8-2016

Inhibitory and Synergistic Effects During Biodegradation of Mixed Contaminants at an Industrial Site in South America

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INHIBITORY AND SYNERGISTIC EFFECTS DURING BIODEGRADATION OF MIXED CONTAMINANTS AT AN INDUSTRIAL SITE IN SOUTH AMERICA

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Environmental Engineering and Science

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by
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August 2016

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ABSTRACT

In spite of decades of remediation activities, there are still thousands of industrial sites worldwide that are in need of clean-up. As remediation technologies have advanced, numerous sites have been successfully addressed. Many of the sites that still need attention are those at which complex mixtures of contaminants are present, making the development of clean-up strategies more challenging. The site (Area P) that is the subject of this thesis is located in one of the largest industrial facilities in South America. The area is characterized by comingled environmental impacts caused by petrochemical industries.

The main objective of this research was to evaluate the interaction among chemicals of concern in Area P during biodegradation under aerobic and anaerobic conditions, as well as the potential impact of chemical oxidation products on biodegradation. The specific objectives were:

1) To evaluate the impact of 2,4-DNT, 4-IPA, 1,2-DCA, 1,4-dioxane, and products from chemical oxidation of source zone contaminants on the aerobic biodegradability of CB;

2) To evaluate the impact of 2,4-DNT, 4-IPA, 1,2-DCA, 1,4-dioxane, and products from chemical oxidation of source zone contaminants on the aerobic biodegradability of 1,2-DCB;

3) To evaluate the impact of 2,4-DNT, 4-IPA, 1,2-DCA, 1,4-dioxane, and products from chemical oxidation of source zone contaminants on the anaerobic biodegradability of 4-NT; and

4) To evaluate the impact of 2,4-DNT, 4-IPA, 1,2-DCA, 1,4-dioxane, and products from chemical oxidation of source zone contaminants on the anaerobic biodegradability of 2,6-DNT.

The experimental approach was to develop enrichment cultures that aerobically biodegrade CB and 1,2-DCB and anaerobically biodegrade 4-NT and 2,6-DNT, and then expose these cultures to low and high concentrations of 2,4-DNT, 4-IPA, 1,2-DCA, 1,4-dioxane, and products of chemical oxidation from source zone contaminants.

Based on the results of this research, the following conclusions are offered:

1) Aerobic biodegradation of CB and 1,2-DCB was demonstrated in microcosms using soil and groundwater from an industrial site in South America. The microcosms served as inoculum to develop enrichment cultures, which were subsequently used to assess the effect of co-contaminants on the rate and extent of CB and 1,2-DCB biodegradation.

2) Anaerobic biodegradation of 2,6-DNT and 4-NT was demonstrated in microcosms using soil and groundwater from an industrial site in Brazil. Lactate served as the electron donor and nitro group reduction was the only transformation observed. The microcosms served as inoculum to develop enrichment cultures; the 2,6-DNT enrichment was subsequently used to assess the effect of co-contaminants on the rate and extent of 2,6-DNT biodegradation. The rate of 4-NT transformation was too slow to permit development of the 4-NT enrichment to the point needed to evaluate co-contaminants.

3) Alkaline activated persulfate was effective in chemical oxidation of the contaminants at their maximum concentrations. The treatment that employed a

stoichiometric dose was used to simulate the effect of chemical oxidation groundwater on biodegradation of CB, 1,2-DCB, 2,6-DNT, and 4-NT. Although higher than stoichiometric doses achieved more complete removal, the stoichiometric dose (28 g/g contaminant) is at the high end of what is deployed in situ.

4) 2,4-DNT, 4-IPA, 1,4-dioxane, and 1,2-DCA did not inhibit the rate or extent of aerobic CB biodegradation when these co-contaminants were present at their target high concentrations. Temporary inhibitory effects on the rate of CB biodegradation were observed in the presence of 10% (v/v) of the chemical oxidation groundwater from the stoichiometric treatment. The source of inhibition is not yet known but may be related to the organic products from partial chemical oxidation of the contaminants. COD analysis of the chemical oxidation groundwater suggests that the extent of contaminant mineralization was minor.

5) CB serves as a primary substrate for aerobic cometabolism of 2,4-DNT and 4-IPA, but not 1,4-dioxane or 1,2-DCA. This suggests that the aromatic oxygenases that are required for metabolism of CB are also reactive with 2,4-DNT and 4-IPA. This is an example of a positive co-occurrence of contaminants.

6) 2,4-DNT, 1,4-dioxane, and 1,2-DCA did not inhibit the rate or extent of 1,2-DCB biodegradation of 1,2-DCB when these co-contaminants were present at their target high concentrations. A temporary decrease in the rate of 1,2-DCB biodegradation occurred in the presence of 4-IPA at its target high concentration and with the 10% (v/v) chemical oxidation groundwater from the stoichiometric treatment.

7) 4-IPA did not inhibit the rate or extent of 1,2-DCB biodegradation when 4-IPA was present at its target low concentration.

8) 1,2-DCB serves as a primary substrate for cometabolism of 2,4-DNT and 4-IPA. This suggests that the aromatic oxygenases that are required for metabolism of 1,2-DCB are also reactive with 4-IPA. This is an example of a positive co-occurrence of contaminants.

9) No inhibitory effects were observed in the rate or extent of anaerobic biodegradation of 2,6-DNT when 2,4-DNT and 4-IPA were added as co-contaminants at the target high concentrations. Minimal inhibitory effects were observed when 1,4-dioxane and 1,2-DCA were added as co-contaminants. Temporary inhibitory effects on the rate of 2,6-DNT degradation were observed when adding 10% of the chemical oxidation groundwater from the stoichiometric treatment.

DEDICATION

This thesis is dedicated to God, my mother Magola, my father Jairo José, my brother Jairo Alejandro, my wonderful aunt Alix María and my boyfriend Matt; for their endless love, unending support and encouragement in all my endeavors. Also to my friends from Colombia and all the new friends I have found in Clemson.

ACKNOWLEDGMENTS

Firstly, I would like to thank my advisor Dr. David Freedman for his vote of trust, for his patience and availability at all times to help me out and share his knowledge, and for the passion he inspires in us, his students.

I would also like to express sincere gratitude to my mentor, James Henderson, for his advice and support, for teaching me that dreams can come true, and for opening so many doors full of opportunities that have changed my life.

I thank my fellow labmates for the countless times they helped me out. Especially to Alejandro Ramos for his unconditional support, for all the late nights and weekends he stayed at the Lab to help me out and offer a ride back home. I'd also like to thank Rong Yu, for all the help she gave me feeding my enrichment cultures while I was away, and for completion of the qPCR analysis.

I would like to acknowledge the DuPont Corporate Remediation Group for the funding provided to perform this research.

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LIST OF ABBREVIATIONS

1,2-DCB	1,2-Dichlorobenzene
1,2-DCA	1,2-Dichloethane
1,4-Diox	1,4-Dioxane
2,4-DAT	2,4-Diaminotoluene
2,4-DNT	2,4-Dinitrotoluene
2,6-DAT	2,6-Diaminotoluene
2,6-DNT	2,6-Dinitrotoluene
2A6NT	2-Amino-6-Nitrotoluene
2A4NT	2-Amino-4-Nitrotoluene
2N4AT	2-Nitro-4-Aminotoluene
4-AT	4-Aminotoluene
4-IPA	4-Isopropylaniline
ABS	Absorbance
BTEX	Benzene, Toluene, Ethylbenzene, Xylenes
BSM	Basal Salt Media
CETESB	Companhia Ambiental Do Estado De São Paulo (Environmental Company of São Paulo State)
CB	Chlorobenzene
CONAMA	Conselho Nacional do Meio Ambiente (National Environmental Council)
DDI	Deionized Water
GC	Gas Chromatography
HPLC	High Pressure Liquid Chromatography
ISCO	In-Situ Chemical Oxidation
KHP	Potassium Hydrogen Phthalate
MCL	Maximum Contaminant Level
MSM	Mineral Salts Medium
PS	Persulfate
RSL	Regional Screening Level
SRB	Sulfate Reducing Bacteria

CHAPTER ONE

1.0 INTRODUCTION AND OBJECTIVES

In spite of decades of remediation activities, there are still thousands of industrial sites worldwide that are in need of clean-up. As remediation technologies have advanced, numerous sites have been successfully addressed. Many of the sites that still need attention are those at which complex mixtures of contaminants are present, making the development of clean-up strategies more challenging.

The site that is the subject of this thesis is located in one of the largest industrial facilities in South America. The area is characterized by comingled environmental impacts caused by petrochemical industries. The client owns two properties at the industrial pole: Area N and Area P. Both properties are impacted with volatile organic compounds (VOCs) and semi volatile organic compounds (SVOCs) in the soil and groundwater. Area P is the main subject of this work. The site started operations in 1987 and shut down in 2014.

Table 1.1 summarizes the principal contaminants found at the site. These fall into eight categories: monoaromatics (i.e., benzene, toluene, ethylbenzene, and xylenes; BTEX), chlorobenzenes, phenols, nitrotoluenes, anilines, cumenes, isocyanates, and others not in the previous categories. Among the 26 chemicals identified, six stand out based on their elevated concentrations and/or regulatory limits: chlorobenzene (CB), 1,2-dichlorobenzene (1,2-DCB), 4-nitrotoluene (4-NT), 2,6-dinitrotoluene (2,6-DNT), 4-isopropylaniline (4-IPA), and 1,2-dichloroethane (1,2-DCA). All have been detected in the part per million range across the site groundwater. In addition, 2,4-dinitrotoluene

(2,4-DNT) is of particular concern based on its energetic properties and 1,4-dioxane is of particular concern to the client based on its occurrence at numerous other sites.

2-Nitrotoluene (2-NT) and 3-nitrotoluene (3-NT) are also present in Area P groundwater at ppm levels (Table 1.1). However, for the purpose of this research project, they were not included in the experimental design (see below) since they have similar properties to 4-NT, and 4-NT is found at higher concentrations.

The current remediation strategy calls for performing active intervention in the source areas (e.g., by chemical oxidation) coupled with monitored natural attenuation or enhanced bioremediation away from the source areas. The potential for biodegradation away from the source areas has not been fully assessed. Implementation may be significantly influenced by inhibitory and/or synergistic interactions among the mixture of contaminants present at the site, as well as by products created by source zone treatment that are likely to move downgradient from the source zones. In order to predict these types of interactions, experimental data is needed. Outlined below is a summary of what is known from the literature on interactions among the contaminants of concern.

1.1 Interactions during Biodegradation of Contaminants Found at Area P

Numerous studies have been conducted to assess the aerobic and anaerobic biodegradability of the target compounds listed in Table 1.1. However, much less is known about potential interactive effects. In many cases, the interaction can be expected to result in inhibition, e.g., when a compound exhibits a toxic effect or causes competitive inhibition for a shared enzyme. Conversely, synergistic interactions are also possible. For example, under aerobic conditions aromatic compounds are typically metabolized via

an initial oxygenase attack. Other compounds are subject to oxygenase activity but do not serve as growth substrates. Consequently, it is conceivable that one aromatic compound may serve as the primary substrate for cometabolic biodegradation of a non-growth substrate compound (Elango et al. 2011). It is essential that these inhibitory and/or synergistic activities be understood in order to have confidence that natural attenuation or enhanced bioremediation will be effective in more dilute parts of the contaminant plume.

Table 1.2 summarizes previous studies that were performed with at least two of the target compounds at Area P present at the same time. A diagonal separates the lower left cells from the upper right. Entries were made only in cells above the diagonal, to avoid repetition. It is immediately apparent that no studies were found that evaluated mixtures across the five groups listed in Table 1.2 (e.g., chlorobenzenes and anilines, or nitrotoluenes and anilines). Studies have been performed within groups (e.g., many studies have included various types of chlorinated benzenes), but not across them.

1.1.1 Interactions during Biodegradation of Chlorobenzenes

A commonly evaluated mixture includes CB and 1,2-DCB. Studies have been performed with both types of chlorobenzenes under aerobic (Haigler et al. 1992; Leahy et al. 2003), anaerobic (Fung et al. 2009; Nelson et al. 2011), and aerobic/anaerobic conditions (Kurt and Spain 2013; Elango et al. 2010). Haigler et al. (1992) reported that CB grown cells also oxidized 1,4-DCB, ethylbenzene, and all of the substituted catechols tested. This pattern suggested that CB induces a nonspecific dioxygenase and catechol

oxygenase that catalyzes the initial attack on the aromatic ring. Leahy et al. (2003) showed that many aromatic utilizing bacteria are capable of degrading a structurally diverse group of hydrocarbons in complex mixtures, and that the co-oxidation of aromatic hydrocarbons can be supported by the presence of growth substrates which act as inducers and sources of carbon, energy, and reducing power. Furthermore, findings clearly suggested the overlapping and complementary substrate specificities of aromatic oxygenases, which should facilitate the biodegradation of hydrocarbon mixtures by naturally occurring bacterial consortia.

Kurt and Spain (2013) investigated CB degradation to below detection limits in the capillary fringe, with rates of 21±1 mg of CB/m^2·d, 3.7±0.5 mg of 1,2-DCB/m^2·d, and 7.4±0.7 mg of 1,4-DCB/m^2·d. This study did not reveal any inhibitory impacts among the contaminants and demonstrated that natural attenuation can prevent migration of CB, 1,2-DCB, and 1,4-DCB vapors. The results also revealed a substantial biodegradation capacity for chlorinated aromatic compounds at the oxic/anoxic interface and illustrate the role of microbes in creating steep redox gradients.

Fung et al. (2009) and Nelson et al. (2011) reported that anaerobic dehalogenation of CB by *Dehalobacter* spp. was considerably slower and less robust than 1,2- or 1,3-DCB dehalogenation and that in some cases degradation did not occur at all unless an initial dose of DCBs was added. This indicates a complementary interaction during anaerobic reductive dechlorination of chloroaromatic compounds.

Elango et al. (2010) reported that of the three DCB isomers, 1,2-DCB was the most extensively dechlorinated to CB in soil microcosms. CB was typically the terminal

product of reductive dechlorination of polychlorinated benzenes and no significant anaerobic transformation of benzene or CB in any of the microcosm treatments occurred. Interactions among the contaminants under anaerobic conditions were not reported.

Heidrich et al. (2004) studied an aquifer contaminated with large quantities of chlorinated aliphatic compounds, in addition to chlorobenzenes and BTEX. In this strictly anaerobic environment, geochemical indications for several microbial processes were found, including methanogenesis, sulfate and iron reduction as well as reductive dechlorination of the chlorinated hydrocarbons. Direct evidence for the latter degradation reaction was observed along the flowpath due to the appearance of intermediates and an increase in the degree of dechlorination.

In spite of the extensive studies that have been performed on aerobic and anaerobic biodegradation of chlorinated benzenes, no literature was found on the impact of chlorinated benzenes on the biodegradability of nitrotoluenes, anilines, chlorinated ethanes, or 1,4-dioxane (Table 1.2). Under aerobic conditions, a positive interaction with nitrotoluenes and anilines seems possible, since oxygenase activity is involved in the initial attack on all of these compounds. Nevertheless, this could also lead to competitive inhibition. Under anaerobic conditions, it is conceivable that the same types of *Dehalococcoides* that are responsible for dihaloelimination of 1,2-DCA to ethene could also respire chlorinated benzenes to benzene, but this has yet to be tested. Reducing conditions should favor reduction of nitrotoluenes, although it is unclear if this will negatively impact reduction of chlorinated benzenes and 1,2-DCA.

1.1.2 Interactions during Biodegradation of Nitrotoluenes

Nitrotoluenes and dinitrotoluenes mixtures have also been evaluated, under aerobic (Lendenmann et al. 1998, Nishino et al. 2000, Leungsakul et al. 2005, Hudcova et al. 2011) and anaerobic (Shin et al. 2005) conditions.

Lendenman et al. (1998) reported that *Pseudomonas* sp. DNT successfully degraded 2,4-DNT in a fixed-bed bioreactor. Strains that are able to use 2,6-DNT as the sole growth substrate were also isolated. However, 2,6-DNT at concentrations higher than 20 mg/L inhibited growth of both 2,4- and 2,6-DNT degrading strains. Therefore, the degradation of isomeric mixtures was not successful in batch cultures where initial DNT concentrations were high. Nishino et al. (2000) also reported that although 2,6-DNT prevented the degradation of 2,4-DNT by 2,4-DNT-degrading strains, the effect was not the result of inhibition of 2,4-DNT dioxygenase by 2,6-DNT or of 4-methyl-5-nitrocatechol monooxygenase by 3-methyl-4-nitrocatechol. The results also indicated that high concentrations of either isomer of DNT inhibit growth of DNT-degrading strains on simple substrates such as succinate.

Leungsakul et al. (2005) investigated 2,4-DNT biodegradation via dioxygenases from *Burkholderia* sp. strain DNT (DDO) which catalyzes the initial oxidation of 2,4-DNT to form 4-methyl-5-nitrocatechol and nitrite. However, there was significantly less activity on other dinitrotoluenes and nitrotoluenes.

Shin et al. (2005) studied the anaerobic biodegradation of four dinitrotoluene isomers, (2,3-, 2,4-, 2,6- and 3,4-DNT) using *Lactococcus lactis* subsp. *lactis* strain 27, which was isolated from the intestines of earthworms. *L. lactis* strain 27 was capable of

reducing 2,4-, 2,6-, 2,3-, and 3,4-dinitrotoluenes up to 173.6, 66.6, 287.1, and 355 µM, respectively, during 12 h of incubation. However, biodegradation of aromatic nitrated compounds produced more toxic dinitroazoxytoluenes *in vitro*.

