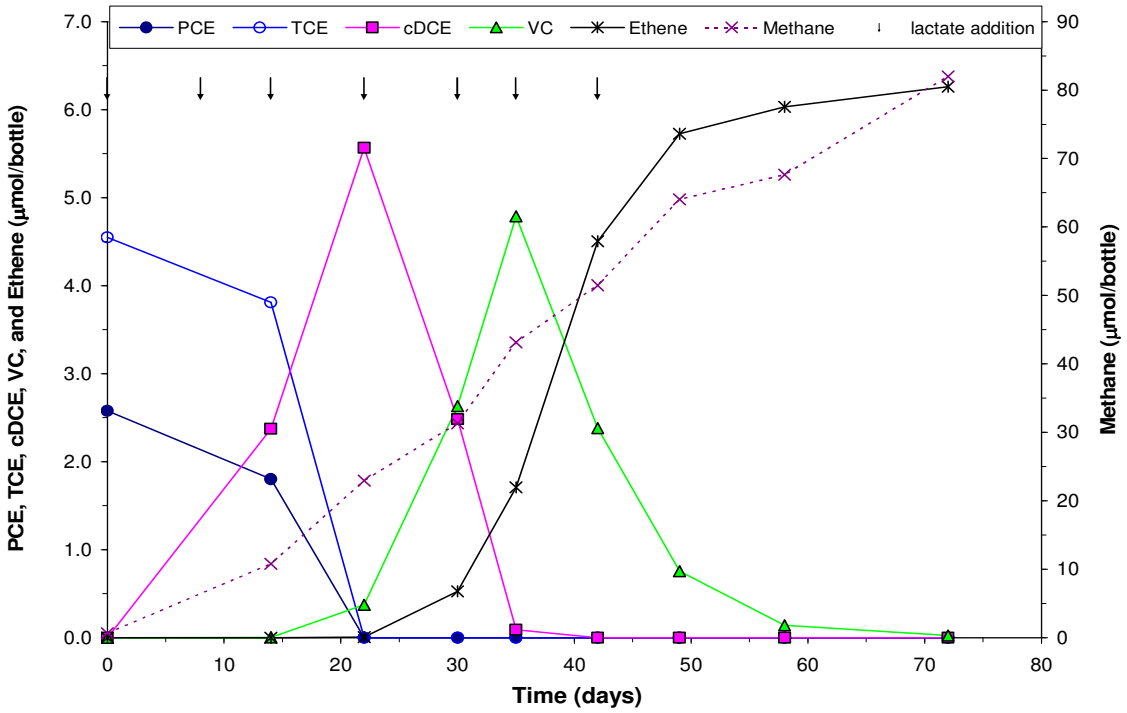

Figure E.2 Results for the single bottle exposed to air for 12 hours in Phase 1 of oxygen tolerance experiment.

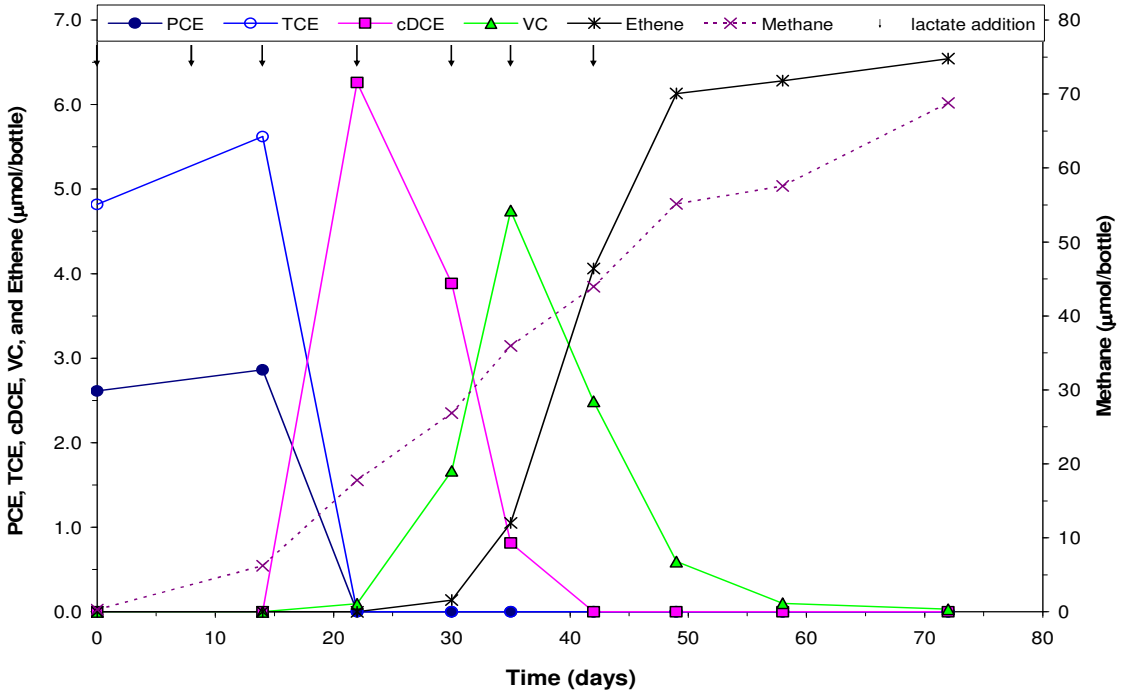

Figure E.3 Results for the single bottle exposed to air for 24 hours in Phase 1 of oxygen tolerance experiment.

Appendix F

GC RFs for Halogenated Alkenes and Alkanes in Serum Bottles

Table F.1 GC response factors for chlorinated ethenes, halogenated ethanes, ethene, ethane, and methane.

Compound	GC RT	Response Factor		Conversion Factor[a]		Min. Detection Limit
	(min)	(µmol/bottle/PAU)	R^2	(µmol/bottle)	(µmol/bottle to mg/L)	(µmol/bottle)
Methane	0.49	1.8837E-06	0.99810	0.0140	0.0002	3.97E-03
Ethene	0.70	1.0873E-06	0.99999	1.8713	0.0524	5.60E-04
Ethane	0.90	9.9400E-07	0.99998	0.8984	0.02700	4.17E-04
VC	2.60	2.3983E-06	0.99991	6.2345	0.3897	2.54E-04
1,1-DCE	6.00	2.4717E-06	0.99973	6.3184	0.6129	2.99E-04
tDCE	6.84	6.6197E-06	0.99977	8.2832	0.8035	9.93E-04
cDCE	6.84	1.1510E-05	0.99922	9.2198	0.8943	5.56E-03
TCE	10.02	5.6814E-06	1.00000	8.2692	1.0866	8.64E-04
PCE	14.64	3.8933E-06	0.99958	7.2111	1.1958	6.42E-04
CA	3.90	4.4833E-06	0.99917	7.9490	0.5128	5.07E-04
VB	4.90	4.0797E-06	0.99985	7.6824	0.8216	1.03E-02
Bromoethane	5.60	6.9476E-06	0.99983	8.4621	0.9221	1.61E-03
1,1-DCA	6.60	1.0539E-05	0.99979	8.9045	0.8812	3.13E-01
1,2-DCA	8.10	4.0967E-05	0.99986	9.6856	0.9585	2.37E-02
1,1,1-TCA	8.30	4.6400E-06	0.99975	7.2557	0.9680	4.02E-02
EDB	12.10	9.6590E-05	0.99944	9.8515	1.8422	1.97E-02

[a] Based on liquid volume of 100 mL, gas volume of 60 mL, and 23°C.

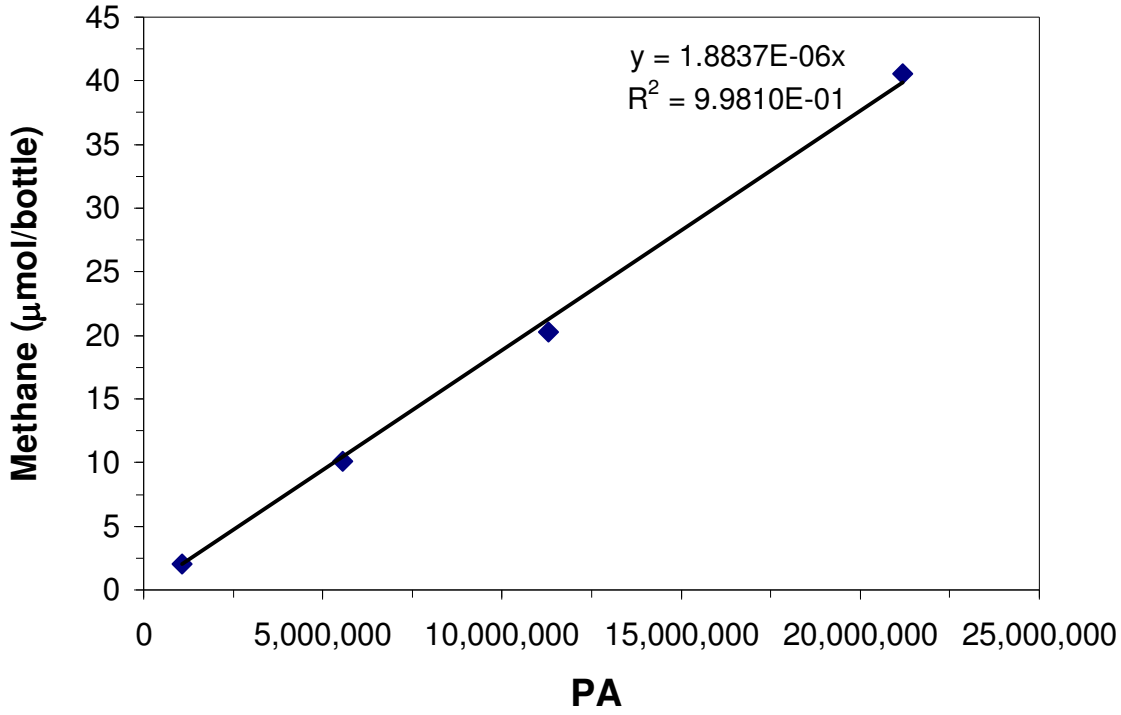

Figure F.1 GC response curve for methane.

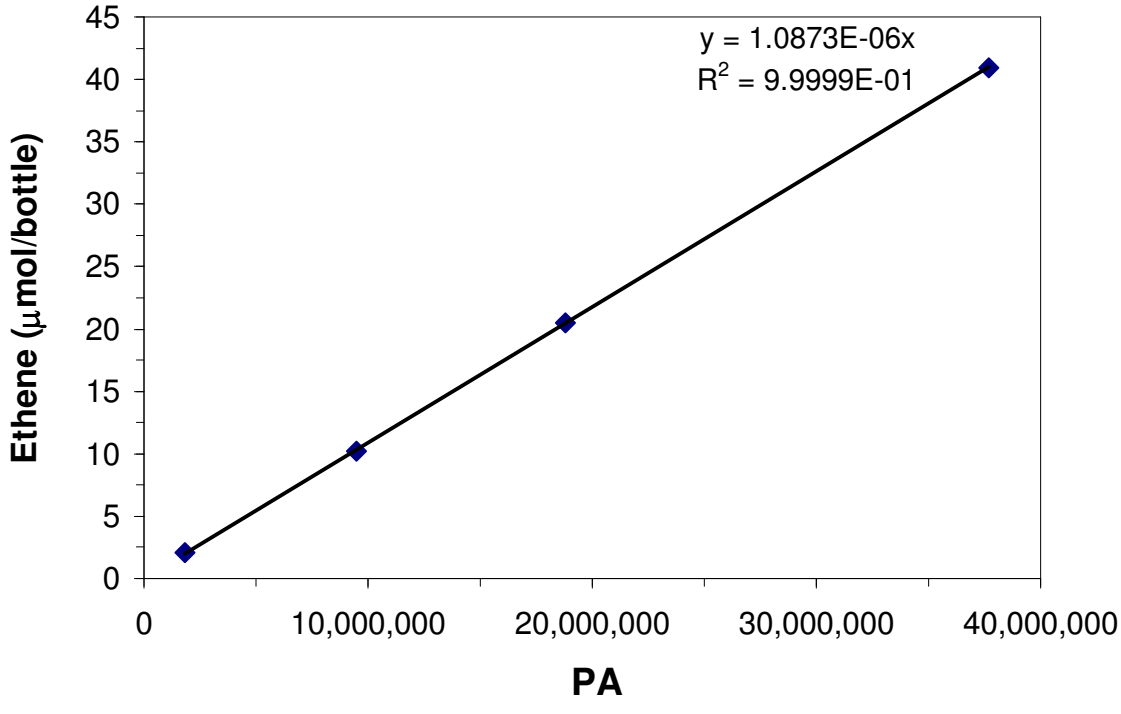

Figure F.2 GC response curve for ethene.