Hudcova et al. (2011) investigated the degradation efficiencies of isomeric mono-nitrotoluenes (2- and 4-NT) and dinitrotoluenes (2,4- and 2,6-DNT) by either individual bacterial strains (*Bacillus cereus* NDT4; *Pseudomonas putida* NDT1; *Pseudomonas fluorescens* NDT2; and *Achromobacter* sp. NDT3) or their mixtures, using submerged batch cultivations. The presence of both readily degradable 2-NT (or 4-NT) and poorly degradable 2,6-DNT in the medium negatively affected 2,4-DNT biodegradation. However, the mixed bacterial culture still effectively degraded 2,4-DNT with only slightly lower rates under these unfavorable conditions.

Spiess et al. (1998) reported the anaerobic degradation of 4-NT. 6-Amino-*m*-cresol was identified as an intermediate of 4-NT degradation when resting cells, pregrown with 4-NT and succinate, were incubated in an argon atmosphere with a 4-NT solution. The conversion of 4-NT to 6-amino-*m*-cresol was stoichiometric; the metabolite was identified unequivocally by comparison of its chromatographic properties and UV spectrum with those of authentic 6-amino-*m*-cresol.

No literature was found on the potential interactive effects among nitrotoluenes, chlorobenzenes, anilines, chlorinated ethanes, or 1,4-dioxane, under aerobic or anaerobic conditions (Table 1.2).

1.1.3 Interactions during Biodegradation of Anilines, 1,2-DCA, and Other Compounds

Biodegradation of anilines and chlorinated anilines has been studied extensively under aerobic conditions (Scheunert and Reuter 2000; Zhang et al. 2012; Hongsawat and Vangnai 2011; Nitisakulkan et al. 2014). However, no literature was found that examined biodegradation of anilines in the presence of chlorobenzenes, nitrotoluenes, or chlorinated ethanes (Table 1.2). Anilines are generally considered to be refractory under anaerobic conditions, since they are not readily amenable to reductive processes. No literature was found that explored the potential for anilines to inhibit anaerobic biodegradation of other categories of contaminants (Table 1.2).

1.1.4 Impacts Resulting from Source Zone Chemical Oxidation

Chemical oxidation is an aggressive form of treatment that is non-specific in terms of the target organic compounds. Contaminants are oxidized along with the natural organic matter that is also present. Chemical oxidation can result in a major alteration of subsurface conditions, including the oxidation potential and pH. These changes have generally been regarded as neutral or complimentary to subsequent efforts to remove residual contaminants with aerobic biodegradation. However, mobilization of metals can create inhibitory conditions for biodegradation. Chemical oxidation is generally regarded as non-complimentary to subsequent treatment by anaerobic biodegradation, due to the highly oxidized environment. Nevertheless, a surprisingly large number of anaerobes are able to survive the elevated redox level (at least for the relatively short periods of time when oxidants are applied) and subsequently reestablish low redox conditions.

Furthermore, at least one study has shown that in situ chemical oxidation benefited subsequent anaerobic bioremediation. Droste et al. (2002) speculated that this was a consequence of supplying sulfate (since persulfate was used as the oxidant), reduction of which may have helped to reestablish low redox conditions; generation of simpler organic carbon by degrading naturally occurring complex organic carbon, thereby increasing electron donor supply; and/or making VOCs more bioavailable by breaking down adsorption sites (e.g., naturally occurring complex organic carbon).

Given the potential for both negative and positive outcomes from chemical oxidation of the source zones at Area P with respect to biodegradation of remaining contaminants away from the source, it is important to evaluate the potential impacts on biodegradation of specific compounds. No literature was found on how chemical oxidation may impact subsequent biodegradation of contaminants of concern at Area P.

1.2 Objectives

It is apparent that negative interactions, positive interactions, and no interactions are possible among the contaminants present at Area P. It is also apparent from this analysis that very little is known about the interactions of concern among these contaminants (Table 1.2), as well as potential impacts from the products of chemical oxidation. The main objective of this research will be to evaluate the interaction among chemicals of concern in Area P during biodegradation under aerobic and anaerobic conditions, as well as the potential impact of chemical oxidation products on biodegradation. The specific objectives are:

1) To evaluate the impact of 2,4-DNT, 4-IPA, 1,2-DCA, 1,4-dioxane, and products from chemical oxidation of source zone contaminants on the aerobic biodegradability of CB;

2) To evaluate the impact of 2,4-DNT, 4-IPA, 1,2-DCA, 1,4-dioxane, and products from chemical oxidation of source zone contaminants on the aerobic biodegradability of 1,2-DCB;

3) To evaluate the impact of 2,4-DNT, 4-IPA, 1,2-DCA, 1,4-dioxane, and products from chemical oxidation of source zone contaminants on the anaerobic biodegradability of 4-NT; and

4) To evaluate the impact of 2,4-DNT, 4-IPA, 1,2-DCA, 1,4-dioxane, and products from chemical oxidation of source zone contaminants on the anaerobic biodegradability of 2,6-DNT.

The experimental approach (described in section 2) was to develop enrichment cultures that aerobically biodegrade CB and 1,2-DCB and anaerobically biodegrade 4-NT and 2,6-DNT, and then expose these cultures to low and high concentrations of 2,4-DNT, 4-IPA, 1,2-DCA, 1,4-dioxane, and products of chemical oxidation from source zone contaminants.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Sample Locations

It was not possible for the client to collect soil and groundwater samples from Area P because of restricted access to the site. Consequently, soil and groundwater samples from Area N were used, as these had already been collected and were shipped to the United States. The client understands the limitations and constrains associated with the applicability of the data obtained from this investigation to draw conclusions for the Area P site. The enrichment cultures and microcosms were set up with Area N samples, which have a different set of contaminants of concern (i.e., chlorinated anilines, dichloronitrobenzenes), although some of them overlap (i.e., chlorobenzenes). Nevertheless, given the proximity of Areas P and N, the assumption was made that the microbial community composition in Area N is similar enough to Area P so that it was reasonable to make use of samples from Area N for this research.

Samples of groundwater and soil cores were collected by CH2M Hill on March 31, 2015. The samples were stored at 4 °C until they were shipped in refrigerated storage containers to the CH2MHILL in Corvallis, Oregon. There, the samples were split and sent to various laboratories, including Clemson University. Groundwater samples were received at Clemson University on September 29, 2015. Soil samples were received on October 2, 2015.

The sampling points selected are located away from the Area N source zones. For the aerobic experiments, groundwater (15 L) was collected in plastic bottles (1 L) from

well PM-26. Soil cores were collected in 120 cm acrylic sleeves from locations N035 and N031, at the depth of the groundwater table. The cores were cut into 30 cm long sections and the ends were sealed with rubber caps before shipping to the United States.

For the anaerobic experiments, groundwater was collected in the same manner from well PM-20. Soil samples were collected in the same manner from location N037, 2 m below the groundwater table.

Upon arrival at Clemson University, the groundwater and soil cores were stored at 4 ºC, until the microcosms were prepared.

2.2 Chemicals and Medium

The types and sources of contaminants, daughter products, and electron donor used in this research are summarized in Table 2.1. Additions of basal salts medium (Hareland et al., 1975) and anaerobic salts medium (Chen, 2012) were used as sources of nutrients to support growth during the development of aerobic and anaerobic microcosms and enrichments, respectively.

The components of the mineral salt media are listed in Tables 2.2, 2.3 and 2.4. The procedures followed to prepare both types of media are described in Appendix A.

Water-saturated solutions of CB, 1,2-DCB, and 1,2-DCA were prepared by equilibrating an excess of neat compound in contact with DDI water (so that a non-aqueous phase was present along with the water) for at least one week in a sealed bottle, to allow the compound to saturate the water.

Stock solutions for 2,4-DNT (solid), 2,6-DNT (solid), 4-NT (solid) and 4-IPA (liquid) were prepared by adding neat compound to DDI water, and stirring for 72 hours.

Because of the low solubility of the compounds in water, significant volumes of the water-saturated solutions were needed.

2.3 Experimental Design and Microcosm Preparation

The experimental plan for the proposed research is outlined in Table 2.5. Four "parent" compounds were selected for evaluation: CB and 1,2-DCB under aerobic conditions and 4-NT and 2,6-DNT under anaerobic conditions. Using samples of soil and groundwater from the industrial site, microcosms were prepared. Once biodegradation of the parent compounds was established, the contents of one or more microcosms was used as inoculum to develop an enrichment culture, by diluting the inoculum in groundwater and medium. Once the enrichment cultures were established, they were used according to the list of treatment in Table 2.5 to evaluate how 2,4-DNT, 4-IPA, 1,2-DCA, 1,4-dioxane, and products from chemical oxidation of the source area material impacted the rate and extent of biodegradation of the parent compound. Using enrichment cultures is a step removed from microcosms and is therefore not as representative of actual in situ conditions. However, this approach allowed for a more robust assessment of how the presence of other compounds impacts biodegradation of the parent compounds, i.e., it was more direct to associate the presence of the additional contaminant on the rate of biodegradation of the parent compound.

2.3.1 Preparation and Evaluation of the Soil and Groundwater

Upon receipt, soil was removed from the core samples and the initial concentrations of target contaminants present in soil and groundwater were assessed.

Using a sterilized steel rod and spoon, the soil cores from the aerobic locations (N035 and N031) were discharged into a sterile Tupperware container and then aggressively homogenized with the spoon. The same procedure was followed for the soil cores from the anaerobic location (N037), except that removal of soil occurred in an anaerobic chamber. To estimate the initial concentrations of target contaminants present in soil, sets of triplicate sterilized serum bottles were prepared by adding 20 g of mixed soil from the aerobic locations and 100 mL of DDI water, and sealing them with Teflon-faced butyl rubber septa and aluminum crimp caps. Similarly, triplicate bottles were set with the mixed soil from the anaerobic location and DDI water. The serum bottles were incubated for three days on an orbital shaker, at which point liquid and headspace samples were removed for analysis. This procedure was not intended to provide a rigorous solvent-based extraction of the soil; instead, the intent was determine the approximate extent of desorption that may occur in the microcosms while the soil was in equilibrium with the groundwater.

None of the target contaminants (Table 2.5) were detected in water that was in contact with the soil samples. In the aerobic soil, a volatile unknown was detected on the gas chromatograph (GC) used to analyze CB and 1,2-DCB, at a retention time of 19.7 min (after CB at 11.0 min and before 1,2-DCB at 21.5 min). Nevertheless, the magnitude of the peak area (4.0 units) was minor relative to the peak areas resulting from addition of CB and 1,2-DCB to the microcosms (3000 and 150 units, respectively). Several unknown peaks were detected using the high performance liquid chromatograph (HPLC) used to analyze for 4-IPA, 4-NT, 2,4-DNT, and 2,6-DNT, although the peak areas were close to

the detection limit. In the anaerobic soil, the same volatile unknown detected in the aerobic soil was present (retention time = 19.7 min), and with a similarly low peak area (5.2). As with the aerobic soil, several unknown peaks were detected using the HPLC, although the peak areas were close to the detection limit. The results from this analysis are shown in Appendix B.

The plastic bottles that contained the groundwater samples from the aerobic location (PM-26) were opened in the anaerobic chamber. To measure the initial concentrations of target contaminants present in the groundwater, triplicate bottles were prepared by adding 100 mL of groundwater, capping them and sealing them with Teflon-faced butyl rubber septa. Similarly, triplicate bottles were set with groundwater from anaerobic locations (PM-20). The serum bottles were incubated for 12 h on an orbital shaker, at which point liquid and headspace samples were removed for analysis. None of the target compounds (Table 2.5) were detected. Several unknown peaks were observed during GC analysis of headspace samples, including the peak at 19.7 min and another VOC that eluted at 23.1 min (Tables 2.6 and 2.7). Unknowns were also detected during HPLC analysis, although the size of the peaks suggested that the concentrations of these compounds were low.

2.3.2 Chemical Oxidation Laboratory Scale Test

The experimental design calls for use of water from chemical oxidation treatment of the source zone contaminants present in Area P. Since groundwater from the site that had been treated by chemical oxidation was not available, it had to be generated in the laboratory. For this purpose, the treatments outlined in Table 2.8 were prepared.

Persulfate was selected as the oxidant since previous testing of chemical oxidation with Area P soil and groundwater used this approach (Gray et al. 2014).

Alkaline activated Klozur® persulfate was applied per the recommendations of site personnel. Klozur® is a widely used technology capable of remediating most common contaminants of concern. In addition to the oxidative radical species typically formed during the activation of persulfate, alkaline activated persulfate benefits from the formation of reductants and nucleophiles. These additional reactive species result in the treatment of chlorinated methanes, such as carbon tetrachloride, and chlorinated ethanes, such as 1,1,1-trichloroethane, in addition to many other compounds typically reactive with activated persulfate (Peroxychem, 2016).

The groundwater needed to assess the potential impact of chemical oxidation was generated by preparing microcosms with soil (20 g) from the mixed aerobic locations and groundwater (100 mL) from PM-26 in sterilized 160 mL serum bottles, according to the treatments listed in Table 2.8. The stoichiometric dose was based on the target compounds (Table 2.5) and their maximum concentration in Area P groundwater (Table 1.1). The maximum dose applied (4.2 times stoichiometric) was based on an assumed limit on the volumetric amount of persulfate and hydroxide, such that their combined volume would represent no more than 10% of the liquid in the serum bottles (i.e., 10 mL in a total of 100 mL of liquid). The amount of groundwater added was based on the quantity needed to reach a total volume of 100±1 mL. To obtain the contaminant concentrations, it was necessary to add neat compounds or saturated water solutions (Table 2.9).

For alkaline activation of Klozur® persulfate, the pH of the soil and groundwater in contact with the persulfate had to be maintained above 10.5. NaOH was used to achieve this. The demand for NaOH arises from the natural demand from soil and groundwater plus the generation of acid during the decomposition of Klozur persulfate. The demand for NaOH due to contaminant decomposition was estimated as two moles per mole of Klozur persulfate.

The initial pH in all bottles was increased to 10.5 or above by adding NaOH (9.2 M). The bottles were then sealed, placed on an orbital shaker table for 1 h, and rechecked for pH to confirm that it was still above 10.5. Next, the contaminants were injected into the serum bottles (Table 2.9). Lastly, Klozur® and NaOH (for reaction) were injected to each treatment according Table 2.9. The time zero concentrations were determined based on contaminant levels in the treatment with no persulfate added. Headspace and liquid samples were removed from all bottles periodically over 29 days of incubation to determine the concentration of the target compounds that remained. At that time, samples of groundwater from the stoichiometric treatment were used in the first set of inhibition tests. The chemical oxidation bottles were then stored at 4 °C and later used in additional inhibition tests.

2.3.3 Preparation of the Aerobic Microcosms

One day prior to preparing the aerobic microcosms, one of the 1 L plastic bottles containing groundwater from PM-26 was moved from storage (4 ºC), placed in the anaerobic chamber, allowed to warm overnight and poured into a 1 L media bottle. The

intent of preparing aerobic microcosms in the anaerobic chamber was to adjust the initial oxygen concentration in the headspace to 5% by subsequently adding pure oxygen (see below). The pH was adjusted from 4.6 to 7.0 using NaOH (9.2 M) and resazurin was added (1 mg/L). Resazurin is a colorimetric redox indicator that turns from pink to colorless below an E_h of -110 mV (Jacob, 1970)

Aerobic microcosms for evaluation CB biodegradation were prepared in triplicate 160 mL sterilized serum bottles with homogenized soil from the aerobic locations (20 g) and groundwater from PM-26 (100 mL). Another set was prepared with 1,2-DCB as the parent compound. After sealing the serum bottles with Teflon-faced butyl rubber septa, they were removed from the anaerobic chamber and the headspace of each bottle was sparged for 1 min with N_2, to remove H_2 present in the atmosphere of the anaerobic chamber. Then pure oxygen was added to the headspace to achieve an initial level of 5%, to favor the development of microaerotolerant bacteria. Considering that none of the target contaminants were present in the soil and groundwater samples used for this study, CB and 1,2-DCB were added to the microcosms using saturated solutions in DDI water to achieve the low target concentrations shown in Table 2.9. The amount added was gradually increased to the target maximum once biodegradation commenced. Neat compound was added in place of the water saturated solutions when the mass needed could be delivered in a volume in excess of 1 µL.

After allowing the headspace and liquid phases to equilibrate for one hour following the addition of CB or 1,2-DCB, a headspace sample (0.5 mL) from each triplicate bottle was analyzed by GC.

Water controls consisted of CB and 1,2-DCB at their low target concentration, plus a volume of glass beads that displaced the same volume of water as the soil added to the live microcosms. This ensured that the water controls had the same ratio of headspace to liquid as the microcosms. The water controls were prepared in the anaerobic chamber with sterilized serum bottles. When not being monitored, all of the serum bottles were placed in a horizontal position (liquid in contact with the septa) inside boxes and stored on an orbital shaker at room temperature.

2.3.4 Preparation of the Anaerobic Microcosms

The day prior to preparing the anaerobic microcosms, one of the 1L plastic bottles containing groundwater from PM-20 was moved from storage (4ºC) to the anaerobic chamber, allowed to warm overnight and poured into a 1 L media bottle. The pH was adjusted from 5.5 to 7.3 using NaOH (9.2 M) and resazurin was added (1 mg/L).

Two sets of triplicate anaerobic microcosms were prepared in 160 mL sterilized serum bottles with mixed soil from anaerobic locations (20 g) and groundwater from PM-26 (100 mL). After being capped with Teflon-faced butyl rubber septa, the bottles were removed from the anaerobic chamber and the headspace of each bottle was sparged for 1 min with N_2 to remove H_2 present in the atmosphere of the anaerobic chamber. Considering that none of the target contaminants were present in the soil and groundwater samples used for this study, 2,6-DNT was added to one set and and 4-NT to the other set, to achieve the low target concentration shown in Table 2.9.

Lactate was used as the electron donor to promote nitro group reduction, based on the ease with which it is fermented. The initial dose of lactate was stoichiometric with respect to reduction of the nitro groups, plus a safety factor of 10. Sodium lactate was added using a stock solution (112.8 mg/L). After incubating the microcosms for 1 h to allow the 2,6-DNT and 4-NT to equilibrate, a liquid sample (1 mL) was removed from each bottle and analyzed by HPLC.

Water controls consisted of 2,6-DNT and 4-NT at their low target concentration, plus a volume of glass beads that displaced the same volume of water as the soil added to the live microcosms. This ensured that the water controls had the same ratio of headspace to liquid as the microcosms. The water controls were prepared in the anaerobic chamber with sterilized serum bottles. When not being monitored, all of the serum bottles were placed in a horizontal position (liquid in contact with the septa) inside boxes and stored on an orbital shaker at room temperature.

2.4 Preparation of Enrichment Cultures

2.4.1 Aerobic Enrichment Culture

After biodegradation of the parent compounds started in the microcosms, they added several more times at increasing concentrations, so that a high rate of biodegradation was established. A transfer was then made to fresh groundwater along with addition of the parent compounds, in 5 L bottles. The day prior to preparing the enrichments, eight of the 1 L plastic bottles containing groundwater from PM-26 were moved from storage (4ºC) and allowed to warm overnight. The pH was adjusted from

5.2 to 7.4 for the CB enrichment, and from 4.9 to 6.8 for the 1,2-DCB enrichment. Resazurin was added (1 mg/L) to the pH-adjusted groundwater.

The contents of CB microcosm #1 and 1,2-DCB microcosm #2 (100 mL of groundwater and 20 g solids) were emptied separately in sterilized 5 L media bottles. The pH-adjusted groundwater was then added to a total volume of 3500 mL and the bottles were capped with a Teflon faced septa and a perforated plastic cap to facilitate headspace sampling. The headspace of each bottle was then sparged for 10 min with N_2 and then 5% of oxygen was added to the headspace, to favor the development of microaerotolerant bacteria.

CB (17 µL of neat compound) and 1,2-DCB (2.8 µL of neat compound) were added to achieve the target low concentrations listed in Table 1.1. After allowing 1 h for the headspace and liquid phases to equilibrate, a headspace sample (0.5 mL) was analyzed by GC. Water controls consisted of both CB and 1,2-DCB in DDI at the target high contaminant concentrations listed in Table 1.1. The water control was prepared on the bench top in a sterilized 5 L media bottle.

When not being monitored, the bottles were placed in a horizontal position (liquid in contact with the septa) inside boxes and stored on an orbital shaker, at room temperature.

After biodegradation started in the enrichments, the CB and 1,2-DCB were added repeatedly, so that a high rate of biodegradation was established. The contents of the enrichment were then used to evaluate the effect of other compounds on biodegradation of the parent compounds.

2.4.2 Anaerobic Enrichment Culture

After biodegradation of the parent compounds started in the microcosms, they were added several more times at increasing concentrations, so that a high rate of biodegradation was established. A transfer was then made to fresh groundwater along with addition of the parent compounds, in 5 L bottles. The day prior to preparing the enrichments, ten of the 1 L plastic bottles containing groundwater from PM-20 were moved from storage (4ºC) to the anaerobic chamber and allowed to warm overnight. The pH was adjusted from 4.3 to 7.2 for the 2,6-DNT enrichment, and from 5.4 to 7.2 for the 4-NT enrichment. Resazurin was added (1 mg/L) to the pH-adjusted groundwater.

The contents of 2,6-DNT microcosms #2 and #3, and 4-NT microcosms #1 and #2 were emptied into sterilized 5 L media bottles. pH-adjusted groundwater was then added to a total liquid volume of 4,000 mL. 2,6-DNT (24 mg of neat compound) was added to the 2,6-DNT enrichment, and 4-NT (13 mg of neat compound) was added to the 4-NT enrichment, in order to achieve the target low concentration listed in Table 1.1. The bottles were then capped with Teflon faced septa and a perforated plastic cap. Lactate was injected as an electron donor. After 1 h of preparation, a liquid sample (1.0 mL) was analyzed by HPLC.

Water controls consisted of 2,6-DNT and 4-NT in DDI water at the target high concentrations listed in Table 1.1. The water controls were prepared in the bench top in a sterilized 5 L media bottle. When not being monitored, all enrichments were placed in the anaerobic chamber with a stirring bar to facilitate mixing and dissolution of the solid neat compound, at room temperature.

After biodegradation started in the enrichments, 2,6-DNT and 4-NT were added repeatedly, so that a high rate of biodegradation was established. The contents of the enrichment were then used to evaluate the effect of other compounds on biodegradation of the parent compounds.

2.5 Inhibition Tests

2.5.1 Chlorobenzene Inhibition

The day prior to preparing the inhibition experiment, two of the 1 L plastic bottles containing groundwater from PM-26 and from PM-20 were moved from storage (4ºC) to the bench top and allowed to warm overnight. Equal amounts of groundwater from PM-26 (900 mL) and PM-20 (900 mL) were mixed. The pH was adjusted from 3.8 to 6.9 and resazurin was added (1 mg/L). Groundwater was then added to sterile 160 mL serum bottles in order to set up the high concentration treatments listed in Table 2.5 for the CB inhibition experiment.

The amount of groundwater used for each treatment was determined based on the quantity needed to reach a total volume of 100±1 mL per bottle, after accounting for addition of CB, co-contaminants or the chemical oxidation water, and the enrichment culture inoculum. Groundwater (10 mL) from the chemical oxidation test at stoichiometric dosing was then added to the CB + chemical oxidation treatment. This volume of chemical oxidation groundwater was selected in order to simulate dilution as source zone groundwater moves downgradient. Further assessment and modeling needs

to be conducted in order to determine the extent of dilution that is most likely to occur under field conditions.

All of the serum bottles were sealed with Teflon-faced butyl rubber septa. Then, the pH of the CB + chemical oxidation treatment was re-adjusted from 11.4 to 6.8 with HCl (0.5 N). The headspace was then flushed with N_2, except for the CB + chemical oxidation treatments, to avoid loss of VOCs still present in the chemical oxidation groundwater. Oxygen (13%) was then injected to the headspace, according to the stoichiometric required amount of oxygen needed for degradation of the CB.

CB (neat) and the co-contaminants (saturated solutions and stock solutions) were added according to Table 2.9 to reach the high concentrations listed in Table 1.1. One treatment received only the parent compound and thereby served as the positive control (CB Live Controls). Water controls consisted of each of the target compounds (CB, 2,4-DNT, 4-IPA, 1,4-dioxane and 1,2-DCA) at the highest concentrations listed in Table 1.1. The water controls were prepared on the bench top in sterilized 160 mL serum bottles containing 100 mL of DDI water.

After allowing the headspace and liquid phases to reach equilibrium for one hour, headspace samples (0.5 mL) were analyzed by GC and liquid samples (1 mL) from the DDI controls, CB + 2,4-DNT, CB + 4-IPA, CB + 1,4-dioxane and CB + chemical oxidation treatments were centrifuged and filtered (0.22 µm) prior to analysis by HPLC and GC. These date were used to establish the time zero conditions. The next day, all of the serum bottles (except the water controls) were inoculated with the enrichment culture (1% v/v). A low dose of inoculum was used because of the high rate of CB

biodegradation in the enrichment culture; higher doses would have resulted in CB biodegradation that was too fast to detect on a time scale of several days.

As the results will show, none of the co-contaminant mixtures at the highest concentration was inhibitory to biodegradation of CB. Consequently, experiments with lower doses were not performed.

2.5.2 1,2-Dichlorobenzene Inhibition

The protocol used to prepare the 1,2-DCB inhibition experiment was very similar to that used for CB, as described above. The day prior to preparing the experiment, two of the 1 L plastic bottles containing groundwater from PM-26 and from PM-20 were moved from storage (4º C) to the bench top and allowed to warm overnight. Equal amounts of groundwater from PM-26 (900 mL) and PM-20 (900 mL) were mixed. The pH was adjusted from 6.2 to 7.2 and resazurin was added (1 mg/L). Groundwater was then added to sterile 160 mL serum bottles in order to set up the treatments listed in Table 2.5 for the 1,2-DCB inhibition experiment.

The amount of groundwater used for each treatment was determined based on the quantity needed to reach a total volume of 100±1 mL per bottle, after accounting for addition of 1,2-DCB, co-contaminants or the chemical oxidation water, and the enrichment culture inoculum. Groundwater (10 mL) from the chemical oxidation test at stoichiometric dosing was then added to the 1,2-DCB + chemical oxidation treatment. The pH of the 1,2-DCB + chemical oxidation treatment was adjusted from 11.5 to 7.1 with HCl (0.5 N). The headspace of the bottles was supplied with 5% oxygen, which was

sufficient for stoichiometric degradation of the 1,2-DCB. 1,2-DCB (saturated solution) and the co-contaminants (saturated solutions and stock solutions) were injected according to Table 2.9 to reach the high concentrations listed in Table 1.1. One treatment received only 1,2-DCB and thereby served as the positive control.

Water controls consisted of each of the target compounds (1,2-DCB, 2,4-DNT, 4-IPA, 1,4-dioxane and 1,2-DCA) at the highest concentrations listed in Table 1.1. As with CB, a low dose of inoculum was used because of the high rate of 1,2-DCB biodegradation in the enrichment culture; higher doses would have resulted in 1,2-DCB biodegradation that was too fast to detect on a time scale of several days. BSM (1 mL) was also added to support growth.

As the results will show, the high concentration treatment with a mixture of 1,2-DCB and 4-IPA experienced inhibition of 1,2-DCB biodegradation. Consequently, another experiment was prepared using a lower dose of 4-IPA. The procedures used were the same as those described above for the higher concentrations, except that the low concentration of 4-IPA was used (Table 1.1).

2.5.3 2,6-Dinitrotoluene Inhibition

The day prior to preparing the inhibition experiment, one of the 1 L plastic bottles containing groundwater from PM-20 was moved from storage (4 ºC) to the bench top and allowed to warm overnight. The pH was adjusted from 5.9 to 6.9. Considering that the additions of 2,6-DNT, 2,4-DNT and 4-IPA involved volumes on the order of milliliters, the stock solutions were placed in the anaerobic chamber overnight with the cap loose to

allow facilitate deoxygenation, with the intent of minimizing impacts on the redox level of the inoculum. Groundwater was then added to sterile 160 mL serum bottles in order to set up the treatments listed in Table 2.5 for the 2,6-DNT high concentration inhibition experiment.

Since the rate of 2,6-DNT in the enrichment culture was considerably slower than the rate of CB and 1,2-DCB biodegradation in the aerobic enrichments, a much higher inoculum dose (78 mL) was used. The balance of liquid added to the serum bottles consisted of the contaminants, chemical oxidation groundwater, and groundwater, such that the total liquid volume was 100±1 mL per bottle. The pH of the serum bottles containing the chemical oxidation groundwater was adjusted from 12.3 to 6.9.

2,6-DNT and the co-contaminants were added according to Table 2.9 to reach the high concentrations listed in Table 1.1. One treatment received only the 2,6-DNT and thereby served as the positive control. Resazurin (1 mg/L) and lactate were added. Water controls consisted of the target compounds (2,6-DNT, 2,4-DNT, 4-IPA, 1,4-dioxane and 1,2-DCA) at the highest concentrations listed in Table 1.1. They were prepared in the anaerobic chamber. Sampling and analysis for VOCs and soluble compounds was performed as described above.

2.5.4 4-Nitrotoluene Inhibition

As the results will show, development of the 4-NT enrichment culture lagged behind development of the others. Consequently, time did not permit evaluation of the 4-NT treatments.

2.6 Analytical Procedures

2.6.1 VOCs and Oxygen

CB and 1,2-DCB were analyzed by injecting headspace samples (0.5 mL) onto a HP 6890 Series II Plus GC equipped with an RTX 5 column (30-m×0.53-mm×1.5-µm film; Restek Corp.) and flame ionization detector (Elango et al., 2010). The injector and detector temperatures were 250°C and 325°C, respectively. The oven temperature program was 50 °C for 4 min, rise at 10 °C per min to 80 °C, hold 10 min, rise at 10 °C per min to 150 °C, and hold for 1 min. Hydrogen (5 mL/min) was used as the carrier gas and nitrogen (30 mL/min) as the makeup gas. The retention times were 11.0 min for CB and 21.4 min for 1,2-DCB. Standard curves for CB and 1,2-DCB (referred to as GC-Method 1) are presented in Appendix C.

In order to decrease the time for headspace analysis during the inhibition experiments, CB, 1,2-DCB and 1,2-DCA were analyzed with a different method (GC-Method 2) on the same instrument described above. The injector and detector temperature were 225 °C and 250 °C, respectively. The oven temperature program was 80 °C for 2 min, rise at 12 °C per min to 110 °C, and hold for 4 min. Hydrogen (5.1 mL/min) was used as the carrier gas and nitrogen (60 mL/min) as the makeup gas. The retention times were 1.8 min for 1,2-DCA, 4.0 min for CB and 8.0 min for 1,2-DCB. Standard curves for GC-Method 2 are presented in Appendix C. The detection limits for CB, 1,2-DCB and 1,2-DCA using this method were approximately 0.12, 0.42 and 1.0 mg/L, respectively.

GC Method 3 was used for analysis of 1,2-DCA and CB during the anaerobic inhibition experiments, given limited availability of the GC used for Methods 1 and 2. Headspace samples (0.5 mL) were injected onto an HP 5890 Series II GC equipped with a 60/80 Carbopak B column (Supelco) and flame ionization detector, using an isothermal program at 200º C. Nitrogen (30 mL/min) was used as the carrier gas. The detector and injector temperatures were 200 °C. The retention times were 2 min for 1,2-DCA and 14.1 min for CB. The standard curves for 1,2-DCA and CB are presented in Appendix C. The detection limits for CB and 1,2-DCA using this method were approximately 0.50 and 0.11 mg/L, respectively.

Assuming the headspace and aqueous phases were in equilibrium, the aqueous phase concentration of VOCs was determined based on the total mass in the bottle and the distribution between the headspace and liquid according to Henry's Law:

$$C_l\,(mg/L) = \frac{V_{stock} \times C_{stock} \times M_l}{V_l} \quad (1)$$

$$M_l = \frac{V_l}{V_l + V_g * H} \quad (2)$$

where C_l = concentration in the aqueous phase (mg/L); V_{stock} = volume of stock solution added to a standard (mL); C_{stock} = concentration of contaminant in a stock solution (mg/mL); M_l = fraction of contaminant in the liquid phase based on Henry's Law; V_l = volume of the liquid in the bottle (L); V_g = volume of the headspace in the bottle (L); and

H = Henry's constant (dimensionless) at 23 °C. Table 2.11 lists the values for the Henry's constants and the values for M_l when V_l = 0.1 L and V_g = 0.06 L.

Oxygen was analyzed by injecting a headspace sample (0.5 mL) onto a GC (HP 5890 Series II) equipped with thermal conductivity detector and molecular sieve 5A 60/80 column (1.8 m×3.1 mm; Alltech) (Elango et al., 2010). The detector, oven, and injector temperatures were set at 120, 70 and 120 °C, respectively. Nitrogen (30 mL/min) was used as the reference gas and carrier gas. The elution time for oxygen was 3.4 min. Room air was used to develop a response factor (i.e., percent oxygen per GC peak area unit) before every monitoring event. Since the detector response is linear over the range that was tested (i.e., 0-21% O_2), a one point calibration was considered acceptable. Response factors are presented in Appendix C.

2.6.2 1,4-Dioxane

1,4-Dioxane was monitored by GC analysis of filtered aqueous samples (0.2 µm) on a Hewlett Packard 5890 Series II gas chromatograph, equipped with a flame ionization detector and a 60-m x 0.32-mm ZB-624 capillary column (Phenomenex). Hydrogen was delivered at 1.75 mL/min as the carrier gas. The temperature program was 40 ºC for 5 min, then increased to 90 ºC at 6.0 ºC/min and held for 5 min. The injector and detector temperatures were set at 180 ºC and 260 ºC, respectively. The retention time was 10.2 min. The standard curve for 1,4-dioxane is presented in Appendix C and is referred to as GC-Method 4. The detection limit for 1,4-dioxane using this method was approximately 0.54 mg/L.