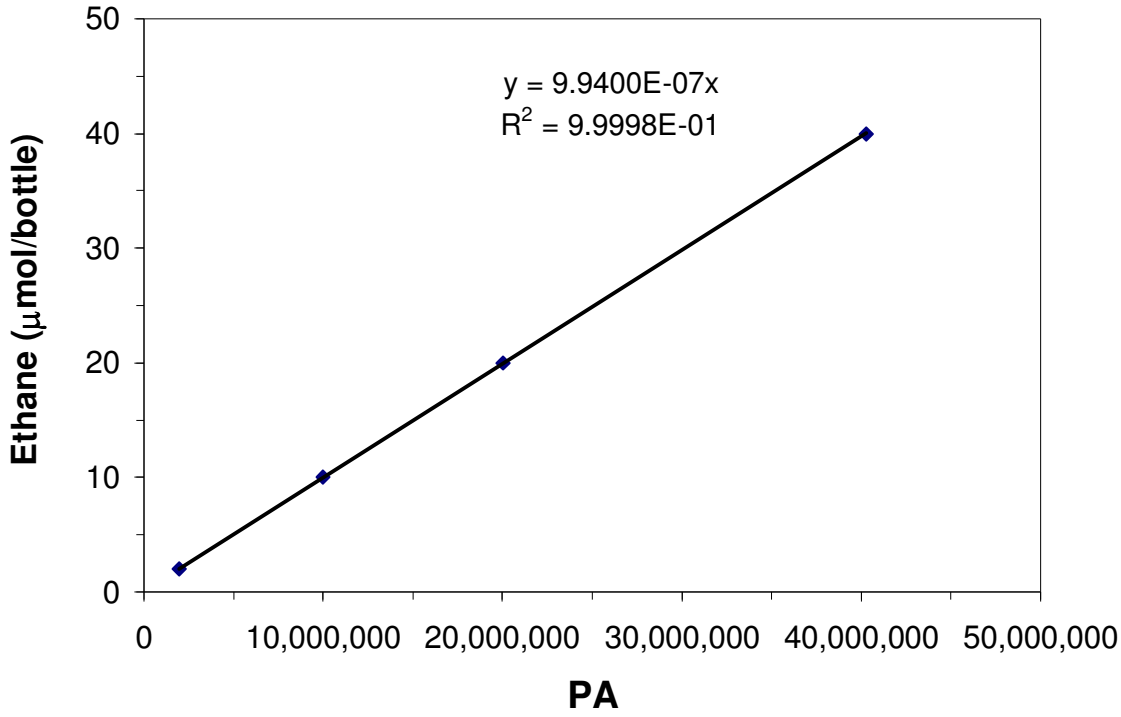

Figure F.3 GC response curve for ethane.

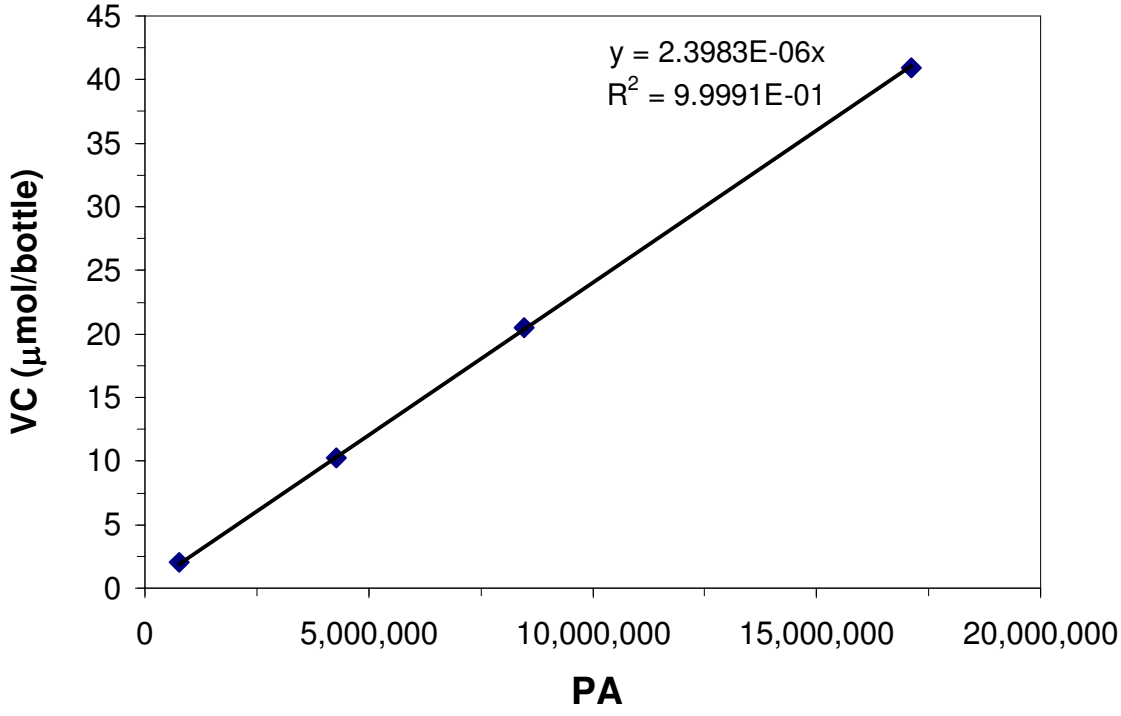

Figure F.4 GC response curve for VC.

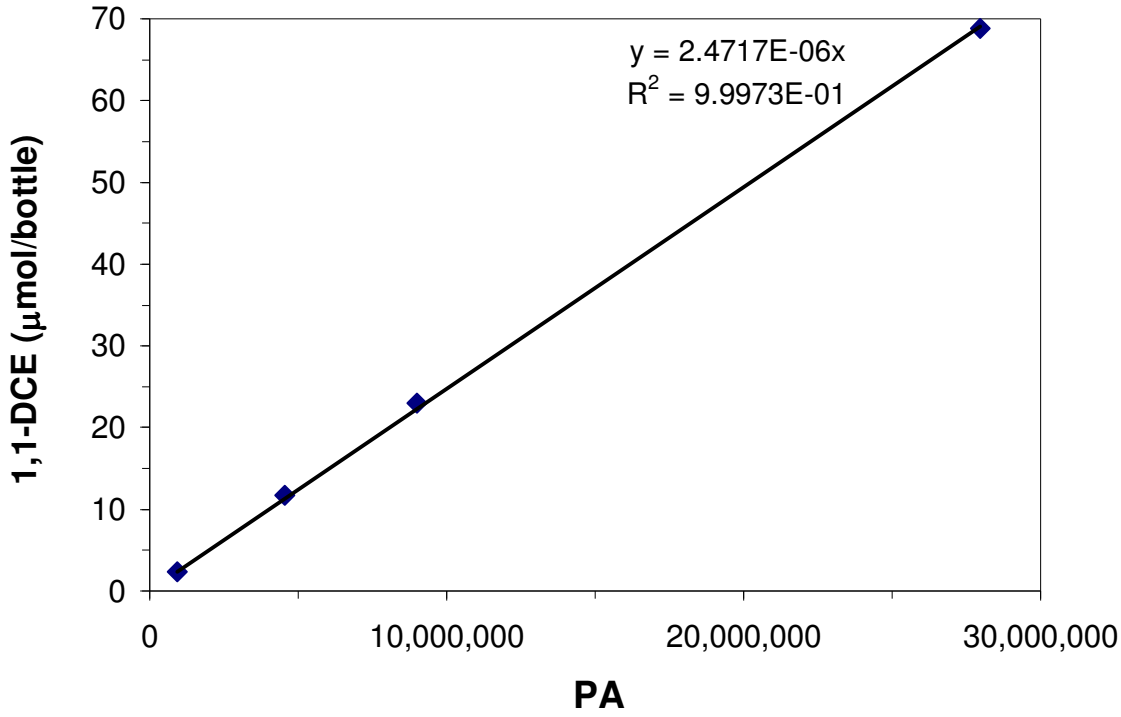

Figure F.5 GC response curve for 1,1-DCE.

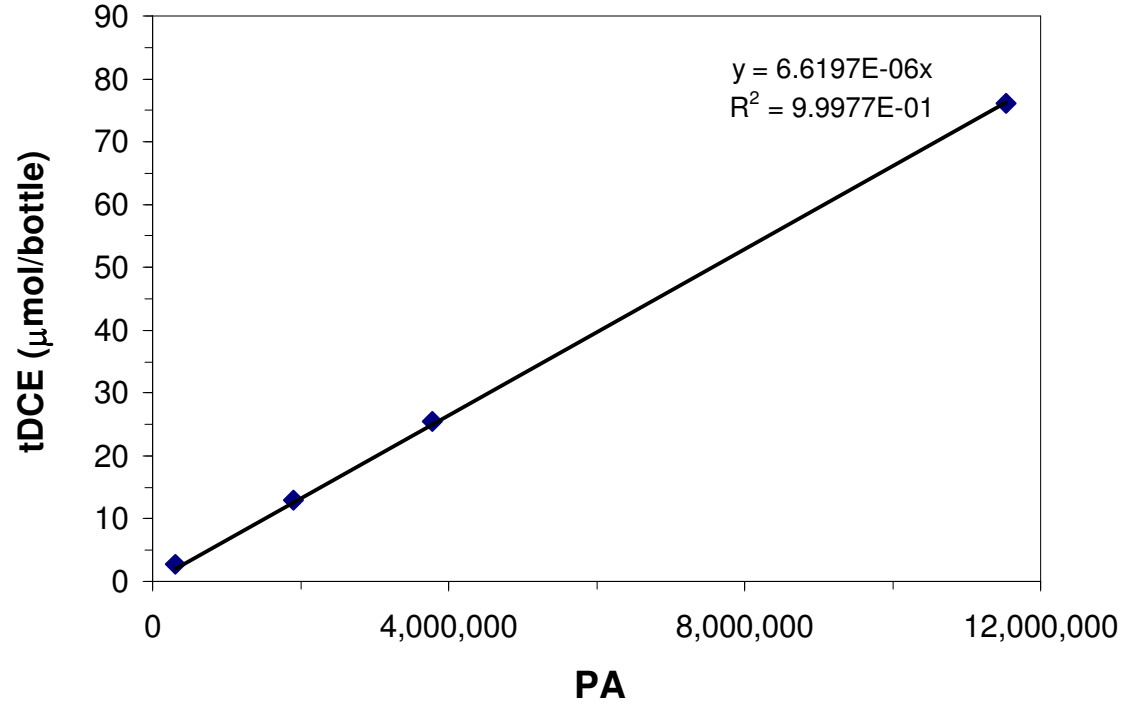

Figure F.6 GC response curve for tDCE.

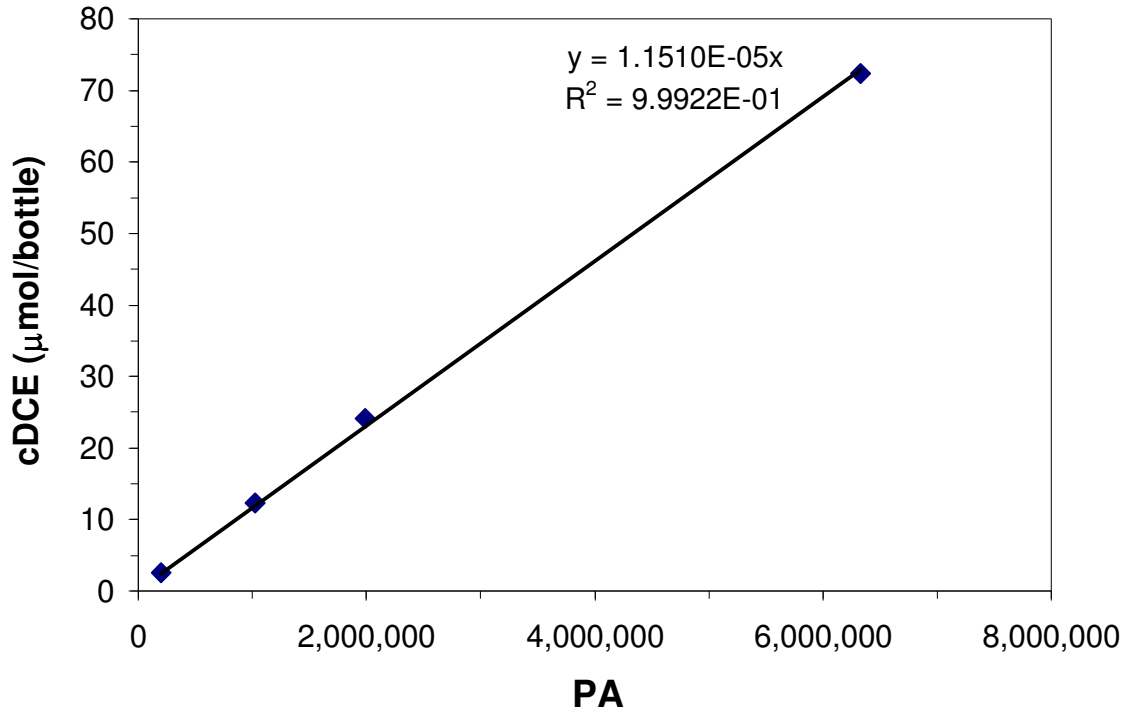

Figure F.7 GC response curve for cDCE.