2.6.3 HPLC Methods

2,4-DNT, 2,6-DNT, 4-NT, their daughter products (2,6-DAT, 2,4-DAT, 4-AT, 2A6NT, 2N4AT and 2A4NT) and 4-IPA were analyzed by HPLC with a Dionex 3000 Ultimate series equipped with a Kinetex® 5 µm EVO C18 LC column (molecular sieve 100A, 250 mm x 4.6 mm) and a multiple wavelength UV detector. For the analysis of 2,4-DNT, 2,6-DNT, 4-NT and 4-IPA, when only one of the dinitrotoluene isomers was present, the eluant consisted of DDI (45%) and methanol (55%), using an isocratic program at a flow rate of 1 mL/min and a constant temperature (30 ºC). This is referred to as HPLC-Method 1. The retention times were 6.1 min for 4-IPA (240 nm), 7.0 min for 2,4-DNT (250 nm), 7.0 min for 2,6-DNT (240 nm) and 8.3 min for 4-NT (268 nm). A typical column pressure was 2700 psi. Standard curves for HPLC-Method 1 are presented in Appendix C. The detection limits for 4-IPA, 2,4-DNT , 2,6-DNT and 4-NT using this method were approximately 0.04, 0.02, 0.06 and 0.08 mg/L, respectively. It was not possible to use HPLC-Method 1 when both dinitrotoluene isomers were present because they co-eluted.

HPLC-Method 2 was used when 2,4-DNT and 2,6-DNT were present and for the analysis of daughter products. The eluant consisted of DDI (65%) and methanol (35%), using an isocratic program at a flow rate of 1 mL/min and a constant temperature (30 ºC). The retention times were 2.7 min for 2,6-DAT (240 nm), 3.0 min for 2,4-DAT (240 nm), 7.6 min for 4-AT (240 nm), 9.6 min for 2A6NT (240 nm), 10.6 min for 2N4AT (240 nm), 11.4 min for 2A4NT (240 nm), 22.7 min for 2,6-DNT (240 nm) and 23.3 min for 2,4-DNT (250 nm). A typical column pressure was 2700 psi. Standard curves for HPLC-

Method 2 are presented in Appendix C. The detection limits for 2,4-DNT and 2,6-DNT using this method were approximately 0.01 and 0.04 mg/L, respectively. For the metabolites, the approximate detection limits were 0.48 mg/L for 2,6-DAT, 0.49 mg/L for 2,4-DAT, 0.50 mg/L for 4-AT, 0.50 mg/L for 2A6NT, 0.53 mg/L for 2N4AT, and 0.22 mg/L for 2A4NT.

For all samples except the water controls, it was necessary to allow solids to settle before removing 1 mL of the liquid phase, which were prepared for HPLC analysis by filtering (pore size 0.2 μm, PTFE, VWR International). The first 0.5 mL was discarded. The next 0.3 mL was discharged into HPLC sample vials (2 mL, borosilicate, VWR) containing an insert (0.5 mL, borosilicate, VWR). For HPLC-Methods 1 and 2, the injection volume was 100 μL.

A test was performed in order to confirm that the target compounds analyzed in this study were not adsorbed by the filter (PTFE) used for preparation of liquid samples. Stock solutions with known concentrations of 2,4-DNT, 2,6-DNT, 4-NT, 4-IPA, 2,4-DAT, 2,6-DAT, 4-AT, 2A6NT, 2N4AT and 2A4NT were prepared for HPLC analysis. A set of triplicates was filtered, and a second set of triplicates was not filtered. Both sets were analyzed by HPLC and compared. The results are presented in Appendix C. Based on a Student's *t*-test, the concentration of analytes in filtered and non-filtered samples was not statistically different ($\alpha = 0.05$). This indicated that there was no adsorptive loss to the PTFE filters.

2.6.4 Other Methods

COD was measured in 5 mL vials according the manufacturer's instructions (Bioscience Accu-TEST low range, 5-150 mg/L). A protocol is given in Appendix D, including a calibration curve.

Total bacterial 16S rRNA gene copies were quantified in the enrichment cultures by qPCR using universal primers. Samples from each enrichment culture (50 mL) were centrifuged (4º C, 4000x*g*, 10 min, Eppendorf centrifuge 5804R). The liquid was discarded and DNA was extracted from the pellets using a PowerSoil® DNA Isolation Kit (Catalog #12888-50) following the manufacturer's protocol (MO BIO Laboratories). The qPCR reaction mix was prepared as shown in Appendix E.

CHAPTER THREE

3.0 RESULTS

3.1 Chemical Oxidation Laboratory Scale Test

Chemical oxidation was tested at different oxidant and activator doses in order to evaluate the percent removal of contaminants and produce groundwater for use in the inhibition experiments. As evidenced in Figures 3.1, 3.2 and 3.3, there was a modest decrease in the parent compounds in the no-persulfate controls, most likely due to adsorption or diffusive losses. The average decreases over the 26 day incubation period were 5.8% for 1,2-DCB, 19 % for 4-NT, 8.6% for 4-IPA, 21% for 2,6-DNT and 35% for 2,4-DNT.

Table 3.1 summarizes the extent of contaminant removal for different oxidant and activator doses after 26 days of monitoring. As expected, the extent of removal increased with dose, except for CB and 1,2-DCB at 4.2X stoichiometric, which resulted in somewhat lower removal compared to 3.4X stoichiometric. 1,4-Dioxane was completely removed at all doses, while 1,2-DCA, 4-IPA and the nitrotoluenes were completely removed only at the higher doses; CB and 1,2-DCB persisted even at the higher doses. Considering that the stoichiometric treatment achieved contaminant removals ≥50%, and that 28 g persulfate per g contaminant is somewhat higher than what is typically used in practice (20 g of persulfate per g of contaminant), this dose was selected for use in the inhibition experiments.

To assess the extent of oxidation, the COD of the groundwater was measured; details are presented in Appendix D. The average COD of the groundwater without

contaminants added was 14 mg/L. The calculated COD of the contaminants added was 133 mg/L, resulting in an initial COD with the contaminants present of 147 mg/L. In the stoichiometric treatment, the COD of the groundwater 26 days after exposure to chemical oxidation was 148 mg/L. The COD of the remaining contaminants (i.e., all of the compounds except 1,4-dioxane and 2,4-DNT, which were completely removed; Table 3.1) was calculated to be 51.5 mg/L, indicating that 96.5 mg/L of the remaining COD was attributable to incomplete oxidation products. The composition of these products was not evaluated.

3.2 Chlorobenzene Microcosms

Figure 3.4 summarizes the water control results used for comparison to the aerobic microcosms. At the conclusion of the evaluation period (65 days) there was no net decrease in CB, indicating there were no losses through the septa or due to adsorption.

Results for the triplicate CB microcosms are presented in Figure 3.5. Biodegradation started after 22 days. When the CB was completely consumed, the bottles were re-spiked with CB saturated solution (Table 2.10) to reach the target low concentrations (Table 1.1). The amount of CB added was increased until the target maximum concentration was reached (35 mg/L). The rate of biodegradation subsequently decreased. At all times the headspace oxygen concentration was maintained at ~5%, so that it was not the limiting factor. However, the pH level had decreased to 5.8 to 6.9. After re-adjusting the pH (9.2 M NaOH) to circumneutral conditions, the rate of CB biodegradation did not respond. To address a potential limitation in nutrients, 5 mL of BSM was added and CB biodegradation resumed at a high rate. On day 67, the contents

of microcosm #1 was used to prepare the enrichment culture; #2 and #3 were stored at 4 °C.

3.3 Chlorobenzene Enrichment

Figure 3.6 shows the results for the single 5 L water control bottle, used for comparison to the CB enrichment bottle. At the conclusion of the evaluation period (53 days) CB decreased by 25%, indicating losses did occur through the septum or via adsorption. Nevertheless, the magnitude of this loss was minor compared to the biodegradation activity that developed in the enrichment culture (Fig. 3.7). Biodegradation of the first dose of CB was complete by day 4. Several doses of CB (17 μL neat) were consumed at the target low concentration (Table 1.1) and then the amount added was gradually increased to the target maximum (121 μL). The rate of biodegradation decreased between days 25 and 30; pH adjustment (6.0 to 6.7) and addition of BSM (100 mL) restored the high rate of CB biodegradation. At all times oxygen concentration in the headspace was maintained at ~8% so that it was never limiting. To maintain the high rate of biodegradation, each addition of CB beyond day 31 was accompanied by sodium bicarbonate (1.1 mL, 1 M) and BSM (10 mL). The need for alkalinity is a consequence of HCl release during biodegradation, while the need for BSM reflects the use of CB as a growth substrate. A routine respiking schedule was implemented, consisting of Monday, Wednesday and Friday.

On day 78, the enrichment culture was mistakenly fed with 21 μL of 1,2-DCB. The measured concentration of 1,2-DCB after its addition was 4.1 mg/L. In 24 hours, the

1,2-DCB was completely consumed by the CB enrichment, indicating that it is capable of degrading both chlorinated compounds.

Figure 3.7b compares the average concentration of CB measured by GC (34.5 mg/L) to the expected concentration (35.0 mg/L) based on the amount of neat CB added. Maximum, minimum and quartile values are also shown. There was good agreement between the measured and expected concentrations of CB.

DNA was extracted from duplicate samples of the CB enrichment culture in order to estimate the total Bacterial 16S rRNA gene copies by qPCR; each sample was analyzed in triplicate. The average Bacterial 16S rRNA gene copies obtained from sample 1 was $9.6X10^{13}$ copies/mL (standard deviation = $1.3x10^{10}$), and for sample 2 it was $2.2X10^{13}$ copies/mL (standard deviation = $1.5X10^{12}$).

3.4 Chlorobenzene High Concentration Inhibition Experiment #1

Figure 3.8 summarizes the water control results for the first CB inhibition test. There was no appreciable loss of CB, 4-IPA, 2,4-DNT, 1,2-DCA, or 1,4-dioxane, indicating that the serum bottles successfully retained these compounds. Results for the treatments with the enrichment culture are shown in Figure 3.9. Using a 1% inoculum, CB was completely consumed in all treatments after 6 days of incubation, with the exception of the CB + 2,4-DNT and CB + ChemOx treatments. In the CB + 2,4-DNT set, bottles #1 and #2 consumed the CB in 6 days; bottle #3 finished in 8 days. In the CB + ChemOx set, the average concentration of CB was 34.1 mg/L after 40 days of incubation, indicating that a 10% (v/v) addition of groundwater from the stoichiometric chemical oxidation bottles was inhibitory.

Figure 3.10a shows how fast CB was degraded in the CB + 2,4-DNT treatment in comparison with the CB-only treatment. As mentioned above, consumption of CB in bottle #3 of the CB + 2,4-DNT set took 2 additional days to complete. Only a minor decrease in 2,4-DNT occurred (7.8%) over a similar incubation time. Similar results were obtained with the 4-IPA treatment (Fig. 3.10b), although with more uniformity in the CB results among the triplicates. 4-IPA decreased by only 12.7%.

From Figure 3.11, it is apparent that the presence of 1,4-dioxane and 1,2-DCA did not inhibit CB biodegradation in comparison to the CB-only treatment. There was also a minor amount or no consumption of the 1,4-dioxane and 1,2-DCA, respectively. In contrast, groundwater from the chemical oxidation bottles resulted in significant inhibition of CB biodegradation (Fig. 3.12). The 1,2-DCB, 4-NT, and 2,6-DNT that were present in the chemical oxidation groundwater also persisted over the first 6 days of incubation, but were not subsequently analyzed. Since these compounds were not tested individually for their potential to inhibit CB biodegradation, it is possible that their presence contributed to the inhibition of CB degradation observed in the chemical oxidation treatment. Another factor potentially contributing to CB inhibition in the ChemOx bottles was a high pH level, which averaged 11.3 at the end of the incubation period. This was a consequence of using alkaline activated persulfate for chemical oxidation.

The results obtained from CB inhibition experiment #1 were considered incomplete since, in most of treatments, all or nearly all of the CB was consumed by the second sampling event. A second experiment was therefore performed with the intent of

taking more measurement of CB over time. In addition, to rule out the effect of pH in the chemical oxidation treatment, the pH was adjusted prior to adding the enrichment culture.

3.5 Chlorobenzene High Concentration Inhibition Experiment #2

Figure 3.13 summarizes the water control results for CB inhibition experiment #2. There was no appreciable loss of CB, 4-IPA, 2,4-DNT, 1,2-DCA, or 1,4-dioxane, indicating that the serum bottles successfully retained these compounds. Throughout the evaluation period (101 days) the concentrations of CB, 2,4-DNT, 4-IPA, and 1,2-DCA decreased (15-39%), indicating losses via diffusion and/or adsorption. As will be shown below, these losses were minor in comparison to the amounts that have been biodegraded. There was no decrease the concentration of 1,4-dioxane.

The overall results for CB are presented in Figure 3.14a. Using a 1% inoculum, CB was completely consumed in all treatments after 7 days of incubation, with the exception of the CB + ChemOx treatment. It is important to note that after day 3, CB consumption stalled. Oxygen was ruled out as a factor, as was pH, which averaged 6.3 across all of the treatments. BSM (1 mL) was added to all bottles on day 5; the rapid resumption of CB biodegradation indicates that nutrient limitation was the main reason for the stall in CB biodegradation. The response of the CB-only bottles is shown in Figure 3.14b; CB consumption was complete by day 7.

Based on the results in Figure 3.15, it is apparent that 2,4-DNT did not inhibit CB biodegradation, in comparison to the CB-only treatment. On the time scale shown, there was on a minor level of decrease in the 2,4-DNT (5.1%). In contrast, in one of the three bottles in the treatment with CB and 4-IPA, the presence of 4-IPA significantly slowed

the rate of CB biodegradation (Fig. 3.16a). Nevertheless, the inhibition was transient, since biodegradation of CB was complete in bottle #1 by day 12. Over this interval, 4-IPA decreased ~20%.

The presence of 1,4-dioxane was not inhibitory to CB biodegradation (Fig. 3.17), although 1,4-dioxane persisted over the incubation period. Similar results were obtained for the treatment with 1,2-DCA, which also did not undergo degradation (Fig. 3.18).

The inhibition of CB biodegradation caused by the addition of groundwater from the chemical oxidation treatment is clearly revealed in Figure 3.19 for each bottle. The inhibition delayed consumption of CB by only 3 days in bottles #1 and #2 (Fig. 3.19a, b). For reasons that are not known, CB degradation was more inhibited in chemical oxidation bottle #3 (Fig. 3.19c). On day 28, the bottle was reinoculated with 1 mL of the CB enrichment culture and CB biodegradation resumed thereafter until consumption was complete by day 33. During the incubation period for each bottle (Fig. 3.19), the 4-NT and 2,4-DNT that came with the chemical oxidation water did not undergo any significant degradation, in spite of the significant level of aerobic degradation of CB. In this experiment, pH did not contribute to the inhibition of CB biodegradation, since it was adjusted and maintained in the circumneutral range.

Although the decreases in 2,4-DNT (Fig. 3.15) and 4-IPA (Fig. 3.16) were modest, they were not zero, as was the case for 1,4-dioxane and 1,2-DCA. The potential that CB was serving as a primary substrate for cometabolism of 2,4-DNT and 4-IPA was explored by continuing to monitor these bottles and making repeated additions of CB. Results are shown in Figure 3.20 (an extension of Fig. 3.15 and 3.16). 2,4-DNT and 4-

IPA steadily decreased as more and more CB was consumed. More of both compounds was added on day 105 without CB; if their biodegradation is cometabolic, then their rate of consumption should gradually decrease and then stop in the absence of CB. Additional monitoring is needed to confirm this.

The same approach was used with the treatments that contained 1,4-dioxane and 1,2-DCA (Fig. 3.21; an extension of Fig. 3.17 and 3.18). In spite of repeated consumption of CB in all bottles, there was no decrease in 1,4-dioxane and only a modest decrease in 1,2-DCA (~28%), possibly exacerbated by repeated removal of headspace samples. Figure 3.22 presents the average amount of CB, 2,4-DNT, 4-IPA, 1,4-dioxane, and 1,2-DCA consumed, both in terms of cumulative concentration and percent removal. These results suggest that CB serves as a primary substrate for cometabolism of 2,4-DNT and 4-IPA, but not 1,4-dioxane or 1,2-DCA.

3.6 1,2-Dichlorobenzene Microcosms

The water controls for the 1,2-DCB microcosms are the same bottles that were used as water controls for CB (Fig. 3.4). Throughout the evaluation period (70 days) there was no net decrease in the concentration of 1,2-DCB, indicating there were no losses via diffusion or adsorption.

Results for the 1,2-DCB microcosms are presented in Figure 3.23. Biodegradation of the first dose was complete by day 16-30, whereupon more 1,2-DCB was added. The amount added was increased to the maximum dose. On day 24, bottle #2 was sacrificed to start the 1,2-DCB enrichment; the others were stored (4 °C) after a high rate of 1,2-

DCB biodegradation was demonstrated. Their slower response may have been due to low pH or limited nutrients; however, neither was evaluated in the 1,2-DCB microcosms.

3.7 1,2-Dichlorobenzene Enrichment

Figure 3.6 summarizes the water control results for the 5 L bottle containing CB and 1,2-DCB. There was not net decrease in 1,2-DCB, indicating there were no losses via diffusion or adsorption.