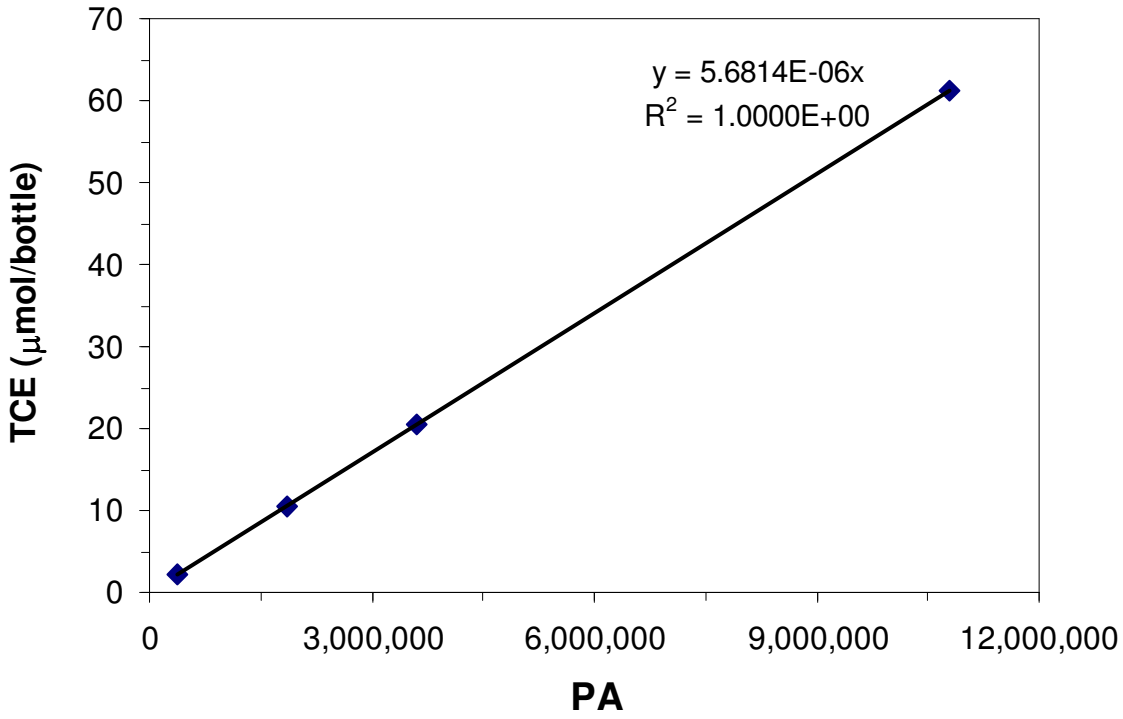

Figure F.8 GC response curve for TCE.

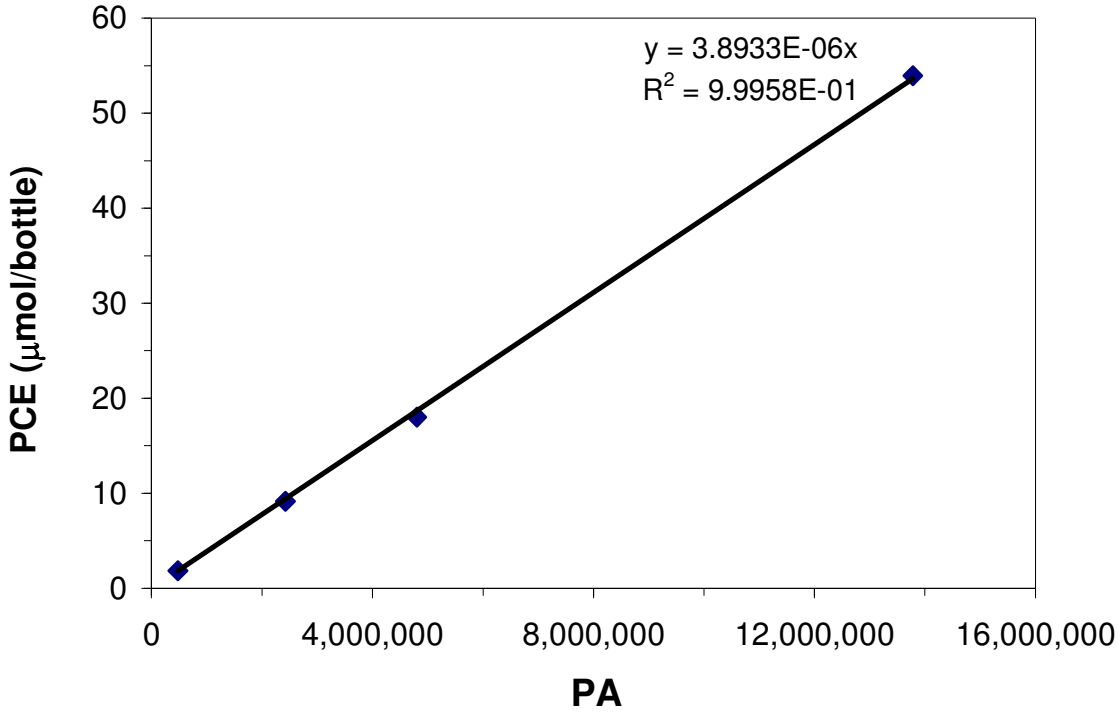

Figure F.9 GC response curve for PCE.

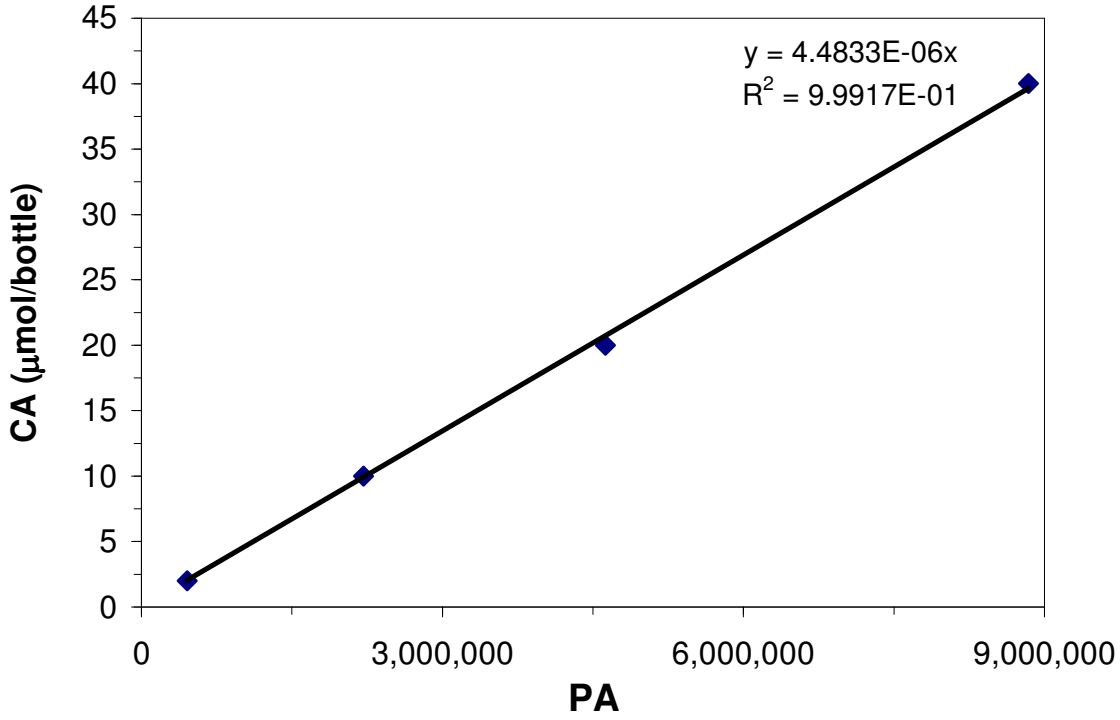

Figure F.10 GC response curve for CA.

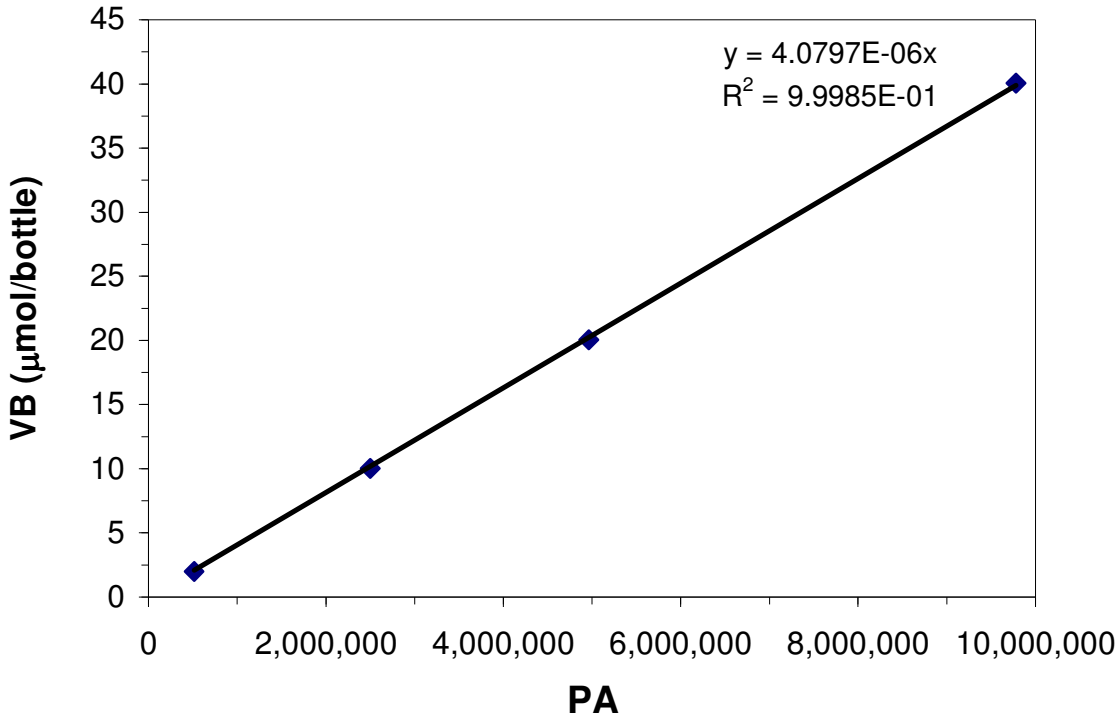

Figure F 11 GC response curve for VB.

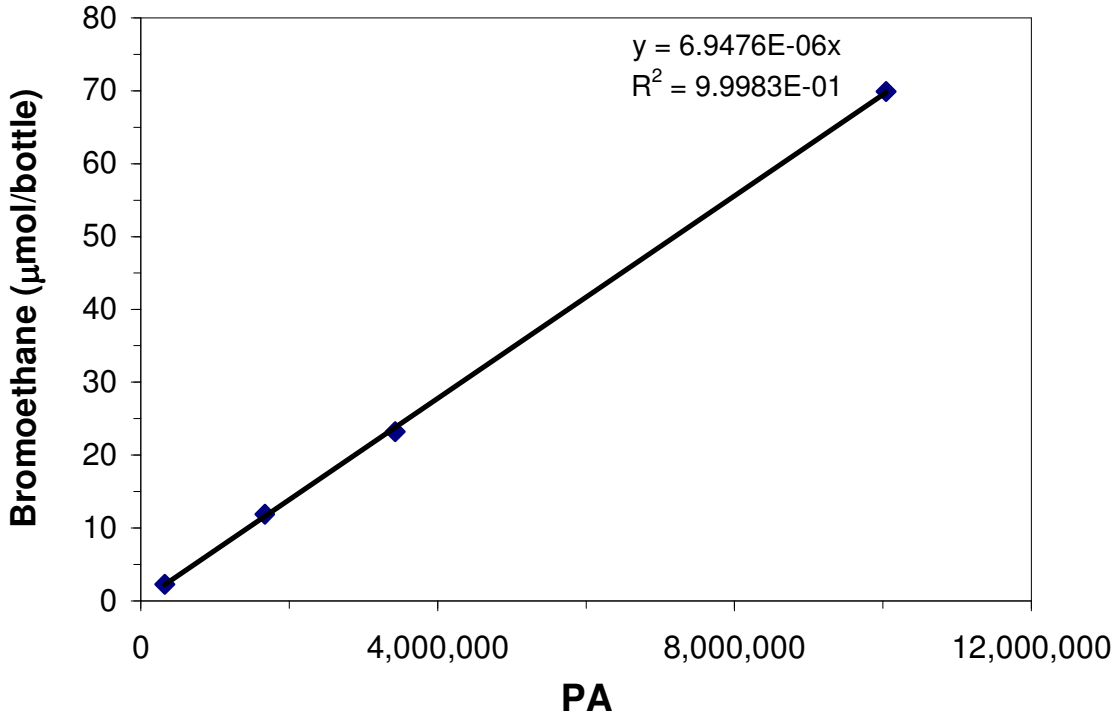

Figure F 12 GC response curve for bromoethane.

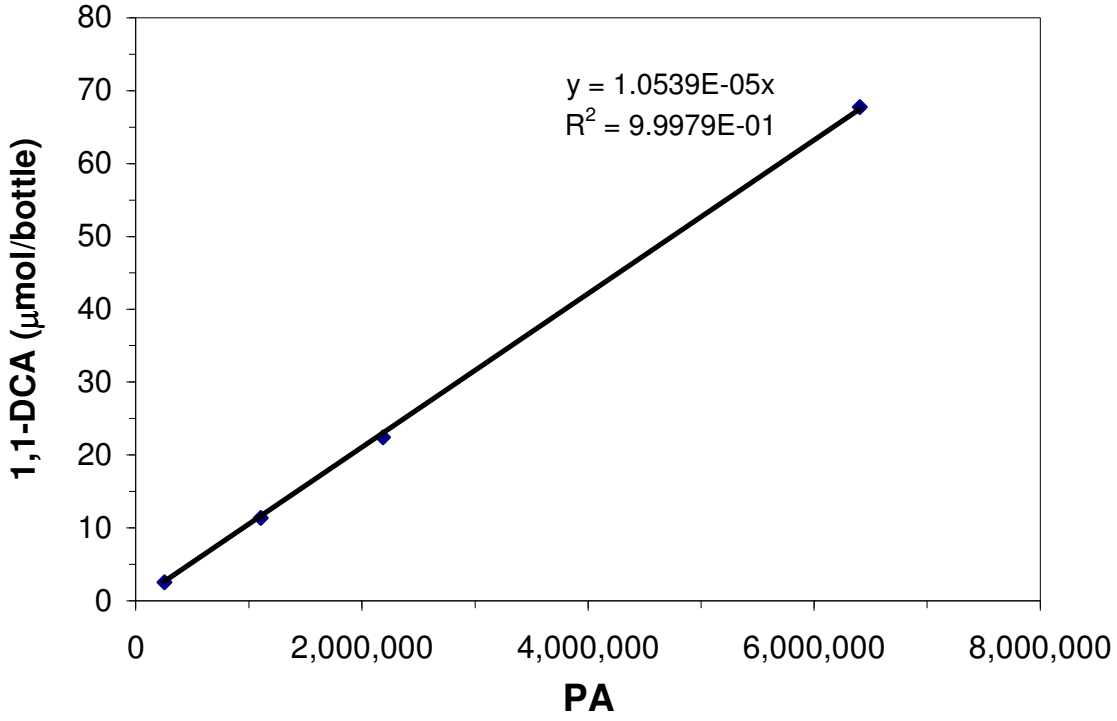

Figure F.13 GC response curve for 1,1-DCA.

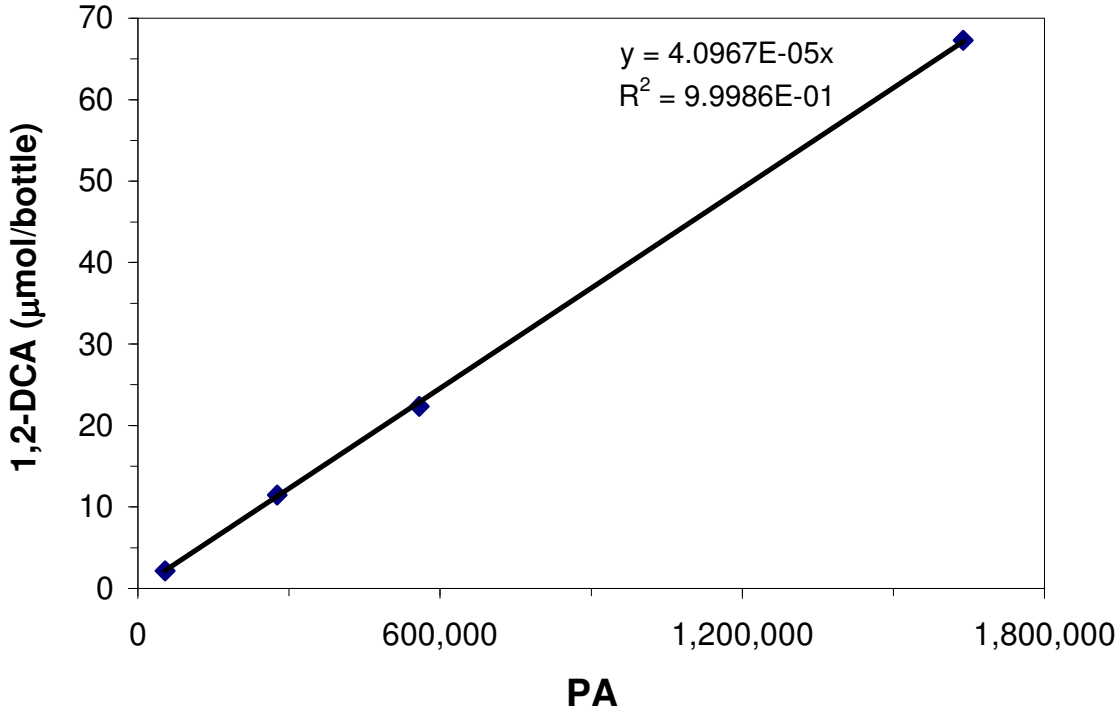

Figure F.14 GC response curve for 1,2-DCA.

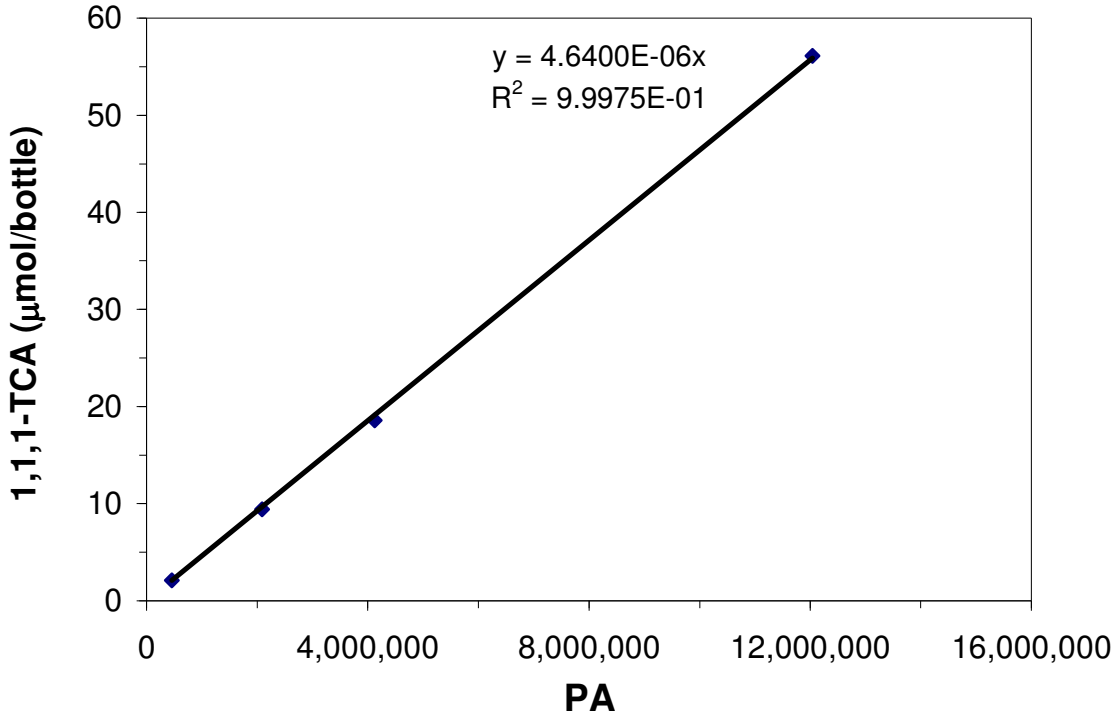

Figure F.15 GC response curve for 1,1,1-TCA.

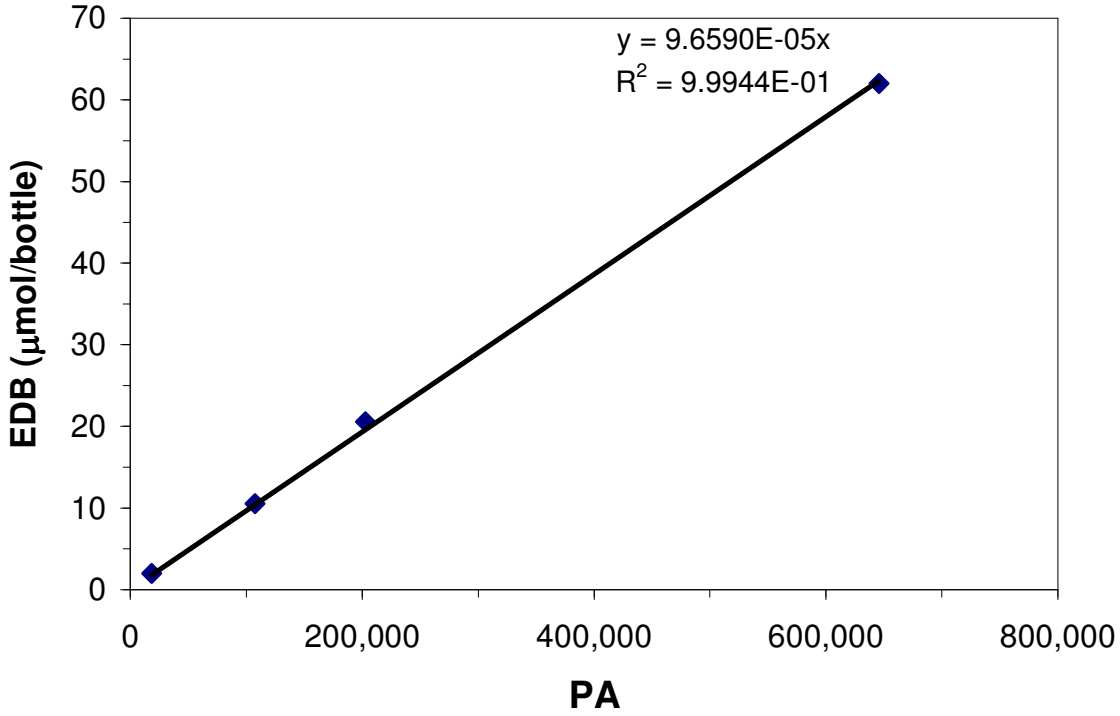

Figure F.16 GC response curve for EDB.

Appendix G

GC RFs for Microcosms

Table G.1 GC response factors for chlorinated ethenes, ethene, and methane used for microcosm evaluation of EOS®.

Compound	GC RT	Response Factor		Conversion Factor[a]		Min. Detection Limit
	(min)	(µmol/bottle/PAU)	R^2	(µmol/bottle to µM)	(µmol/bottle to mg/L)	(µmol/bottle)
Methane	0.49	3.1800E-06	0.99770	0.0085	0.0001	3.05E-01
Ethene	0.70	1.7253E-06	0.99766	1.3042	0.0365	4.72E-03
VC	2.60	2.6963E-06	0.99700	6.6821	0.4176	4.31E-04
cDCE	6.84	7.4977E-06	0.99989	15.6339	1.5165	1.23E-03
TCE	10.02	4.4295E-06	0.99927	11.8295	1.5544	1.39E-03
PCE	14.64	3.7375E-06	0.99694	8.7862	1.4571	6.09E-04

[a] Based on liquid volume of 50 mL, gas volume of 99 mL, and 23°C.

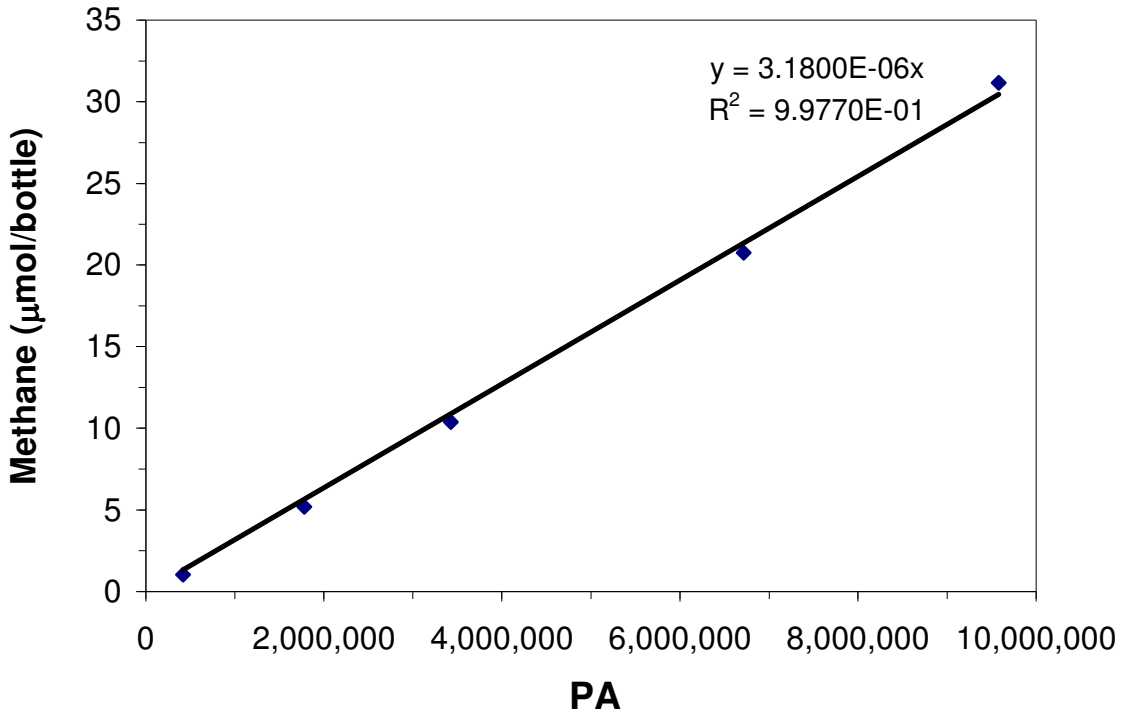

Figure G.1 GC response curve for methane in microcosms.