The results obtained during development of the 1,2-DCB enrichment culture are presented in Figure 3.24a. The first does was consumed within 5 days. The dose was increased to achieve the maximum target concentration, but the rate of biodegradation slowed. At all times the oxygen concentration in the headspace was maintained at concentrations of ~5%, hence oxygen was not expected to be limiting. BSM was added on day 20 (100 mL) and this restored the higher rate of biodegradation. The pH was circumneutral (7.1), so there was no need for adjustment. On average, 7.5 mg/L of 1,2-DCB was consumed in 1-2 days. However, the rate of degradation slowed again after day 49, so another addition of BSM was made. Thereafter, each addition of 1,2-DCB was accompanied by 10 mL of BSM (to provide nutrients on an on-going basis), 0.4 mL of $NaHCO_3$ (1 M) to stochiometrically neutralize the HCl released from each dose, and 28 mL of pure O_2. With these changes, it was possible to maintain a routine respiking and feeding schedule (Monday, Wednesday and Friday), to provide more consistent behavior prior to using the culture for the inhibition experiments.

On day 75, the enrichment was mistakenly fed with 21 μL of CB. The measured concentration of CB after its addition was 8.9 mg/L. In 24 hours, the CB was completely

consumed by the 1,2-DCB enrichment, indicating that it is capable of degrading both chlorinated compounds. As mentioned above, the same phenomenon was observed with the CB enrichment culture, i.e., it is capable of degrading 1,2-DCB.

Figure 3.24b compares the concentrations of 1,2-DCB measured by GC to the expected concentrations based on the mass of 1,2-DCB added. The average measured concentration (5.8 mg/L) was below the expected concentration (7.5 mg/L), although it was close to the measured third quartile. The difference between the measured and expected values may have been a consequence of inadequate equilibration between the headspace and liquid phases, even though the bottle was shaken for one hour after adding the 1,2-DCB. It is also possible that there was immediate update or adsorption of the 1,2-DCB onto cells.

DNA was extracted from duplicate samples of the 1,2-DCB enrichment culture in order to estimate the total Bacterial 16S rRNA gene copies by qPCR; each sample was analyzed in triplicate. The average Bacterial 16S rRNA gene copies obtained from sample 1 was $3.X10^{14}$ copies/mL (standard deviation = $8.5x10^{12}$), and for sample 2 it was $3.5X10^{14}$ copies/mL (standard deviation = $1.7X10^{13}$).

3.8 1,2-Dichlorobenzene High Concentration Inhibition Experiment

Water controls for the 1,2-DCB high concentration inhibition experiments are presented in Figure 3.25. Throughout the evaluation period (82 days), 1,2-DCB decreased by 34.6%, 2,4-DNT by 6.8%, and 4-IPA by 35% (Fig. 3.25a). 1,4-Dioxane and 1,2-DCA were monitored over a shorter time period (25 days) and decreased by 0% and 4%,

respectively. As will be shown below these abiotic losses were minor in comparison to the amounts that were biodegraded.

Overall results for the high concentration inhibition experiment are presented in Figure 3.26. Using a 1% inoculum, 1,2-DCB was completely consumed in all treatments after 6 days of incubation, with the exception of the 1,2-DCB + 4-IPA and 1,2-DCB + ChemOx treatments. To avoid a nutrient limitation, 1 mL of BSM was injected along with the inoculum. The large error bars for the treatment with 4-IPA are a consequence of considerably slower biodegradation of 1,2-DCB in one of the triplicate bottles.

Results for each treatment are presented in Figures 3.27-3.32 covering the time frame for consumption of the first addition of 1,2-DCB. Figure 3.27 shows the behavior of the 1,2-DCB-only bottles. Bottles #1 and #2 degraded 1,2-DCB in 6 and 3 days, respectively; however, biodegradation in bottle #3 lagged considerably and a second addition of inoculum on day 32 was required to reestablish activity. The reason for this behavior is not known. As will be shown below, it appears to be an outlier; when the 1,2-DCB-only treatment was repeated in another experiment, all three bottles consumed 1,2-DCB at a high and similar rate.

Figure 3.28 compares the rate of 1,2-DCB biodegradation in the treatment with 2,4-DNT added to the average of the two high rate bottles with 1,2-DCB-only. The presence of 2,4-DNT had no discernable effect on the rate of 1,2-DCB biodegradation in any of the bottles. Over the 6 day incubation period, there was no apparent change in the concentration of 2,4-DNT in comparison to the water controls.

Figure 3.29 compares the rate of 1,2-DCB biodegradation in the treatment with 4-IPA added to the average of the two high rate bottles with 1,2-DCB-only. The presence of 4-IPA substantially slowed the rate of 1,2-DCB biodegradation in two of the three bottles, with a less pronounced impact in the third. BSM (1 mL) was added to bottles #1 and #3 on day 6; while this seems to have a stimulatory impact in bottle #3, 1,2-DCB continued to persist in bottle #1 until day 28; however, there was an indication of another stall on day 33, so a second dose of inoculum was added. The 1,2-DCB was subsequently consumed to completion. Over the time period when 1,2-DCB was consumed, there was a notable decrease of 4-IPA in all three bottles in comparison to the water controls, suggesting that biodegradation of 1,2-DCB facilitated biodegradation of 4-IPA.

Figures 3.30 and 3.31 compare the rate of 1,2-DCB biodegradation in the treatment with 1,4-dioxane and 1,2-DCA added, respectively, to the average of the two high rate bottles with 1,2-DCB-only. While there was some bottle-to-bottle variability, the presence of 1,4-dioxane and 1,2-DCA did not have a discernable effect on the rate of 1,2-DCB biodegradation. Over the 6 to 7 day incubation period, there was no apparent change in the concentration of 1,4-dioxane and 1,2-DCA in comparison to the water controls.

Groundwater from the simulated chemical oxidation treatment had a significant inhibitory impact on biodegradation of 1,2-DCB, increasing the time required for complete removal by 10-17 days in comparison to the average of bottles #1 and #2 from the 1,2-DCB-only treatment (Fig. 3.32). pH was not a contributor to the inhibition, since

for this experiment the pH of the chemical oxidation groundwater was adjusted to circumneutral. 2,6-DNT, 4-NT and CB are also shown in Figure 3.32, since they were not completely removed by the stoichiometric dose of persulfate and were therefore present in the chemical oxidation groundwater (Table 3.1). Like 2,4-DNT, 2,6-DNT was refractory over the 38 day incubation time. CB was biodegraded in parallel with 1,2-DCB and 4-NT was also consumed, although at a slower rate.

The potential that 1,2-DCB served as a primary substrate for cometabolism of 2,4-DNT and 4-IPA was explored by continuing to monitor these bottles and making repeated additions of 1,2-DCB. Results are shown in Figure 3.33 (an extension of Fig. 3.28) for 1,2-DCB and 2,4-DNT. Each addition of 1,2-DCB (2.0 μL) was accompanied by oxygen (5 mL), BSM (1 mL) and $NaHCO_3$ (36 μL), to ensure that oxygen, nutrients, and pH did not limit the rate of 1,2-DCB biodegradation. The gap in time between when the first dose of 1,2-DCB was consumed and when the second dose was added led to a lag in recovery of biodegradation activity on 1,2-DCB. Once that activity resumed, repeat additions were consumed and were accompanied by a discernable decrease in 2,4-DNT in the triplicate bottles in comparison to the water controls (Fig. 3.34a). The rate of 1,2-DCB consumption in the bottles with 2,4-DNT was very similar to the bottles with only 1,2-DCB. These results suggest that 1,2-DCB serves as a primary substrate for cometabolism of 2,4-DNT; additional monitoring is required to confirm this.

Results for 4-IPA are presented in Figure 3.34 (an extension of Fig. 3.29); the outcome was similar, i.e., repeated biodegradation of 1,2-DCB appeared to promote

cometabolism of 4-IPA, although additional monitoring is required to confirm this. The decrease in 4-IPA was higher than abiotic losses from the water controls (Fig. 3.34b).

Figure 3.35 summarizes the average amount of 1,2-DCB, 2,4-DNT, and 4-IPA, consumed, both in terms of cumulative concentration and percent removal. These results reinforce the presumption that 1,2-DCB serves as a primary substrate for cometabolism of 2,4-DNT and 4-IPA. The amount of 2,4-DNT and 4-IPA consumed in response to repeated consumption of 1,2-DCB was considerably greater than the abiotic loss of 1,2-DCB in the water controls.

3.9 1,2-Dichlorobenzene + 4-IPA Low Concentration Inhibition Experiment

Since the high target concentration of 4-IPA slowed the rate of 1,2-DCB biodegradation (Fig. 3.29), a second experiment was performed at the lower target concentration (Table 1.1). Water controls are shown in Figure 3.36 and the average results for inoculated bottles are shown in Figure 3.37. BSM (1 mL) was added along with the initial dose of 1,2-DCB to prevent a nutrient limitation. At the lower target concentration of 4-IPA, the co-contaminant did not exert an inhibitory effect; instead, the presence of a low concentration of 4-IPA somewhat increased the rate of 1,2-DCB biodegradation.

In contrast to the first experiment (Fig. 3.27), the triplicate 1,2-DCB-only bottles consumed the parent compound at a uniformly rapid rate (4 to 5 days). Figure 3.38 indicates that the individual bottles with 4-IPA present consumed the 1,2-DCB at a slightly faster rate. Over the four days of incubation, there was no decrease in the 4-IPA.

Figure 3.39 compares the rate of 1,2-DCB biodegradation in bottles used for the high and low 4-IPA concentration experiments with the bottles that received only 1,2-DCB. While bottle #3 in the first experiment took 52 days to finish consuming the 1,2-DCB, 3 to 6 days were required in all others. Thus, in 5 of the 6 1,2-DCB –only bottles evaluated, the parent compound was consumed at a high rate, suggesting that bottle #3 from the first experiment was an outlier.

3.10 2,6-Dinitrotoluene Microcosms

Anaerobic microcosms were prepared with 2,6-DNT and 4-NT. Results for the corresponding water controls are shown in Figure 3.40. There was no abiotic loss of these compounds over the 160 days of incubation. Results for the 2,6-DNT microcosms are presented in Figure 3.41. It took several weeks before redox conditions were sufficiently low to cause the resazurin to turn clear. Additional lactate (33 µL of a 113 mg/L stock solution) was added on days 9 and 20, but the bottles remained pink. Consequently, sulfate was added (200 µL of a 107 mg/L stock solution) on day 38 and AASM (5 mL) was added on day 58, to insure that nutrients were not limiting. Between days 64 and 91, the resazurin turned from pink to clear and consumption of 2,6-DNT was complete shortly thereafter. Repeated additions of 2,6-DNT were consumed at a higher rate, even as the concentration was increased to the target maximum (Table 1.1). On day 174, bottles #2 and #3 were sacrificed to start the 2,6-DNT enrichment. Bottle #1 was placed in storage at 4 °C.

3.11 2,6-Dinitrotoluene Enrichment

Results for the 2,6-DNT and 4-NT water control in a 5 L bottle are shown in Figure 3.42. There was no abiotic loss of these compounds over the 82 days of incubation. Results for the 2,6-DNT enrichment culture are presented in Figure 3.43a. Biodegradation of the first dose was completed by day 23. The second dose increased the concentration to the target maximum (Table 1.1). The measured concentrations for the first 6 re-spikes were well below what was expected based on the mass of neat compound added. This was likely a consequence of the low solubility of 2,6-DNT and the slow rate at which the crystals dissolved. Adsorption to biomass may have also been a factor. By day 66, there was closer agreement between the measured and expected concentrations of 2,6-DNT.

Figure 3.43b compares the concentrations of 2,6-DNT measured by HPLC to the expected concentrations based on the mass of 2,6-DNT added. The average measured concentration (3.5 mg/L) was less than one third of the expected concentration (11 mg/L); even the maximum measured concentration was below the expected. The difference between the measured and expected values may have been a consequence of inadequate dissolution of the 2,6-DNT crystals, even though the bottle was shaken for one hour before taking samples.

Consumption of 2,6-DNT was accompanied by an increase in 2,6-DAT and 2A6NT (Fig. 3.44). The molar sum of these products was expected to equal the molar amount of 2,6-DNT; additional work is needed to reconcile the mass balance. Regardless, the appreciable accumulation of predominantly 2,6-DAT in the enrichment

culture meant that this daughter product was also present in the bottles used to perform the inhibition experiment.

One notable feature of the enrichment culture is that a foamy black layer developed over time. Based on the color and the establishment of low redox conditions in the enrichment, this material was likely iron sulfides.

3.12 2,6-Dinitrotoluene High Concentration Inhibition Experiment

Results for the water controls prepared for the high concentration inhibition experiment are shown in Figure 3.45. There was no appreciable loss of the contaminants over the 32 days of monitoring, indicating that abiotic losses were minor.

Figure 3.46a summarizes the results for the inhibition experiment with the 2,6-DNT enrichment culture. Note that each bottle received 78 mL of the enrichment culture, a much higher inoculum level than the aerobic inhibition experiments. The higher volume of inoculum was needed to ensure that 2,6-DNT consumption occurred within several weeks of monitoring. All of the treatments consumed most of the 2,6-DNT by day 32. However, the treatments with 4-IPA and 2,4-DNT were noticeably faster between days 7 and 23 compared to the bottles that received only 2,6-DNT. The treatments with 1,4-dioxane and 1,2-DCA had somewhat slower rates of 2,6-DNT consumption, while the treatment with the chemical oxidation groundwater had the lowest rate of consumption, indicative of some type of inhibition. 2A6NT increased as 2,6-DNT was consumed in all of the treatments (Fig. 3.46b), with no detectable increase in 2,6-DAT (Fig. 3.46c). This is in contrast to the behavior of the enrichment culture, in which 2,6-DAT was the predominant daughter product. This may be a consequence of

the fact that during preparation of the experiment, the color of the liquid in the bottles turned pink, indicative of an increase in the redox level. Even though the bottles were prepared in the anaerobic chamber, the addition of stock solutions and groundwater likely resulted in the elevated redox level. Stronger reducing conditions are needed to reduce 2A6NT to 2,6-DAT. By day 23, the color was a much lighter pink and by the end of the incubation, all of the bottles had turned clear, except for bottle #1 of the 2,6-DNT + 1,2-DCA treatment, which had the slowest rate of 2,6-DNT biodegradation rate in this set. It is likely that additional incubation would have resulted in further nitro group reduction.

Results for the individual bottles within each treatment are shown in Figures 3.47 through 3.51. As mentioned for the overall summary, the presence of 2,4-DNT (Fig. 3.47) and 4-IPA (Fig. 3.48) modestly increased the rate of 2,6-DNT degradation in comparison to the treatment that received only 2,6-DNT. In addition, the 2,4-DNT was also consumed. However, there was no significant change in 4-IPA for the duration of the experiment.

The inhibitory effect of 1,4-dioxane is apparent in two of the three bottles tested, with no consistent change in the concentration of 1,4-dioxane (Fig. 3.49). The presence of 1,2-DCA also had a modest inhibitory effect on the rate of 2,6-DNT reduction, although the concentration in this treatment and in the 2,6-DNT-only bottles converged in two of the three bottles towards the end of the incubation period (Fig. 3.50). The concentration of 1,2-DCA did not change during the experiment. The effect of the chemical oxidation treatment on the rate of 2,6-DNT consumption was transient, with all three bottles nearly catching up in terms of 2,6-DNT degradation by the end of the

incubation period (Fig. 3.51). Since the pH of the groundwater was adjusted before being added to the inoculum, pH did not likely contribute to the inhibition. CB that was present in the chemical oxidation groundwater remained unchanged during the 32 days of incubation.

It is important to highlight that the peak shapes for 2,6-DNT and 2,4-DNT in the 2,6-DNT + 2,4-DNT treatment, were altered in the presence of 2,6-DAT and 2A6NT. The method that separated both dinitrotoluene isomers was the same method used for analysis of the metabolites. 2,6-DAT and 2A6NT were present at higher concentrations than 2,4-DNT and 2,6-DNT; consequently, the peaks for 2,4-DNT and 2,6-DNT were flattened out in the presence of metabolites, making their quantification slightly more difficult. Such behavior was not observed in the water controls, given the absence of metabolites in this treatment. Despite this, both 2,4-DNT and 2,6-DNT were successfully quantified in the 2,6-DNT + 2,4-DNT treatment, yielding measured concentrations close to the expected values.

Despite the degradation of 2,4-DNT in the 2,6-DNT + 2,4-DNT treatment, neither 2,4-DAT nor 2A4NT were observed in the chromatograms from analysis of the metabolites. This is probably a consequence of the low concentrations of 2,4-DNT (yielding low concentrations of daughter products) and the high concentrations of 2,6-DAT and 2A6NT obtained from the degradation of 2,6-DNT in the enrichment culture. It is probable that these peaks may have obscured the presence of 2,4-DAT and 2A4NT.

3.13 4-Nitrotoluene Microcosms

Results for the water controls corresponding to the 4-NT microcosms are shown in Figure 3.40. There was no abiotic loss of these compounds over the 160 days of incubation. Results for the 4-NT microcosms are presented in Figure 3.52. Compared to the 2,6-DNT microcosms, it took even longer to establish low redox conditions in the 4-NT microcosms. Resazurin remained pink one week after the microcosms were prepared. Lactate was added on days 9, 20, and 64; on day 28, sulfate was added (70.4 μL of a 107 mg/L stock solution); on days 58 and 67, 5 mL of AASM was added. By day 91, the color of the resazurin turned clear in bottle #2 . Bottle #3 broke on day 64. The amount of 4-NT added was increased to the target maximum (Table 1.1) with the second or third dose. Bottles #1 and #2 were sacrificed to start the 4-NT enrichment culture on day 216.