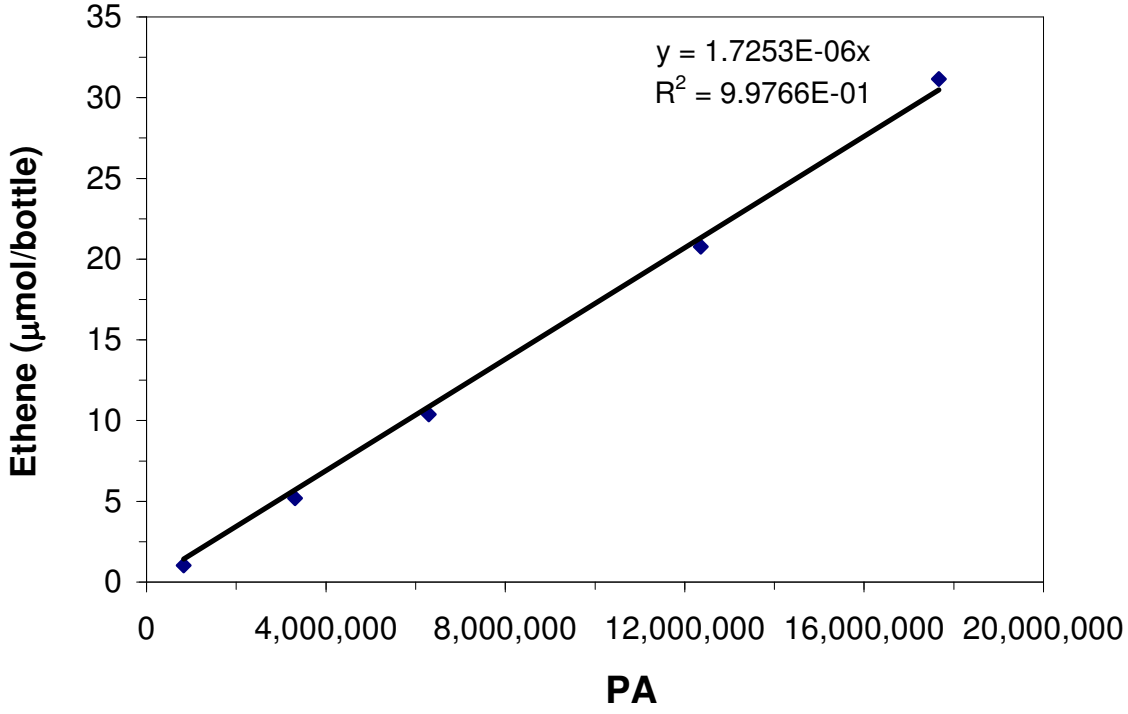

Figure G.2 GC response curve for ethene in microcosms.

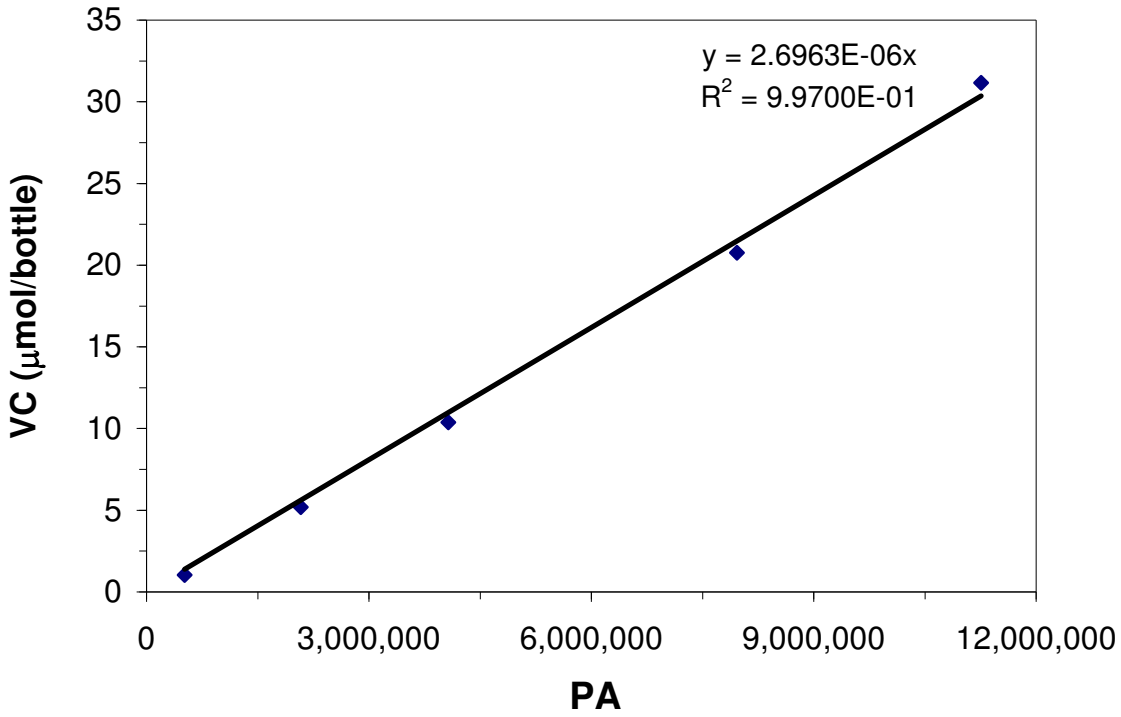

Figure G.3 GC response curve for VC in microcosms.

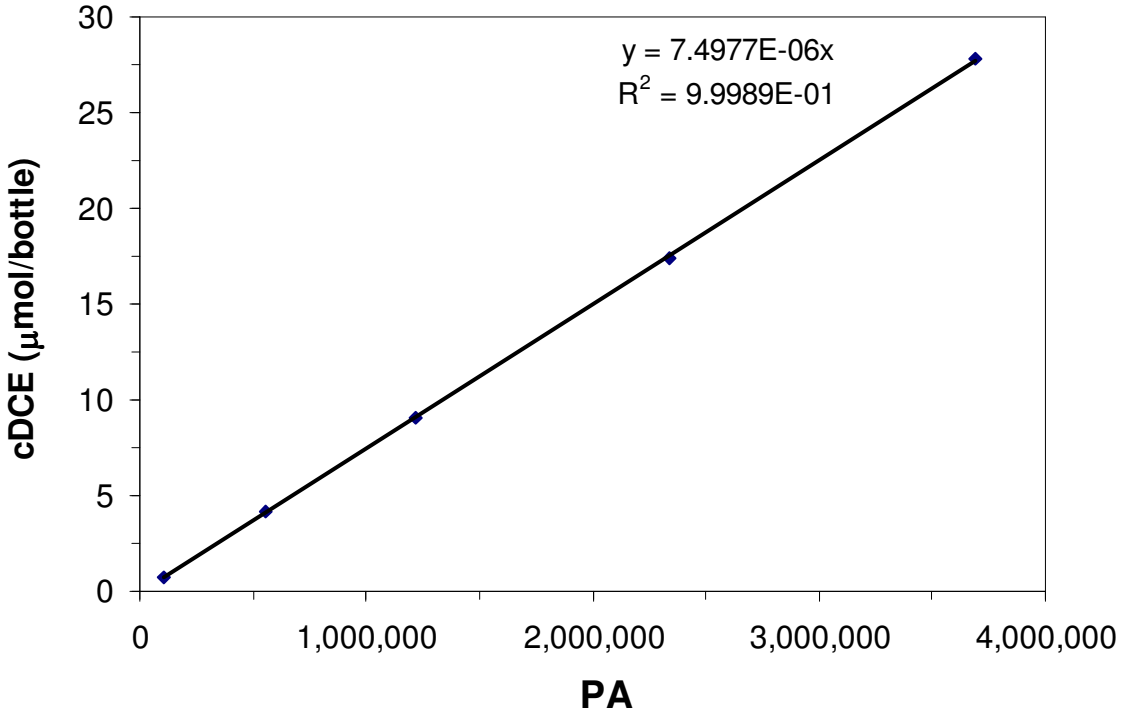

Figure G.4 GC response curve for cDCE in microcosms.

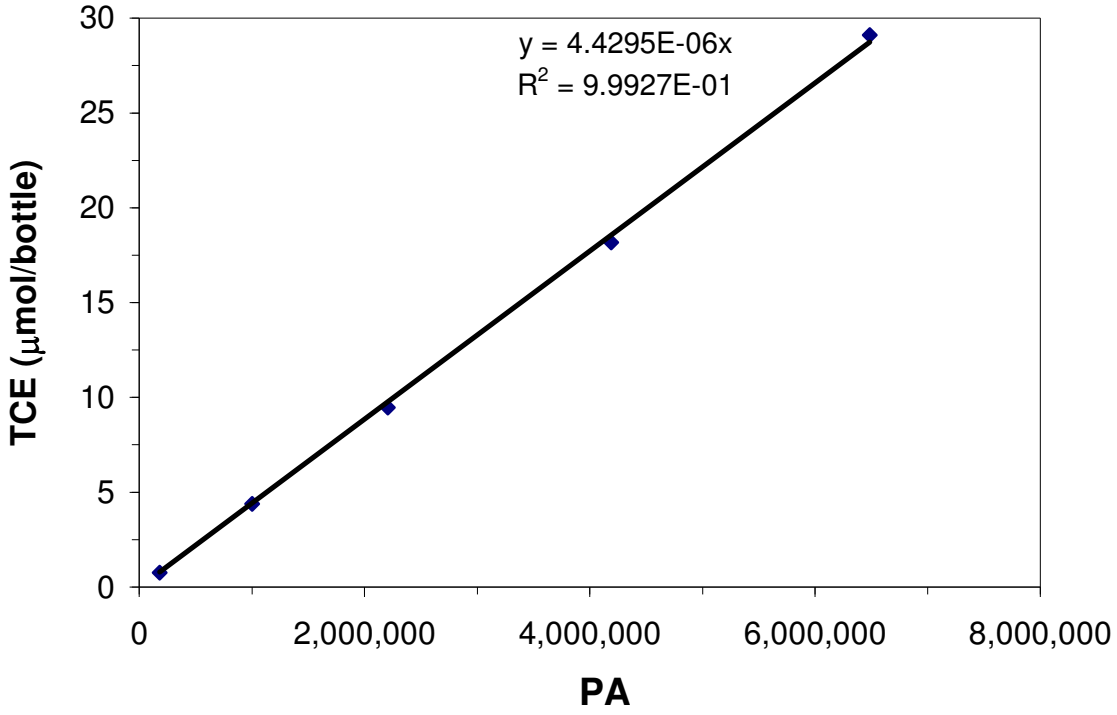

Figure G.5 GC response curve for TCE in microcosms.

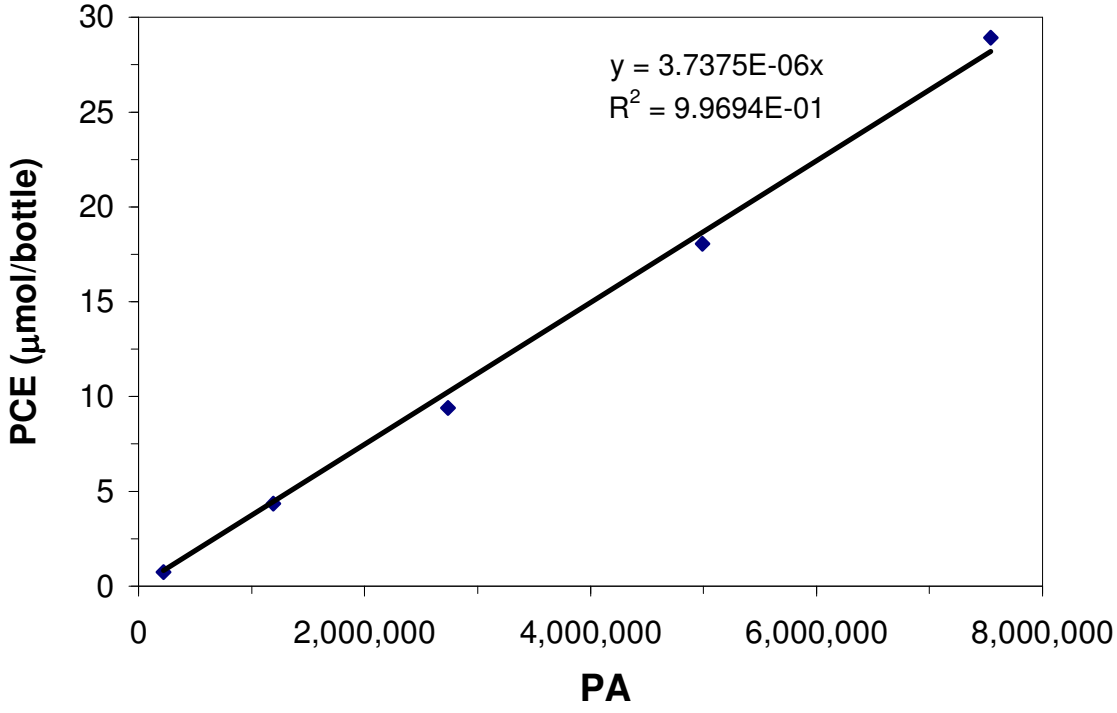

Figure G.6 GC response curve for PCE in microcosms.

Appendix H

GC RFs for Canisters

Table H.1 GC response factors for chlorinated ethenes, ethene, and methane used for canisters.

Compound[a]	GC RT	Adjusted Response Factor		Conversion Factor[b]		Min. Detection Limit
	(min)	(µmol/bottle/PAU)	R^2	(µmol/bottle to µM)	(µmol/bottle to mg/L)	(µmol/bottle)
Methane	0.49	7.7095E-05	0.99910	0.0005	0.0000083	7.47E+01
Ethene	0.70	7.4840E-05	0.99999	0.0338	0.0009	3.33E+02
VC	2.60	3.1674E-04	0.99971	0.0222	0.0007	2.01E-01
cDCE	6.84	2.3456E-03	0.99994	0.0510	0.0032	5.56E+00
TCE	10.02	9.1960E-04	0.99630	0.0549	0.0053	1.32E-01
PCE	14.64	5.7632E-04	0.99978	0.0539	0.0071	1.03E-01

[a] Gas standards (VC, ethene, and methane) were prepared in 160 mL serum bottles and liquid standards (cDCE, TCE, and PCE) were prepared in 70 mL serum bottles.