3.14 4-Nitrotoluene Enrichment

Figure 3.42 summarizes the results obtained for the 4-NT DDI control during the development of the 4-NT enrichment culture. Throughout the evaluation period (80 days) the concentration of 4-NT in the water control did not decrease.

Results for the 4-NT enrichment culture are presented in Figure 3.53a. As a consequence of the slow rate of establishing activity in the microcosms, and the subsequent slow rate in developing the enrichment culture, development of the 4-NT enrichment culture is still in progress. At the point that the target maximum is consumed within several weeks, it will be used in the same manner as the 2,6-DNT enrichment culture to evaluate the effect of co-contaminants.

4-Aminotoluene, the expected reduction product from anaerobic degradation of 4-NT, was detected at increasing concentrations in the 4-NT enrichment (Fig. 3.53b). Unlike the 2,6-DNT enrichment, the mass balance obtained for 4-NT and 4-AT closely matches the expected results, with 1 mole of 4-AT being produced for every mole of 4-NT consumed.

CHAPTER FOUR

4.0 DISCUSSION

4.1 Inhibitory and Synergistic Effects in Mixtures of Contaminants

Table 4.1 summarizes the results for the two sets of experiments completed for CB. No permanent inhibitory effects were observed in the CB inhibition experiments when 2,4-DNT, 4-IPA, 1,4-dioxane, and 1,2-DCA were added as co-contaminants. In the CB + 2,4-DNT and CB + 4-IPA treatment, one out six bottles exhibited temporary inhibition. However, the magnitude of these effects was minor. In the CB + 4-IPA treatment, two bottles exhibited faster biodegradation rates than those observed for the CB-only treatment. Addition of 10% chemical oxidation groundwater had a temporary inhibitory effect on the rate of CB biodegradation. For experiment 1, permanent inhibition was observed in all three bottles, although monitoring was stopped after 40 days of incubation. The pH of this treatment was not adjusted after the addition of the chemical oxidation water, which was subject to alkaline activation with NaOH. The inhibitory effects observed in this experiment may be attributable at least in part to the high groundwater pH. For experiment 2, temporary inhibitory of CB biodegradation was observed in all three of the chemical oxidation bottles, despite lowering the pH to circumneutral prior to adding the inoculum.

After re-spiking all the CB treatments (except for the CB + chemical oxidation set) with the parent compound, the results suggest that CB serves as a primary substrate for cometabolism of 2,4-DNT and 4-IPA, but not 1,4-dioxane or 1,2-DCA.

Table 4.2 summarizes the results obtained per treatment in the two experiments completed to assess the effect of co-contaminants on 1,2-DCB biodegradation. No permanent inhibitory effects were observed when 2,4-DNT, 4-IPA, 1,4-dioxane, and 1,2-DCA were added as co-contaminants. In the 1,2-DCB + 4-IPA treatment, two of three bottles exhibited temporary inhibition at the high concentration of 4-IPA. No inhibitory effects were observed in the 1,2-DCB + 2,4-DNT, 1,2-DCB + 1,4-dioxane and 1,2-DCB + 1,2-DCA treatments. The addition of 10% chemical oxidation groundwater temporarily slowed the rate of 1,2-DCB biodegradation, despite adjustment of the pH to circumneutral prior to adding1,2-DCB. In light of the results for the 1,2-DCB + 4-IPA treatment, a second experiment was conducted at the target low concentration of 4-IPA. No inhibition of 1,2-DCB biodegradation was observed; indeed, 1,2-DCB biodegraded at a faster rate in the 1,2-DCB + 4-IPA low concentration treatment compared to the 1,2-DCB-only treatment. After re-spiking the 1,2-DCB + 2,4-DNT and the 1,2-DCB + 4-IPA treatments with the parent compound several times, the results suggest that 1,2-DCB serves as a primary substrate for aerobic cometabolism of 2,4-DNT and 4-IPA.

Kurt et al. (2013) reported removal of CB and 1,2-DCB to below detection limits at biodegradation rates of 21±1 mg of CB/m^2•day and 3.7±0.5 mg of 1,2-DCB/m^2•day. The experiments were conducted by packing samples from the vadose zone of a site contaminated with CB, 1,2-DCB, and 1,4-DCB in a multiport column to simulate the interface of the vadose zone with an underlying groundwater plume, creating an oxic/anoxic interface and a capillary fringe. The experiments reported in this thesis

confirmed the potential to biodegrade CB and 1,2-DCB at oxygen levels below saturation.

Elango et al. (2011) compared first-order biodegradation rate coefficients for aerobic conditions at low and high levels of TCE and cDCE. For aerobic conditions followed by anaerobic conditions, at low levels of TCE and cDCE, the first order biodegradation rate coefficient was 23 yr^{-1} for CB and 16 yr^{-1} for 1,2-DCB.

Cometabolism of 2,4-DNT and 4-IPA supported by biodegradation of CB and 1,2-DCB has not been reported in the literature. Nevertheless, this result is consistent with the fact that aromatic oxygenases responsible for CB and 1,2-DCB biodegradation likely have an affinity for other mono-aromatic compounds. Aerobic biodegradation of 1,4-dioxane involves a monooxygenase and aromatic oxygenases for toluene are known to cometabolize 1,4-dioxane. However, the oxygenases for CB and 1,2-DCB in this study were not active on 1,4-dioxane. Likewise, one of the pathways for aerobic biodegradation of 1,2-DCA involves an oxygenase, but the aromatic oxygenases for CB and 1,2-DCB exhibited no reactivity with 1,2-DCA.

Table 4.3 summarizes the results for the experiment completed with the 2,6-DNT anaerobic enrichment culture. No permanent inhibitory effects were observed when 2,4-DNT, 4-IPA, 1,4-dioxane, and 1,2-DCA were added as co-contaminants. The presence of 4-IPA and 2,4-DNT resulted in a modest increase in the rate of 2,6-DNT biodegradation compared to the 2,6-DNT-only treatment. 2,4-DNT was completely biodegraded in the 2,6-DNT + 2,4-DNT treatment. The inhibitory effect of 1,4-dioxane and 1,2-DCA is apparent in two of the three bottles tested for each treatment. The addition of 10%

chemical oxidation groundwater temporarily slowed the rate of 2,6-DNT biodegradation, despite adjustment of the groundwater pH to circumneutral. 2A6NT increased as 2,6-DNT was consumed in all of the treatments, with no detectable increase in 2,6-DAT.

Hudcova et al. (2011) reported kinetic parameters for 2,4-DNT degradation by individual strains and mixed cultures known to use 2,4-DNT and NT as growth substrates. The following 2,4-DNT degradation rates were reported for each strain: *Pseudomonas putida* NDT1 (0.67±0.01 mg/L•d), *Pseudomonas fluorescens* NDT2 (0.66±0.01 mg/L•d), *Achromobacter* sp. NDT3 (0.31±0.03 mg/L•d), *Bacillus cereus* NDT4 (0.21±0.02 mg/L•d) and their mixed culture (35.0±0.2 mg/L•d).

In the study by Hudcova et al. (2011), growth on 2,6-DNT was sustained as the sole carbon, energy and nitrogen, although its removal rate was low (0.03 mg/L•d). 2,6-DNT was not degraded by the mixed culture in the presence of 2,4-DNT. Yet, even the most efficient individual 2,4-DNT degrader, *P. putida* NDT1, was not able to degrade 2,6-DNT at all as a pure strain, either as a single substrate or in the mixture with 2,4-DNT.

Hudcova et al. (2011) also reported the influence of an isomeric dinitrotoluene mixture on the rates of biodegardation of each compound. This was evaluated for two 2,6-DNT concentrations and two 2,4-DNT starting concentrations. The observed negative effect of 2,6-DNT on 2,4-DNT degradation could not be explained by catabolic competition because 2,6-DNT was less biodegradable. Perhaps, this effect was due to a higher toxicity of 2,6-DNT to bacterial cells, which could be ameliorated by the presence of 2,4-DNT, a growth substrate. Evidence confirming this hypothesis was obtained while

varying the ratio of the two dinitrotoluenes. When 2,4-DNT was present at a higher concentration (10 mg/L), the eventual complete degradation of 2,4-DNT was observed, although with a two fold lower rate. By contrast, when 2,4-DNT was present at a lower concentration (5 mg/L), its removal efficiency decreased in the presence of increasing 2,6-DNT concentrations, with a concomitant decline in 2,4-DNT removal rates (four fold compared to that with no 2,6-DNT). In addition, the cells were no longer able to remove 2,4-DNT completely, unlike the previous case.

The pH level in the aerobic and anaerobic microcosms, and in the enrichment cultures was adjusted to circumneutral. The average pH measured in groundwater for the site ranged from 4.6 to 5.5, indicating low buffering capacity. The effects of low pH on the growth of microorganisms, and in the rate and extent of biodegradation of parent compounds was not assessed and is recognized as a limitation of this study. Nevertheless, given the high porosity of the aquifer, pH adjustment throughout a treatment zone may be achievable through reinjection of extracted groundwater, with a low risk of clogging.

4.2 Chemical Oxidation

Given the effects of the chemical oxidation groundwater on the biodegradation of CB, 1,2-DCB and 2,6-DNT, this section discusses chemical oxidation as a remedial strategy and reviews the potential causes for slower biodegradation rates with the parent contaminants.

The remediation of groundwater contamination using in-situ chemical oxidation (ISCO) involves injecting oxidants and potentially co-amendments directly into the

source zone and downgradient plume. The intent is for the oxidant chemicals to react with the contaminants, producing innocuous substances such as carbon dioxide, water, and—in the case of chlorinated compounds—inorganic chloride. However, there may be many chemical reaction steps required to reach those end points, and some reaction intermediates. Fortunately, in most cases when an adequate oxidant dose is applied, the reactions proceed to completion, and the end products are reached quickly (ITRC, 2005).

The impact of oxidants on the native microbial population during ISCO is not well understood. As a result, some concerns exist within the remediation community as to the potential for negative impacts that ISCO may have on bioremediation processes, especially its impact on down-gradient plume control via bioremediation and its impact on subsequent natural attenuation processes.

There is a generalized hypothesis that injection of oxidants can ultimately enhance long-term bioremediation and natural attenuation capability. According to this concept, oxidants may actually improve microbial function by reducing contaminants to less toxic concentrations; breaking down contaminants to more useable fragment sizes; and increasing the levels of dissolved naturally occurring organics, which can be utilized as a food source. However, while ISCO is recognized as having potential benefits to subsequent aerobic bioremediation processes, it is also possible that ISCO may be harmful to any subsequent anaerobic bio-processes (Peroxychem, 2009)

4.2.1 Unknown Products from Chemical Oxidation

As explained in section 3.1, stoichiometric dosing with sodium persulfate was used for additions into the parent contaminant inhibition experiments. The COD of the chemical oxidation water was 148 mg/L. Based on the concentration of parent contaminants and co-contaminants spiked in the chemical oxidation treatability test, the expected COD was 133 mg/L. The COD of the groundwater was 14 mg/L. By adding the expected COD after the additions of all the contaminants and the COD measured in groundwater, the approximate COD was 147 mg/L. The COD measured in the stoichiometric treatment can be used as an indicator of the degree of oxidation of the mixture of contaminants after treatment. Comparing these two values, it is apparent that mineralization of the parent contaminants did not occur. Indeed, while the contaminants were transformed, there was virtually no net oxidation, since all of the COD remained in the water after treatment. This suggests that, at stoichiometric dosing, most of the parent contaminants were transformed to unknown products. Such intermediate compounds may have been responsible for the temporary toxicity to the enrichment cultures. Alternatively, the chemical oxidation products may have provided a source of organic compounds that are preferred as substrates over the aromatic compounds and therefore degradation of the aromatics was delayed while these organics were consumed.

Huan et al. (2005) reported the degradability of 59 VOCs by persulfate oxidation. The negative degradation results obtained for some compounds (e.g. chloroethane and chloroform) indicated that they could be intermediates of the decomposition of one or several of the VOCs in the mixture or their daughter products. Base don this, toxic

compounds could still be produced from the incomplete chemical oxidation of some VOCs. No intermediate products from the oxidation of CB and 1,2-DCB were identified in this study; this would be a fruitful avenue for future research.

4.2.2 Sulfate, Sodium, and Nitrite Concentrations After ChemOx Treatment

In general, one mole of the persulfate ($S_2O_8^{-2}$) forms two moles of sulfate (SO_4^{-2}), either through reaction with the contaminant or decomposition. The maximum sulfate concentration and the longevity of augmented sulfate levels in the groundwater are dependent upon many factors, including the groundwater flow rate, and the lithology and population density of sulfate reducing bacteria (SRB), to name a few. Treatment zones with slow to no groundwater flow may be impacted by elevated concentrations of sulfate for extended periods of time (Peroxychem, 2007).

Given the very high dose of oxidant used in this study and its activation with NaOH, the resulting high concentrations of sulfate and sodium could be exerting an inhibitory effect on aerobic degradation of the parent contaminants. Also, oxidation of nitrated compounds in this experiment (2,4-DNT, 2,6-DNT and 4-NT) is expected to produce nitrite, which is also known to cause inhibition at elevated concentrations. Based on the oxidant and NaOH additions, the expected concentrations of these compounds in the stoichiometric treatment are 1597 mg/L for SO_4^{-2}, 574 mg/L for Na^+ and 5.6 mg/L for NO_2^-. After a ten-fold dilution with groundwater in the microcosm bottles used for the inhibition experiments, the expected concentrations are 160 mg/L for SO_4^{-2}, 57 mg/L for

Na^+ and 0.6 mg/L for NO_2^-. These concentrations are sufficiently low that they are not expected to result in an inhibitory effect.

4.2.3 Type of Oxidant

The four major oxidants used for ISCO are hydrogen peroxide, potassium and sodium permanganate, sodium persulfate, and ozone. The effectiveness of some of these oxidants can be enhanced through activation (e.g., Fenton's reagent, activated persulfate) and used in conjunction with other oxidants (perozone) (ITRC, 2005)

While there have been several studies in the literature looking at the impact of various oxidants on microbial viability, there have been few published investigations involving activated persulfate (Peroxychem, 2009).

Droste et al. (2002) reported that application of persulfate and permanganate (sequentially injected) in a pilot field test to treat chlorinated solvents supported evidence of ongoing sulfate-reducing bacterial activity post-injection. In fact, these results indicated that the reductive dechlorination of TCE may actually have been enhanced by the oxidant application, based on their assessment of TCE to vinyl chloride ratios. This conclusion may indicate that even for subsequent anaerobic bioremediation processes, the benefits of reduced contaminant loading and increased natural dissolved organics more than offset the impacts of increased oxygen content on the anaerobic population (Peroxychem, 2009).

Tsitonaki et al. (2008) published one of the few peer-reviewed studies on the impact of persulfate, in this case activated by heat (40ºC), on soil microorganisms. They

investigated the effects on indigenous microorganisms as well as soils spiked with *P. putida*, at persulfate concentrations ranging from 0.1 to 10 g/L over a period of 14 days. Their work indicated that the microbial populations of spiked samples were reduced greatly by the application of activated persulfate, which is consistent with other studies referenced in their paper showing that "spiked" microbes are very susceptible to chemical oxidation. However, the indigenous microbial populations in their soil samples showed a high degree of resistance in terms of cellular integrity (Peroxychem, 2009).

Bou-Nasr et al. (2006) also reported little impact on indigenous cell concentrations when exposed to iron-activated persulfate. Crimi et al. (2007) reported that the biomass was not altered significantly and the sulfate-reducing bacteria were present and remained active when the soil samples were treated with Klozur® persulfate, activated by either Fe-EDTA, Fe-citrate or high pH (Peroxychem, 2009).

A laboratory study by Peroxychem (2009) examined the effect of Klozur CR (calcium peroxide and sodium persulfate designed to provide both ISCO and long-term oxygen release benefits) on SRBs in sediments contaminated with polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons. The impact on SRBs may be significant, as they are strict anaerobes. Previous studies identified *Desulfovibrio*, *Desulfobacteriaceae* and *Desulfobulbus* as the predominant SRB species present in these soils. Klozur CR was dosed at 4 (dose 1), 50 (dose 2) and 100 g/kg sediment (dose 3). In the control (no Klozur CR added), no significant change in the density of SRBs was observed. Addition of Klozur CR did result in significant decreases in the relative abundance of SRBs, with an increasing loss of population as the dosage increased.

However, within the timescale of the study (8 weeks), the microbial population rebounded in the reactors subjected to doses 1 and 2.

The rebound of microbial activity may have occurred in response to an increase in the sulfate concentration (a by-product of the persulfate reaction) and the accumulation of low molecular weight fatty acids and alcohols, such as acetate, oxalate, propionate and ethanol resulting from the oxidation of the PCBs and aromatic hydrocarbons (Peroxychem, 2009). Analysis of the microbial distribution showed that the three SRBs mentioned above represented approximately 30% of the total bacterial populations, and that the SRB concentrations had a greater decrease as the oxidant dosage increased, commensurate with the relative abundance of all species data.

Studer et al. (2008) presented data from a field application of Klozur CR at a site contaminated with BTEX and fuel constituents. Bio-Trap® monitoring indicated the presence of both aerobic and anaerobic hydrocarbon degraders, and that significant total bacterial and Proteobacetrial populations existed three months post chemical oxidant application. Population levels did decrease by about 20%, but the microbial population remained viable (Peroxychem, 2009).

These previous studies indicate that in the short-term, application of activated persulfate to the subsurface will impact microbial populations, but that they will eventually recover, including anaerobic microbes. Also important is the impact that activated persulfate may have on the substrate (contaminant) utilization efficiency of the microbes, as this is key to bioremediation and natural attenuation processes. Tsitonaki (2008) investigated the acetone consumption of microbes in the presence of heat-

activated persulfate. While that study indicated the indigenous microbial population was not significantly influenced by persulfate in terms of cell membrane integrity, they were vulnerable to the highest level of persulfate concentration (10 g/L) in terms of acetone consumption. One explanation offered was that at the highest persulfate concentrations, the pH of their lab samples was low (pH = 3), which may have affected the proton motive force and thereby influenced the uptake of acetone. In the field study performed by Studer et al. (2008), the inclusion of ^{13}C-labelled benzene in Bio-Traps allowed for an analysis of benzene utilization by the indigenous population. Three months post application of the Klozur CR product, first-order estimated benzene utilization rates ranged from 0.023 to 0.043 mg/day. This indicated that after the chemical oxidation event, benzene utilization still continued and that the oxidant did not destroy the population nor eliminates its ability to utilize and destroy the contaminant. Likewise, the work of Droste demonstrated that application of persulfate did not severely impact the utilization of TCE by native dechlorinators, and in fact may have enhanced the bioremediation of residual contaminant (Peroxychem, 2009).

The transient inhibitory effects observed in the parent compound inhibition experiments for this study are consistent with previous research with chemical oxidation. While biodegradation was retarded, after some period of time the microbial population recovered and finished consuming CB and 1,2-DCB. The same effect was observed in the 2,6-DNT experiment, although biodegradation is on-going.

4.2.4 Effect of Dilution Between the Source Zone and Downgradient Areas

To effectively degrade contaminants, the oxidant must come into contact with the contaminant molecules. Some of the more stable forms of contamination can be oxidized only with the stronger oxidants, but stronger oxidants are consumed quickly in the subsurface, limiting the distance the oxidant can travel. Less reactive oxidants are more stable and can be transported greater distances in the subsurface. Therefore, the volume of aquifer to be treated is an important variable to consider when choosing an oxidant, as well as the dilution that takes place from the source zone treatment with chemical oxidation to downgradient non-source areas. The solubility of the oxidant in water, the usual injection fluid, is also important because it limits the mass of oxidant that can be injected per volume of injection fluid (ITRC, 2005).

An important consideration for all ISCO designs, especially source zones, is the amount of contaminated water displaced from the immediate vicinity. The volume that is injected into the saturated zone displaces same volume of groundwater with mixing occurring at the interfaces. In source areas where groundwater contamination is elevated, this displacement should be minimized and controlled such that adequate contact with the oxidant is obtained. The spatial distribution of both the contaminants and the injected oxidant is also greatly influenced by heterogeneous subsurface geology and the groundwater flow speed/direction (ITRC, 2005).

In order to complete the inhibition experiments, a 10% dilution of the stoichiometric chemical oxidation groundwater was completed. However, based on the site hydrogeological characteristics it is necessary to fully assess the expected dilution

from the source zone where chemical oxidation may be be performed, to downgradient areas where bioremediation and monitored natural attenuation are the likely remedial strategies.

4.3 Enzyme Induction in the Presence of 4-IPA

Given the effects of 4-IPA in the biodegradation of parent compound in the CB + 4-IPA, 1,2-DCB + 4-IPA low concentration and 2,6-DNT + 4-IPA, this section discusses potential mechanisms that could explain the faster biodegradation rates observed for these treatments.

Zhang et al. (2012) reported the biodegradation of isoproturon, an extensively used herbicide and its metabolites by *Sphingobium* sp. YBL2. Isoproturon and its related phenylurea herbicides are degraded mainly through aniline derivatives, such as 4-isoproylaniline and 3,4-dichloroaniline in bacteria and agricultural soils (Sørensen et al., 2001, 2003; Hussain et al., 2009). Aniline and its derivatives are mainly converted to corresponding catechols by aniline dioxygenase (Fukumori and Saint, 1997; Quanfeng; Liang et al., 2005; Kim et al., 2007; Shin and Spain, 2009). Then, the catechols are cleaved through an ortho- or metapathway catalyzed by 1,2-catechol 1,2-dioxygenase and catechol 2,3-dioxygenase, respectively (Lee et al., 2001; Na et al., 2001; Rodarie and Jouanneau, 2001). To investigate whether these pathways also existed in strain YBL2, the activity of aniline dioxygenase, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase of strain YBL2 were analyzed. The authors concluded that aniline dioxygenase and catechol 2,3-dioxygenase activity were induced by isoproturon exposure, while catechol 1,2-dioxygenase was not induced by exposure to isoproturon; it was constitutively

expressed in strain YBL2. These results suggest that the presence of 4-IPA in this study induced the expression of aniline dioxygenase, and then aniline dioxygenase converted 4-IPA to the corresponding catechol, which was opened by catechol 1,2-dioxygenase and catechol 2,3-dioxygenase.

Mohammed et al. (2014) demonstrated that aniline is not a precursor for indole biosynthesis; rather, it induces indole biosynthesis in strain JA2. Their results also suggest a possible stress-induced metabolic re-programming and shift towards synthesis of indoles in the presence of aniline.

4.4 Sorption of DNTs and their Amine Products onto Anoxic Sediment

The difference in the mass balance between 2,6-DNT and 2,6-DAT plus 2A6NT may be explained by abiotic losses in the anoxic environment via adsorption of these amine products to organic material, i.e. sediment and/or biomass. Aromatic amines interact with soil through both reversible and irreversible processes. Reversible sorption includes hydrophobic interactions or cationic exchange (Lee et al., 1997; Fábrega et al., 1998)

Yang et al. (2008) described biotransformation of 2,4-DNT and 2,6-DNT in marine sediment sampled from a shipwreck site near Halifax Harbour. Incubation of either 2,4-DNT or 2,6-DNT in anaerobic sediment slurries (10% w/v) at 10 ºC led to the reduction of both DNTs to their corresponding diaminotoluene (2,4-DAT and 2,6-DAT) via the intermediary formation of their monoamine derivatives (ANTs). The production of diaminotoluene was enhanced in the presence of lactate for both DNT isomers. Using [^{14}C]-2,4-DNT, less than 1% mineralization was observed as determined by liberated

$^{14}CO_2$. Sorption of DNTs, ANTs, and DATs was thus investigated to learn of their fate in marine sediments. Under anaerobic conditions, sorption followed the order: DNTs (K_d = 8.3–11.7 L kg^{-1}) > ANTs (K_d = 4.5–7.0 L kg^{-1}) > DATs (K_d = 3.8–4.5 L kg^{-1}). Incubation of 2,4-DAT in aerobic sediment led to rapid disappearance from the aqueous phase. LC/MS analysis of the aqueous phase and the acetone sediment extract showed the formation of azo- and hydrazo-dimers and trimers, as well as unidentified polymers. Experiments with radiolabelled 2,4-DAT showed a mass balance distributed as follows: 22% in the aqueous phase, 24% in acetone extracts, and 50% irreversibly bound to sediment. Yang et al. concluded that DNT in anoxic marine sediment can undergo in situ natural attenuation by reduction to DAT followed by oxidative coupling to hydrazo-oligomers or irreversible binding to sediment.

In the absence of oxygen, sorption of the tested aromatic compounds followed the order DNTs > ANTs > DATs. The decreasing sorption observed when replacing nitro groups by amino groups under anaerobic conditions is explained by two factors: 1) the diminution of the number of nitro groups available for binding to clays; and 2) the absence of cationic exchange in a medium (pH 7.7) where the neutral form of aromatic amines prevails. DNTs and ANTs were reversibly sorbed to the sediment as supported by the closeness between K_d and $K_{d\text{-}des}$ values as well as the high recoveries obtained after acetonitrile extraction. The fact that most of 2,4- and 2,6-DNT could be recovered in acetonitrile confirms that the rapid initial loss of DNTs in the biotransformation experiments or the total loss in sterile controls was caused by reversible sorption, thus

leaving the two parents compounds available for biodegradation. In contrast, a fraction of the DATs was irreversibly bounded to the sediment ($K_d < K_{d\text{-}des}$, recoveries ≈ 80%).

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Based on the results of this research, the following conclusions are offered:

1. Aerobic biodegradation of CB and 1,2-DCB was demonstrated in microcosms using soil and groundwater from an industrial site in Brazil. The microcosms served as inoculum to develop enrichment cultures, which were subsequently used to assess the effect of co-contaminants on the rate and extent of CB and 1,2-DCB biodegradation.
2. Anaerobic biodegradation of 2,6-DNT and 4-NT was demonstrated in microcosms using soil and groundwater from an industrial site in Brazil. Lactate served as the electron donor and nitro group reduction was the only transformation observed. The microcosms served as inoculum to develop enrichment cultures; the 2,6-DNT enrichment was subsequently used to assess the effect of co-contaminants on the rate and extent of 2,6-DNT biodegradation. The rate of 4-NT transformation was too slow to permit development of the 4-NT enrichment to the point needed to evaluate co-contaminants.
3. Alkaline activated persulfate was effective in chemical oxidation of the contaminants at their maximum concentrations. The treatment that employed a stoichiometric dose was used to simulate the effect of chemical oxidation groundwater on biodegradation of CB, 1,2-DCB, 2,6-DNT, and 4-NT. Although higher than stoichiometric doses achieved more complete removal, the

stoichiometric dose (28 g/g contaminant) is at the high end of what is deployed in situ.

4. 2,4-DNT, 4-IPA, 1,4-dioxane, and 1,2-DCA did not inhibit the rate or extent of aerobic CB biodegradation when these co-contaminants were present at their target high concentrations. Temporary inhibitory effects on the rate of CB biodegradation were observed in the presence of 10% (v/v) of the chemical oxidation groundwater from the stoichiometric treatment. The source of inhibition is not yet known but may be related to the organic products from partial chemical oxidation of the contaminants. COD analysis of the chemical oxidation groundwater suggests that the extent of contaminant mineralization was minor.
5. CB serves as a primary substrate for aerobic cometabolism of 2,4-DNT and 4-IPA, but not 1,4-dioxane or 1,2-DCA. This suggests that the aromatic oxygenases that are required for metabolism of CB are also reactive with 2,4-DNT and 4-IPA. This is an example of a positive co-occurrence of contaminants.
6. 2,4-DNT, 1,4-dioxane, and 1,2-DCA did not inhibit the rate or extent of 1,2-DCB biodegradation of 1,2-DCB when these co-contaminants were present at their target high concentrations. A temporary decrease in the rate of 1,2-DCB biodegradation occurred in the presence of 4-IPA at its target high concentration and with the 10% (v/v) chemical oxidation groundwater from the stoichiometric treatment.
7. 4-IPA did not inhibit the rate or extent of 1,2-DCB biodegradation when 4-IPA was present at its target low concentration.

8. 1,2-DCB serves as a primary substrate for cometabolism of 2,4-DNT and 4-IPA. This suggests that the aromatic oxygenases that are required for metabolism of 1,2-DCB are also reactive with 4-IPA. This is an example of a positive co-occurrence of contaminants.
9. No inhibitory effects were observed in the rate or extent of anaerobic biodegradation of 2,6-DNT when 2,4-DNT and 4-IPA were added as co-contaminants at the target high concentrations. Minimal inhibitory effects were observed when 1,4-dioxane and 1,2-DCA were added as co-contaminants. Temporary inhibitory effects on the rate of 2,6-DNT degradation were observed when adding 10% of the chemical oxidation groundwater from the stoichiometric treatment.

5.2 Recommendations

Recommended actions for further research based on this study include:

1. Further assess the effect of pH on the CB + chemical oxidation treatment, the 1,2-DCB + chemical oxidation treatment, and the 2,6-DNT + chemical oxidation treatment. In the experiments conducted for this thesis, the pH level was adjusted to circumneutral in order to rule out pH as a limiting factor. However, pH adjustment may be challenging under in-situ conditions. Further evaluation of how pH affects the biodegradation rates of the target parent compounds is warranted.
2. Based on the Site Conceptual Model for Area P, evaluate the inhibitory effect of different doses of groundwater subjected to chemical oxidation, considering that

dilution takes place between the source areas and downgradient areas where bioremediation and monitored natural attenuation is the preferred remedial strategy. The 10% volumetric dose used in this study is a starting point and since it consistently caused transient inhibition, it is worthwhile to determine the dilution factor at which no inhibition occurs.

3. Assess the effects of using other oxidants (e.g. hydrogen peroxide, potassium and sodium permanganate, and ozone) and other activation methods for sodium persulfate (e.g., chelated iron and hydrogen peroxide) to produce the chemical oxidation groundwater for the parent compound inhibition experiments.
4. Conduct additional inhibition experiments to assess the effect of 2,6-DNT and 4-NT (present in the chemical oxidation groundwater, due to incomplete oxidation) on the rate and extent of aerobic biodegradation of CB and 1,2-DCB.
5. Further monitor the CB enrichment bottles that received repeated addition of CB, after the final addition of 2,4-DNT and 4-IPA. This will permit a more definitive assessment that their biodegradation is cometabolic.
6. Further monitor the 1,2-DCB enrichment bottles that received repeated addition of 1,2-DCB, after the final addition of 2,4-DNT and 4-IPA. This will permit a more definitive assessment that their biodegradation is cometabolic.
7. Complete a standard addition analysis and obtain a new response factor in order to conciliate the mass balance between to 2,6-DNT and 2,6-DAT and 2A6NT.
8. The method detection limits used in this study are an order of magnitude above the MCL/RSLs established by USEPA, and used as regulatory guidance for this

project in the absence of Brazilian standards for 2,4-DNT, 2,6-DNT, 4-NT and 1,2-DCA (Table 1.1). Consequently, there is a need to achieve lower detection limits for further studies, to determine if biodegradation can achieve treatment goals. The method detection limits for CB and 1,2-DCB are equal to or lower than the CETESB/CONAMA regulations.

9. Switch several anaerobic bottles that reduce 2,6-DNT to 2,6-DAT to aerobic conditions, to assess the potential for complete oxidation.
10. Conduct the 4-NT Inhibition experiments after completing development of the 4-NT enrichment culture.

TABLES

Table 1.1 Principal contaminants present in groundwater (mg/L) at the site.[a]

		Maximum	Low	Brazilian Limit [b]	U.S. Limit [c]
BTEX	Benzene	0.44	0.06	0.005	0.005
	Toluene	0.23	0.06	0.700	
Chlorobenzenes	***Chlorobenzene***	34.47	4.70	0.120*	0.100
	1,3-Dichlorobenzene	0.01	0.00		
	1,4-Dichlorobenzene	0.09	0.02	0.300*	0.075
	1,2-Dichlorobenzene	7.52	1.04	1*	0.600
Phenols	Phenol	0.33	0.33		
	2-Methylphenol	0.01	0.01		
Nitrotoluenes	2-Nitrotoluene	3.93	2.96	NA	0.00031 [b]
	3-Nitrotoluene	1.66	0.63	NA	0.00017 [b]
	4-Nitrotoluene	7.55	3.23	NA	0.0042 [b]
	2,6 Dinitrotoluene	11.27	6.07	NA	0.000048 [b]
	2,4 Dinitrotoluene	0.57	0.24	NA	0.00024 [b]
Anilines	(3+4) Chloroaniline	1.08	0.19		0.00036 [b]
	3,4-Dichloroaniline	0.22	0.08		
	4-Isopropylaniline	5.90	2.83	NA	NA
Cumene	Cumene	0.04	0.03		
	4-Nitrocumene	2.08	0.87	NA	NA
Isocyanate	3,4 Dichlorophenylisocyanate	0.03	0.03		
Others	***1,2-Dichloroethane***	1.52	0.39	0.050	0.005
	Carbon Tetrachloride	0.11	0.04		
	1,4-Dioxane	0.23	0.23		
	Phthalate	0.08	0.03		
	Vinyl Chloride	0.02	0.01		

[a] Contaminants in yellow were considered for inclusion in this study; those in bold red italics were selected for evaluation.
[b] CETESB/CONAMA are regulatory agencies in Brazil.
[c] MCL is the legally enforceable limit in U.S. drinking water; RSL is a regional screening level used by the EPA.

Table 1.2 Summary of previous studies for contaminants of concern at the site.[a]

Target Compounds		Chlorobenzenes		Nitrated Compounds			Anilines	Chl.Ethan	Others
		CB	1,2-DCB	4 NT	2,4-DNT	2,6-DNT	4-IPA	1,2 -DCA	1,4-dioxane
Chlorobenzenes	CB	-	1, 2, 3, 4, 5, 6					12	
	1,2 DCB		-					12	
Nitrotoluenes	4 NT			-	11	11			
	2,4 DNT				-	7, 8, 9, 10, 11			
	2,6 DNT					-			
Anilines	4 IPA						-		
Chlorinated Ethanes	1,2 DCA							-	
Others	1,4-dioxane								-

[a] Yellow indicates the study was performed under aerobic conditions; green indicates anaerobic; grey indicates both. Numbers in the cells indicate the following references:

1. Haigler et al. (1992)
2. Kurt and Spain (2013)
3. Fung et al. (2009)
4. Nelson et al. (2011)
5. Elango et al. (2010)
6. Leahy et al. (2003)
7. Lendenmann et al. (1998)
8. Nishino et al. (2000)
9. Leungsakul et al. (2005)
10. Shin et al. (2005)
11. Hudcova et al. (2011)
12. Heidrich et al. (2004)

Table 2.1 Sources and purity of selected chemicals used.