[b] Based on liquid volume of 18 L, gas volume of 1.6 L, and 23°C.

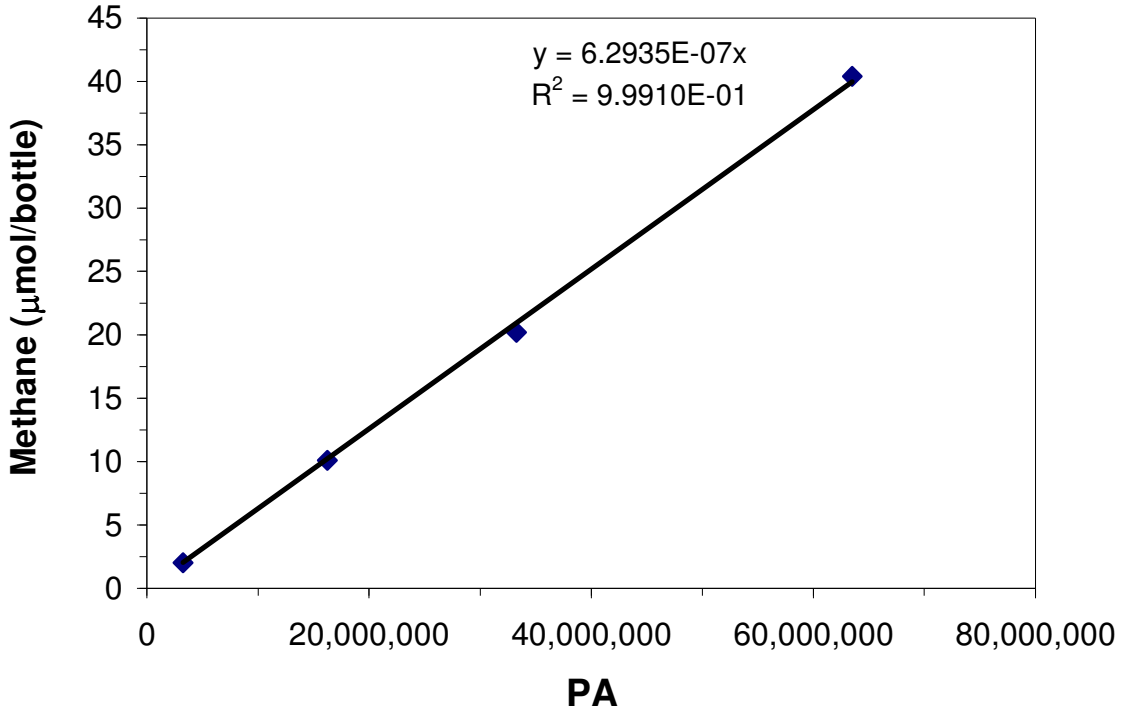

Figure H.1 GC response curve for methane in canisters.

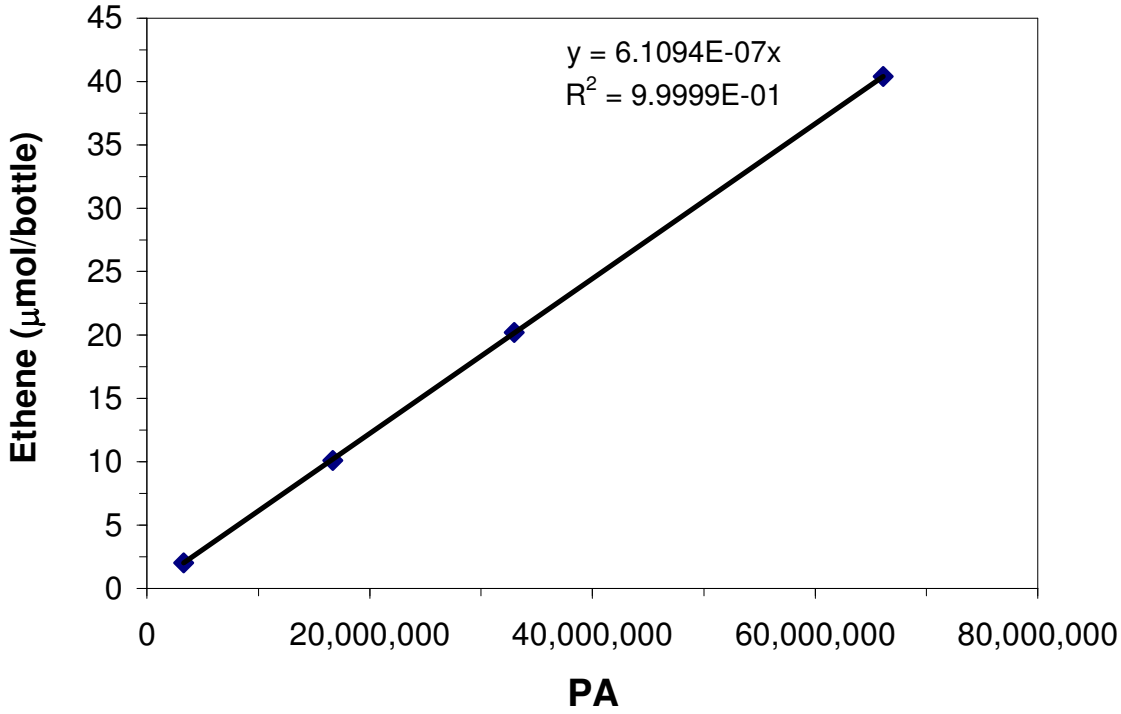

Figure H.2 GC response curve for ethene in canisters.

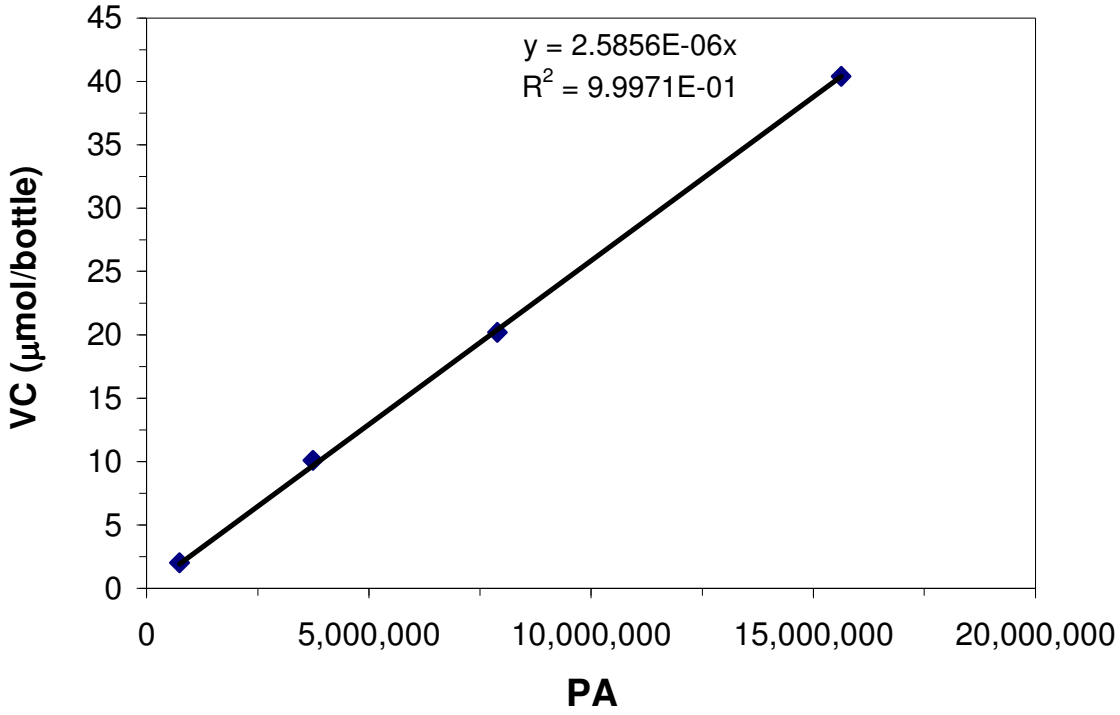

Figure H.3 GC response curve for VC in canisters.

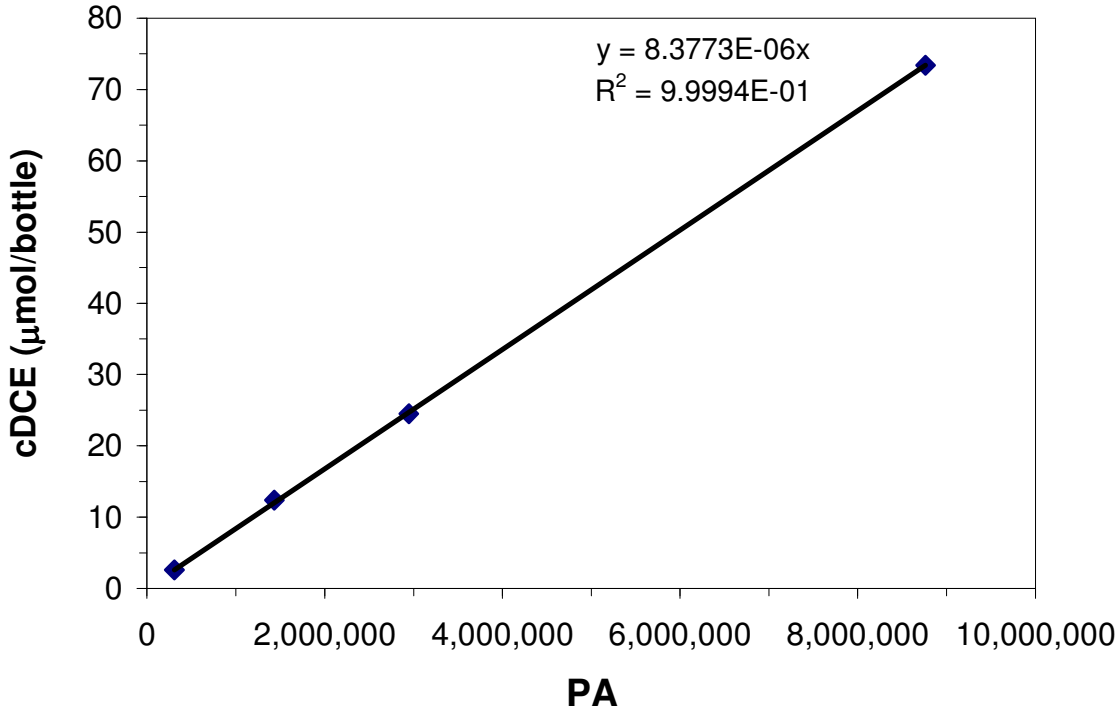

Figure H.4 GC response curve for cDCE in canisters.

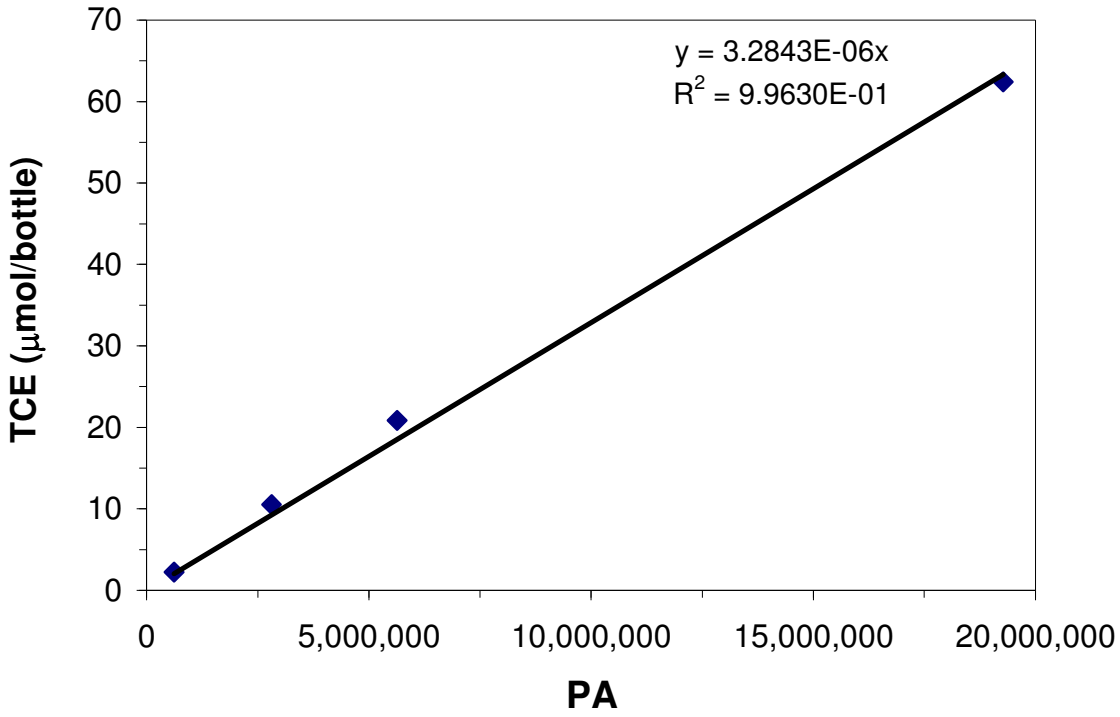

Figure H.5 GC response curve for TCE in canisters.

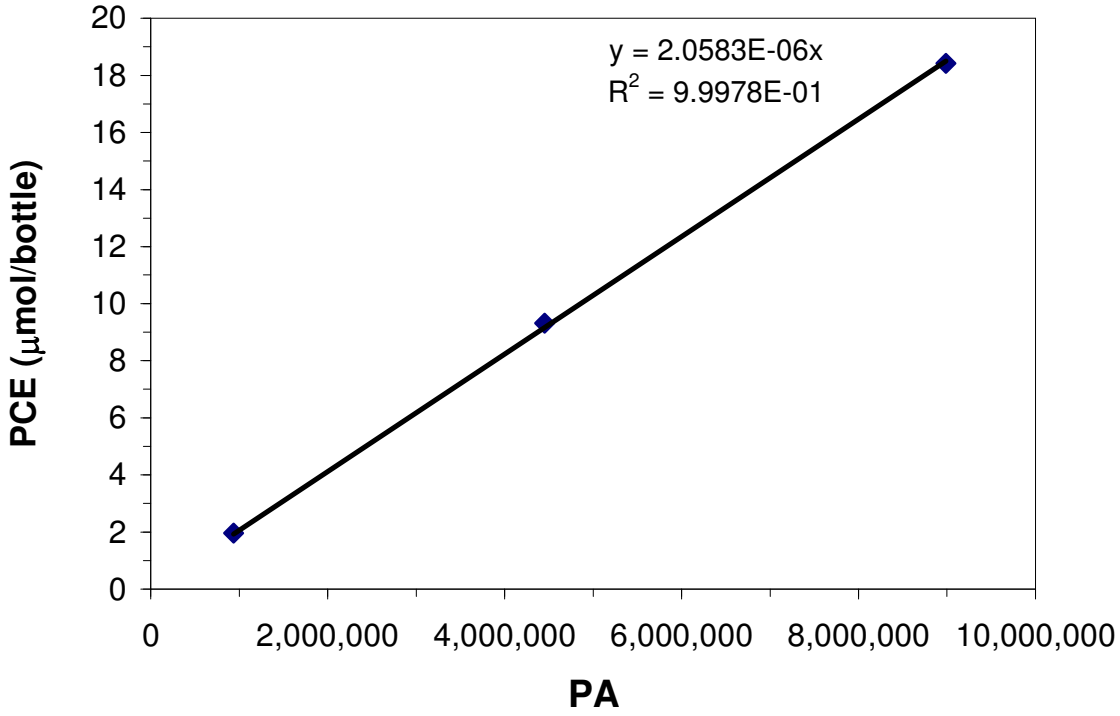

Figure H.6 GC response curve for PCE in canisters.

Appendix I

GC RFs for Chlorinated Benzenes

Table I.1 GC response factors for chlorinated benzenes.

Compound[a]	GC RT	Response Factor		Conversion Factor[a]		Min. Detection Limit
	(min)	(µmol/bottle/PAU)	R^2	(µmol/bottle to µM)	(µmol/bottle to mg/L)	(µmol/bottle)
CB	2.0	7.4729E-06	0.99953	9.1734	1.0326	5.27E+00
1,2-DCB	3.9	2.4425E-05	0.99852	9.5584	1.4051	2.68E+00
1,3-DCB	3.4	1.9570E-05	0.99955	9.2809	1.3643	4.93E+00
1,4-DCB	3.5	1.8357E-05	0.99984	9.3138	1.3691	5.28E-02
1,2,4-TCB	7.4	3.5488E-05	0.99543	9.5441	1.7318	5.55E-02
1,3,5-TCB	4.6[b]	2.9804E-07	0.99791	9.5547	1.7337	4.01E-01
1,2,4,5-TeCB	6.3[b]	5.0256E-07	0.99831	9.7566	2.1063	2.81E-02
PeCB	9.1[b]	1.6270E-07	0.99284	9.8260	2.4596	1.24E-04
HCB	13.9[b]	5.6164E-08	0.99662	9.7468	2.7755	1.48E-05

[a] Based on liquid volume of 100 mL, gas volume of 60 mL, and 23°C.

[b] Retention times using ECD; otherwise, retention times are based on FID analysis.

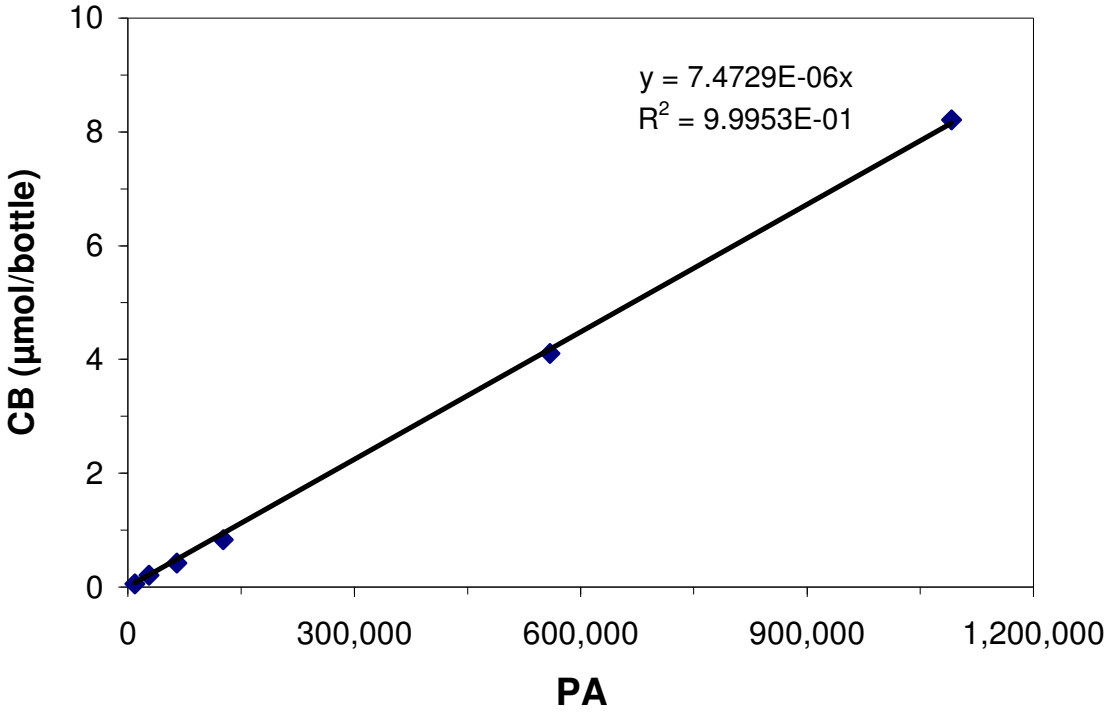

Figure I.1 GC response curve for CB.

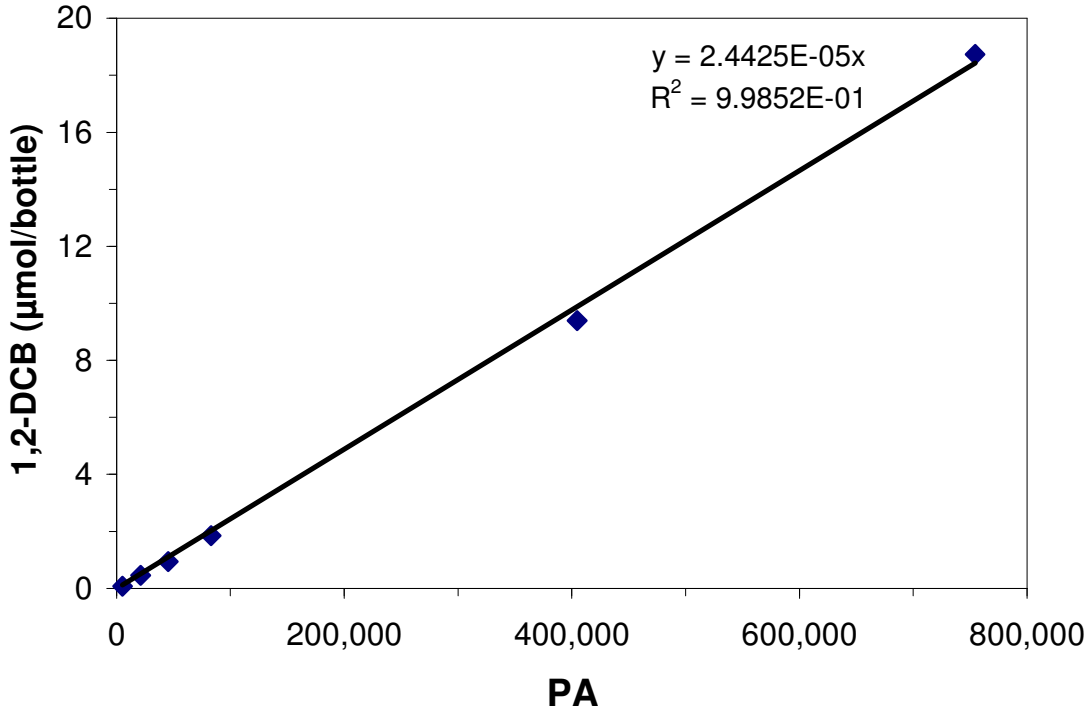

Figure I.2 GC response curve for 1,2-DCB.

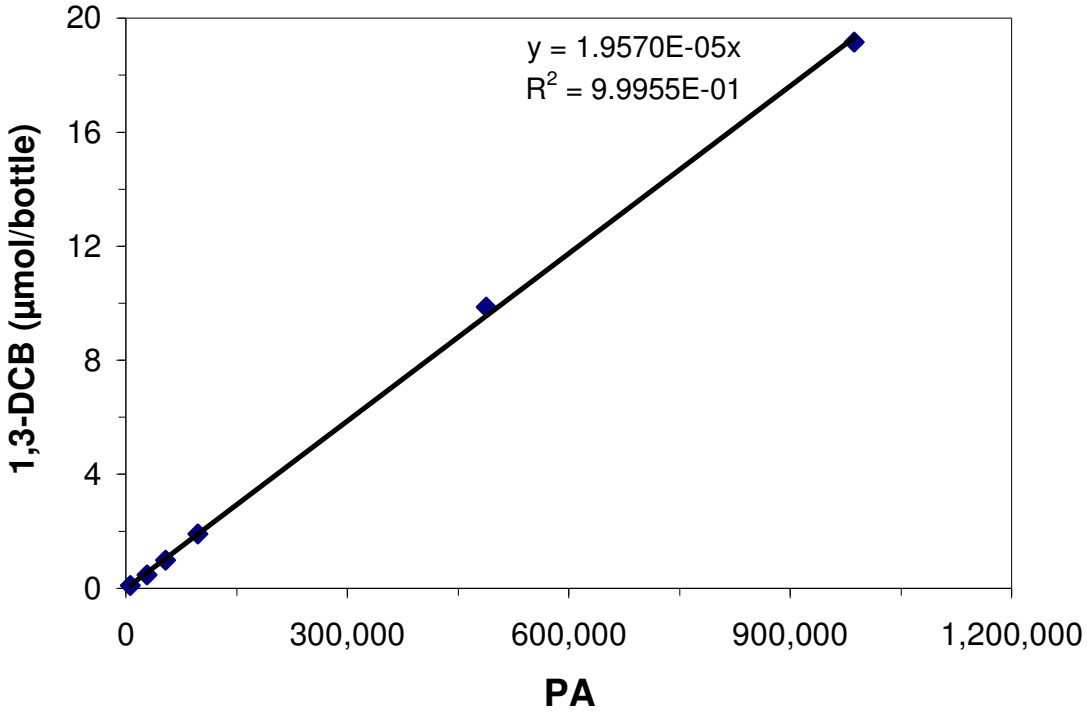

Figure I.3 GC response curve for 1,3-DCB.

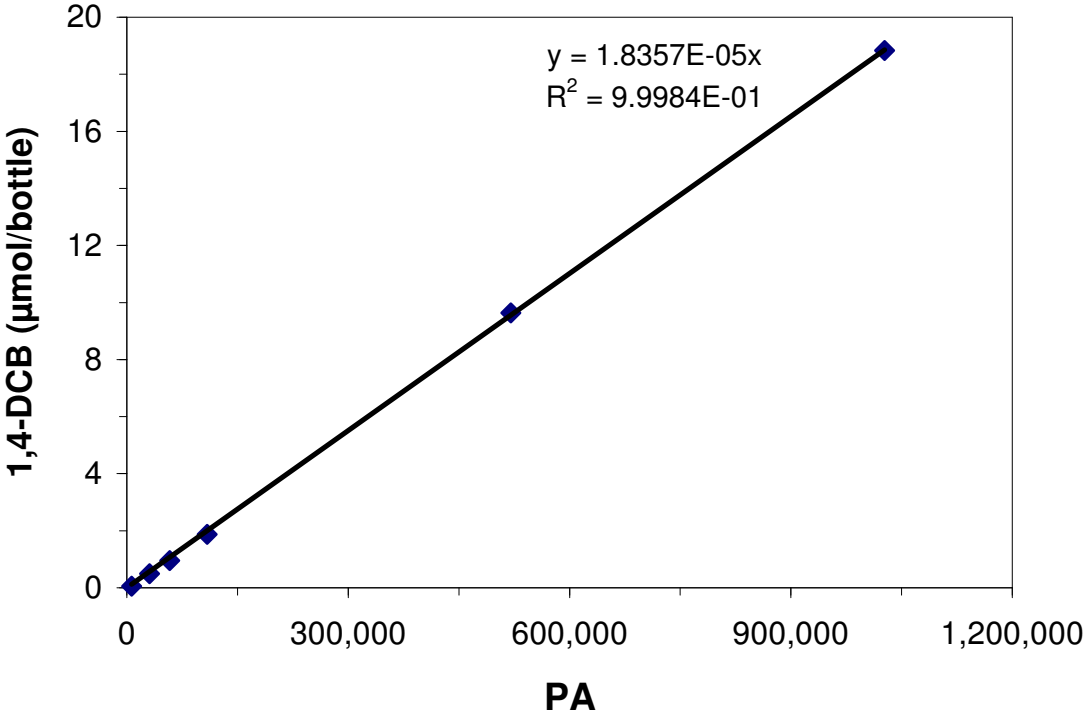

Figure I.4 GC response curve for 1,4-DCB.

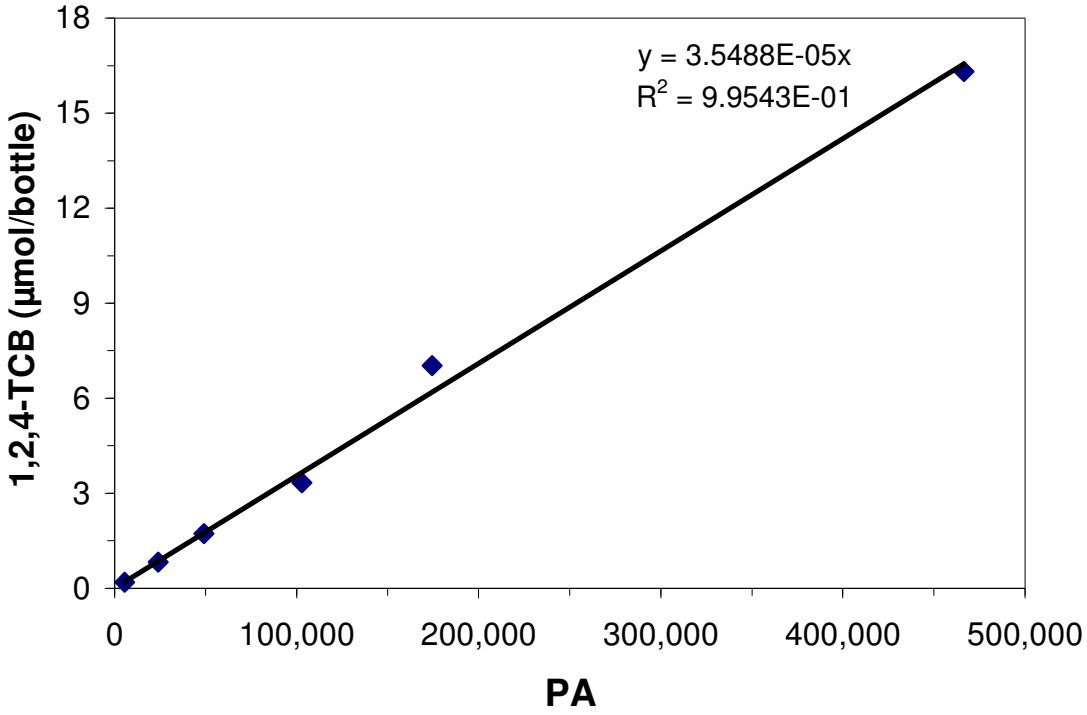

Figure I.5 GC response curve for 1,2,4-TCB.

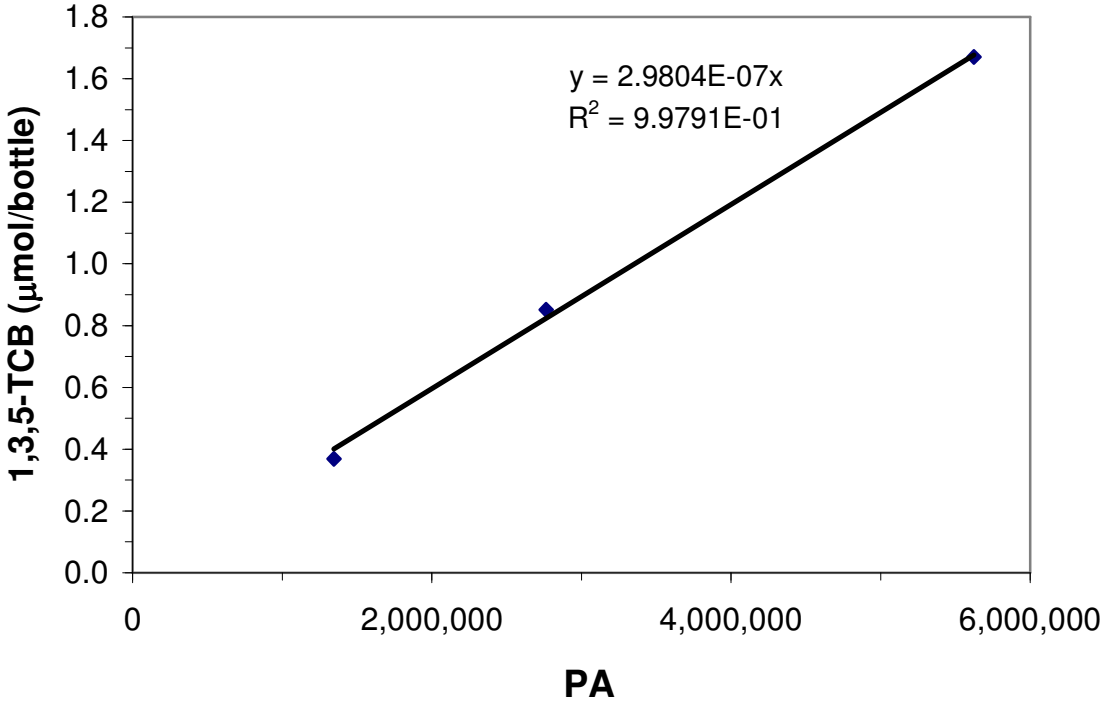

Figure I.6 GC response curve for 1,3,5-TCB.

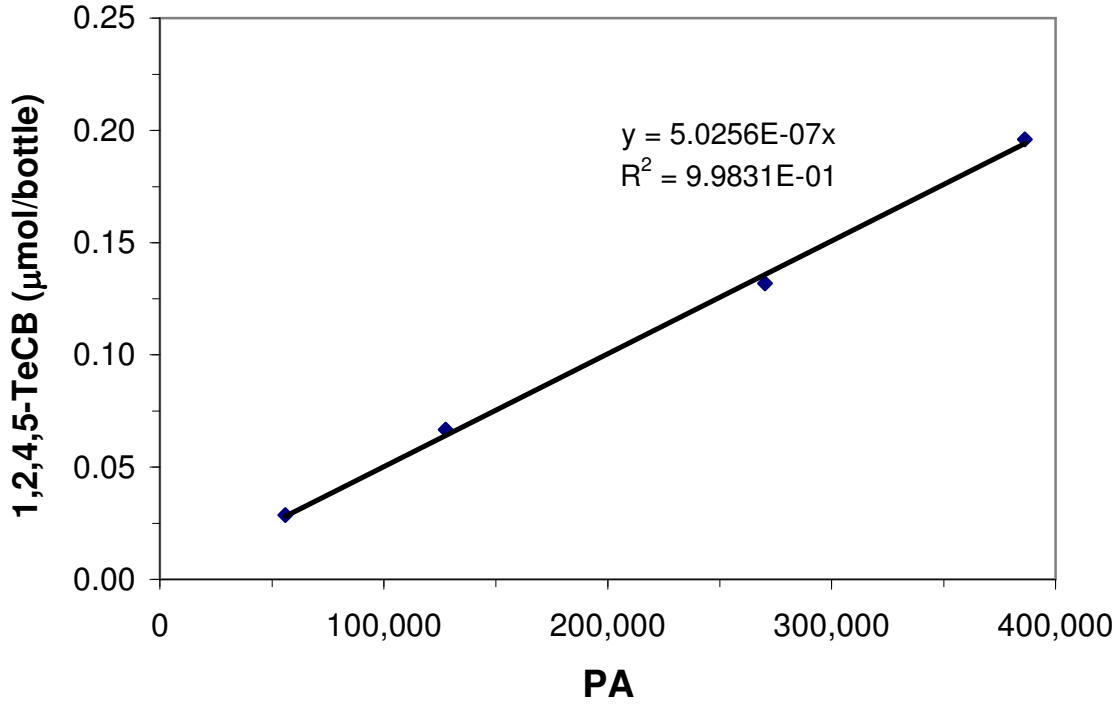

Figure I.7 GC response curve for 1,2,4,5-TeCB.

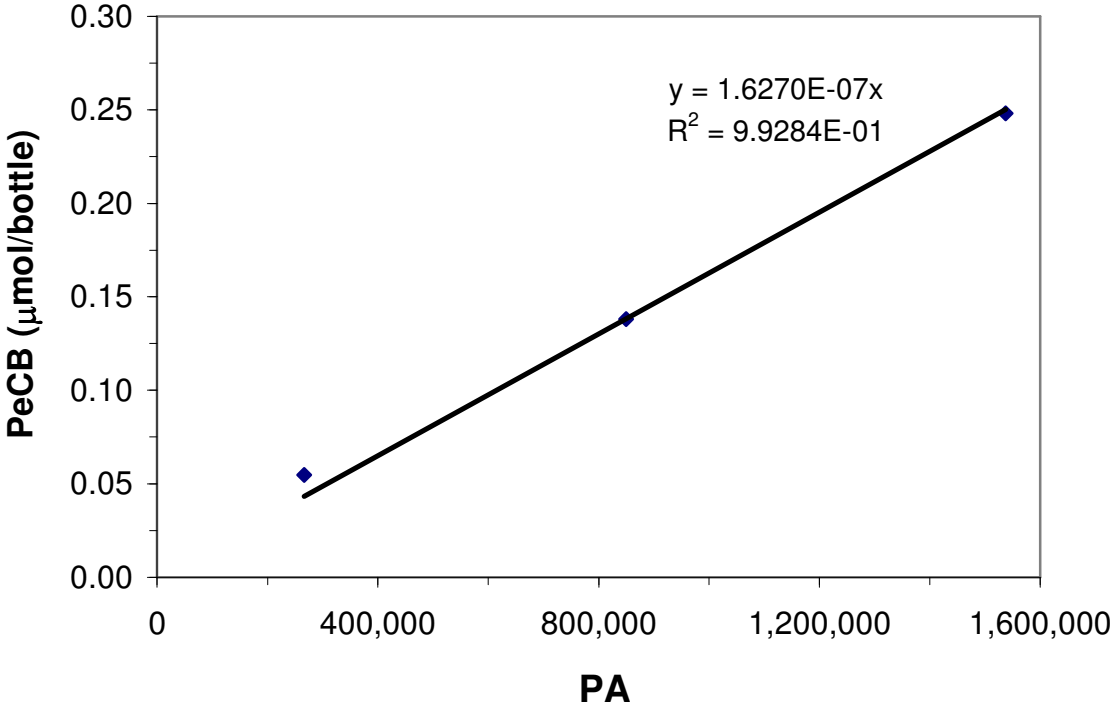

Figure I.8 GC response curve for PeCB.

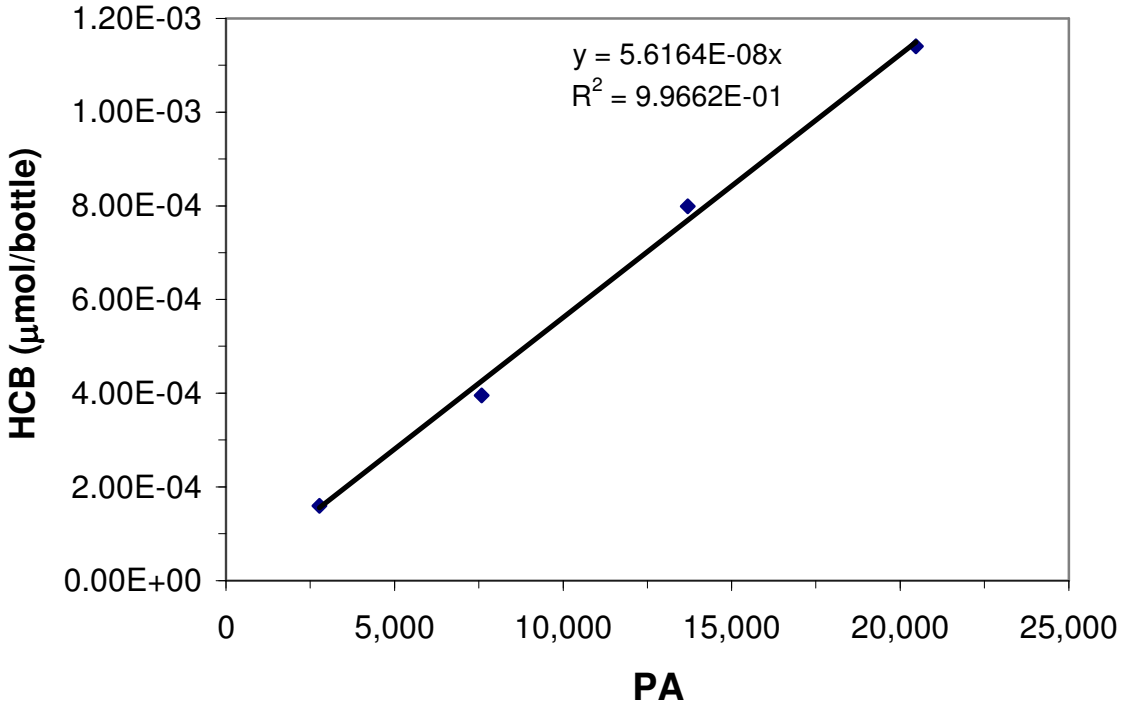

Figure I.9 GC response curve for HCB.

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