Chemical	Purity	Source
Sodium Lactate Syrup	60.0%	E. M. Scientific
CB	99.9%	Sigma Aldrich
1,2-DCB	99.0%	Fluka Analytical
1,2-DCA	100.0%	Mallinckrodt
1,4-Dioxane	>99.0%	Sigma Aldrich
4-IPA	99.0%	Aldrich
2,4-DNT	97.0%	Avocado Research Chemicals Ltd
2,6-DNT	97.0%	Alfa Caesar
4-NT	99.0%	Aldrich
2,6-DAT	97.0%	Aldrich
2,4-DAT	100.0%	Supelco
4-AT	99.6%	Aldrich
2A6NT	97.0%	Aldrich
2N4AT	97.0%	Pfaltz & Bauer
2A4NT	97.7%	Fluka Analytical
2A4NT	97.7%	Fluka Analytical

Table 2.2 Components of the basal salts medium (BSM) used for the aerobic enrichment cultures.

Chemical	Purity	Source
Potassium Phosphate	NA	Macron
Sodium Phosphate	100.0%	Mallinckrodt
Ammonium Chloride	99.5%	BDH
Nitrillotriacetic Acid	99.0%	Sigma
Magnesium Sulfate	98.0%	EMD
Ferrous Sulfate	99.7%	Mallinckrodt
Manganous Sulfate	99.4%	Fisher Scientific
Zinc Sulfate	99.3%	Fisher Scientific
Cobalt Chloride	99.8%	Mallinckrodt

Table 2.3 Components of the anaerobic MSM used for the anaerobic enrichment cultures.

Chemical	Purity	Source
Ammonium Chloride	99.5%	BDH
Dipotassium Phosphate	>99.0%	J.T. Baker
Potassium Phosphate Monobasic	99.0%	BDH
Magnesium Chloride Hexahydrate	99.0%	Mallinckrodt
Sodium Bicarbonate	100.0%	Mallinckrodt
Yeast Extract	NA	Difco
Trace Metal Solution	NA	NA

Table 2.4 Components of the trace metal solution used for preparation of the anaerobic salts medium (AASM).

Chemical	Purity	Source
Boric Acid	99.5%	EMD
Zinc Sulfate Heptahydrate	99.3%	Fisher Scientific
Nickel (II) Chloride Hexahydrate	98.0%	Mallinckrodt
Magnesium Chloride Hexahydrate	99.0%	EMD
Copper (II) Chloride Dihydrate	99.8%	Sigma
Cobalt (II) Chloride Hexahydrate	99.8%	Mallinckrodt
Sodium Selenite Pentahydrate	98.0%	Sigma Aldrich
Aluminum Sulfate	98.0%	Mallinckrodt
Hydrochloric Acid	37.0%	Mallinckrodt

Table 2.5 Experimental design. [a]

Condition	Treatment	Parent Substrate(s)	Co-contaminant	Co-Contaminant Concentration
Aerobic	1	Chlorobenzene	None	None
	2		2,4-Dinitrotoluene	Low
	3			High
	4		4-Isopropylaniline	Low
	5			High
	6		1,2-Dichloroethane	Low
	7			High
	8		1,4-Dioxane	Low
	9			High
	10		GW from chemical ox.	High
	11		Autoclaved Control	None
Aerobic	1	1,2-DCB	None	None
	2		2,4-Dinitrotoluene	Low
	3			High
	4		4-Isopropylaniline	Low
	5			High
	6		1,2-Dichloroethane	Low
	7			High
	8		1,4-Dioxane	Low
	9			High
	10		GW from chemical ox.	High
	11		Autoclaved Control	None
Anaerobic	1	4-Nitrotoluene + Lactate	None	None
	2		2,4-Dinitrotoluene	Low
	3			High
	4		4-Isopropylaniline	Low
	5			High
	6		1,2-Dichloroethane	Low
	7			High
	8		1,4-Dioxane	Low
	9			High
	10		GW from chemical ox.	High
	11		Autoclaved Control	None
Anaerobic	1	2,6-Dinitrotoluene + Lactate	None	None
	2		2,4-Dinitrotoluene	Low
	3			High
	4		4-Isopropylaniline	Low
	5			High
	6		1,2-Dichloroethane	Low
	7			High
	8		1,4-Dioxane	Low
	9			High
	10		GW from chemical ox.	High
	11		Autoclaved Control	None

[a] Each treatment was constructed in triplicate.

Table 2.6 Summary of initial concentrations for the groundwater (PM26) used to prepare the aerobic microcosms.

Instrument and Method	Compound	Retention Time (min)	PM 26					
			Bottle 1		Bottle 2		Bottle 3	
			Peak Area Units	(mg/L)	Peak Area Units	(mg/L)	Peak Area Units	(mg/L)
GC-114 DXELIQ3	1,4-Dioxane	10.0	0.0	0.0	0.0	0.0	0.0	0.0
GC-Ethel 112 Gossett	1,2 DCA	8.2	0.0	0.0	0.0	0.0	0.0	0.0
GC-114 CB013114	CB	11.0	0.0	0.0	0.0	0.0	0.0	0.0
	1,2 DCB	21.5	0.0	0.0	0.0	0.0	0.0	0.0
	GC Unknown 1	19.7	4.6	-	4.6	-	5.9	-
	GC Unknown 2	23.1	5.9	-	3.7	-	6.2	-
HPLC 55% MeOH	4-IPA (240nm)	7.5	0.0	0.0	0.0	0.0	0.0	0.0
	4NT (268 nm)	8.1	0.0	0.0	0.0	0.0	0.0	0.0
	HPLC Unknown 1 (240nm)	2.1	2.4	-	2.4	-	3.0	-
	HPLC Unknown 3 (240nm)	4.4	2.6	-	2.9	-	2.2	-
HPLC 35% MeOH	2,4 DNT (250nm)	23.3	0.0	0.0	0.0	0.0	0.0	0.0
	2,6 DNT (250nm)	24.9	0.0	0.0	0.0	0.0	0.0	0.0
	HPLC Unknown 9 (240nm)	1.9 - 2.2	1.9	-	2.0	-	3.2	-
	HPLC Unknown 10 (240nm)	3.8	0.8	-	0.8	-	0.8	-

Table 2.7 Summary of initial concentrations for the groundwater (PM20) used to prepare the anaerobic microcosms.

Instrument and Method	Compound	Retention Time (min)	PM 20					
			Bottle 1		Bottle 2		Bottle 3	
			Peak Area Units	(mg/L)	Peak Area Units	(mg/L)	Peak Area Units	(mg/L)
GC-114 DXELIQ3	1,4-Dioxane	10.0	0.0	0.0	0.0	0.0	0.0	0.0
GC-Ethel 112 Gossett	1,2 DCA	8.2	0.0	0.0	0.0	0.0	0.0	0.0
GC-114 CB013114	CB	11.0	0.0	0.0	0.0	0.0	0.0	0.0
	1,2 DCB	21.5	0.0	0.0	0.0	0.0	0.0	0.0
	GC Unknown 1	19.7	5.2	-	5.0	-	4.1	-
	GC Unknown 2	23.1	0.0	-	3.9	-	6.3	-
HPLC 55% MeOH	4-IPA (240nm)	7.5	0.0	0.0	0.0	0.0	0.0	0.0
	4NT (268 nm)	8.1	0.0	0.0	0.0	0.0	0.0	0.0
	HPLC Unknown 1 (240nm)	2.1	3.2	-	1.0	-	1.8	-
	HPLC Unknown 2 (240nm)	3.6 - 3.9	0.5	-	0.7	-	0.5	-
HPLC 35% MeOH	2,4 DNT (250nm)	23.3	0.0	0.0	0.0	0.0	0.0	0.0
	2,6 DNT (240nm)	24.9	0.0	0.0	0.0	0.0	0.0	0.0
	HPLC Unknown 9 (240nm)	1.9 - 2.2	2.1	-	1.8	-	2.1	-
	HPLC Unknown 10 (240nm)	3.8	0.2	-	0.2	-	0.2	-

Table 2.8 Required Klozur® and NaOH dosing for the chemical oxidation experiment.

Treatment	Volume of Persulfate (mL)	Volume of NaOH 9.2 M (mL)	Volume of Persulfate + NaOH (mL)	Volume of Groundwater (mL)	mmol persulfate / mmol of contaminants	g PS / g contaminants
No persulfate control	0.0	0.0	0.0	78	-	-
Soil only	6.3	3.6	10	90	-	-
Stoichiometric	1.5	0.9	2.4	75	15.0	27.8
2.1X Stoichiometric	3.2	1.8	5.0	73	31.3	58.3
3.35X Stoichiometric	5.1	2.9	8.0	70	50.0	93.0
4.2X Stoichiometric	6.3	3.6	10.0	68	62.6	116.6

Table 2.9 Required dosing for contaminants added to microcosms and for the inhibition tests.

	Target Concentration (mg/L)		Amount to add for Target Concentration	
Compound	**Low**	**Maximum**	**Low**	**Maximum**
CB	5.0	35	1.1 mL sat. solution	3.5 µL neat
1,2-DCB	1.0	7.5	0.8 mL saturated solution	5.8 mL saturated solution
1,2-DCA	0.4	1.5	4.8 µL saturated solution	18 µL saturated solution
1,4-Dioxane	0.2	2.0	9.0 µL [2196 mg/L] stock solution	90 µL [2196 mg/L] stock solution
4-IPA	3.0	6.0	1.0 mL [300 mg/L] stock solution	2.0 mL [300 mg/L] stock solution
4-NT	3.0	8.0	1.5 mL [205.2 mg/L] stock solution	3.9 mL [205.2 mg/L] stock solution
2,4-DNT	0.2	0.6	0.2 mL [101 mg/L] stock solution	0.6 mL [101 mg/L] stock solution
2,6-DNT	6.0	11.0	6.6 mL [91.4 mg/L] stock solution	12.0 mL [91.4 mg/L] stock solution

Table 2.10 Dimensionless Henry's Law constants for target VOCs.

Compound	**H_c (23ºC)**[a]	***M_l***[b]
CB	0.15	0.917
1,2-DCB	0.07	0.957
1,2-DCA	0.05	0.974

[a] Sander, 1999

[b] M_l is the fraction of contaminant present in the liquid phase, calculated with equation 2, assuming V_l = 0.1 L and V_g = 0.06 L.

Table 3.1 Contaminant removal via chemical oxidation with persulfate and sodium hydroxide.

		Extent of Removal Following 26 Days of Incubation							
Relative PS Dose	**g PS / g contaminants**	**1,2-DCA**	**CB**	**1,2-DCB**	**1,4-Dioxane**	**4-NT**	**4-IPA**	**2,6-DNT**	**2,4-DNT**
Stoichiometric	27.75	65%	60%	57%	100%	84%	50%	54%	100%
2.1X Stoichiometric	58.28	98%	78%	67%	100%	99%	88%	94%	100%
3.4X Stoichiometric	92.98	100%	87%	77%	100%	100%	99%	100%	100%
4.2X Stoichiometric	116.57	100%	80%	68%	100%	100%	100%	100%	100%

Table 4.1 Number of bottles per treatment (in triplicates) that degraded CB at the same rate than the CB only treatments

Treatment	Experiment 1 - High Concentration	Experiment 2 - High Concentration
CB + 2,4-DNT	2/3	3/3
CB + 4-IPA	3/3	2/3
CB + 1,4-dioxane	3/3	3/3
CB + 1,2-DCA	3/3	3/3
CB + ChemOx	0/3	0/3*

*After 5 days and the addition of 1mL of BSM, **two** of the triplicates started biodegrading CB and finished in 10 days.

Table 4.2 Number of bottles per treatment (in triplicates) that degraded 1,2-DCB at the same rate than the 1,2-DCB only treatments

Treatment	Experiment High Concentration	Experiment 4-IPA Low Concentration
1,2-DCB + 2,4-DNT	3/3	-
1,2-DCB + 4-IPA	1/3	3/3
1,2-DCB + 1,4-dioxane	3/3	-
1,2-DCB + 1,2-DCA	3/3	-
1,2-DCB + ChemOx	0/3*	-

*After 6 days and the addition of 1mL of BSM, **one** of the triplicates started biodegrading 1,2-DCB and finished in 14 days.

Table 4.3 Number of bottles per treatment (in triplicates) that degraded 2,6-DNT at the same rate than the 2,6-DNT only treatments

Treatment	Experiment High Concentration
2,6-DNT + 2,4-DNT	3/3
2,6-DNT + 4-IPA	3/3
2,6-DNT + 1,4-dioxane	3/3
2,6-DNT + 1,2-DCA	3/3
2,6-DNT + ChemOx	0/3*

*After 20 days and the addition of more lactate (62 μL) all three bottles started biodegrading 2,6-DNT. On day 32, approximately 77% of the 2,6-DNT spiked had been consumed

FIGURES

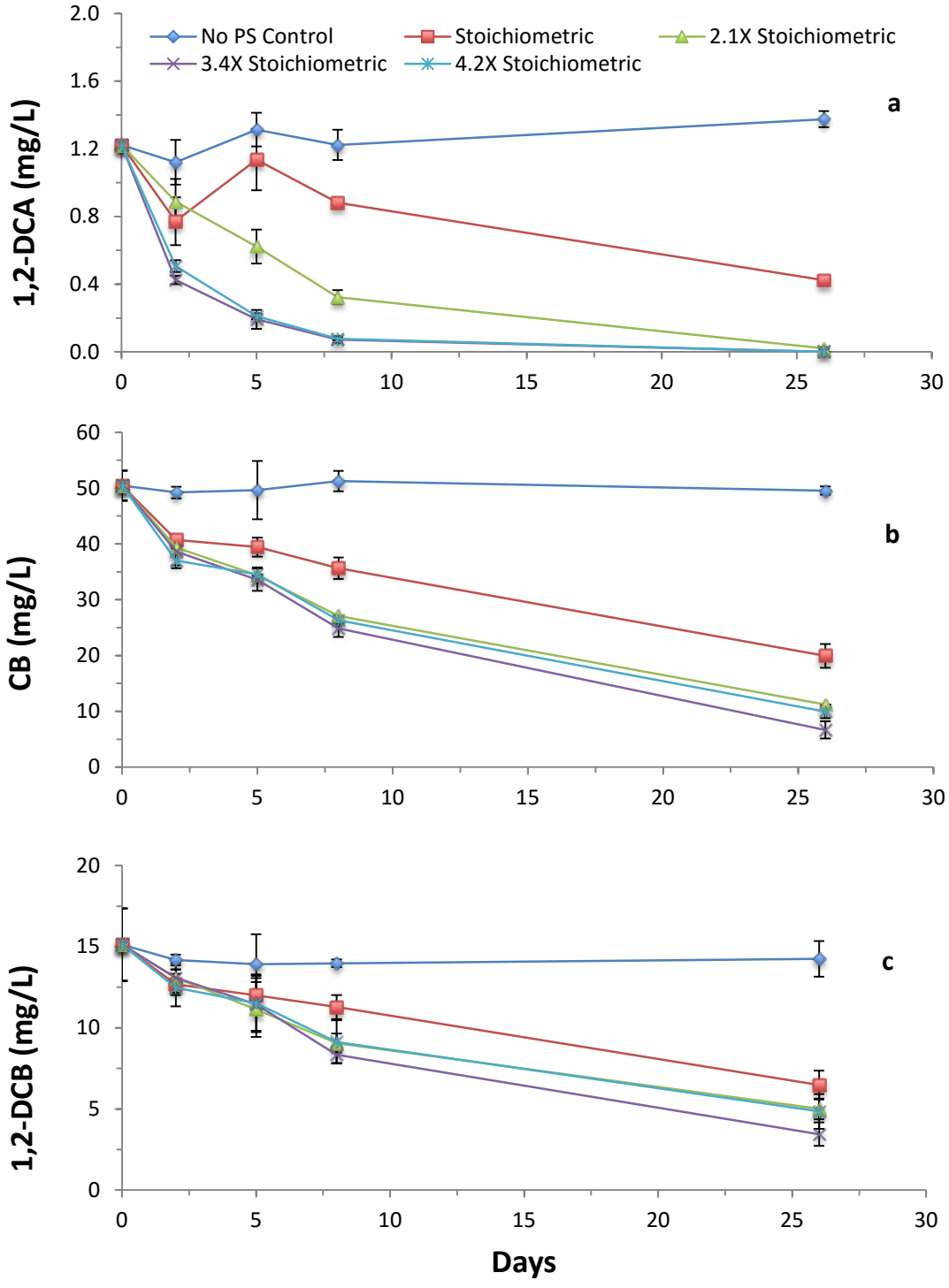

Figure 3.1 Chemical oxidation test results for a) 1,2-DCA; b) CB; and c) 1,2-DCB; PS = persulfate. Error bars are one standard deviation for triplicate bottles.

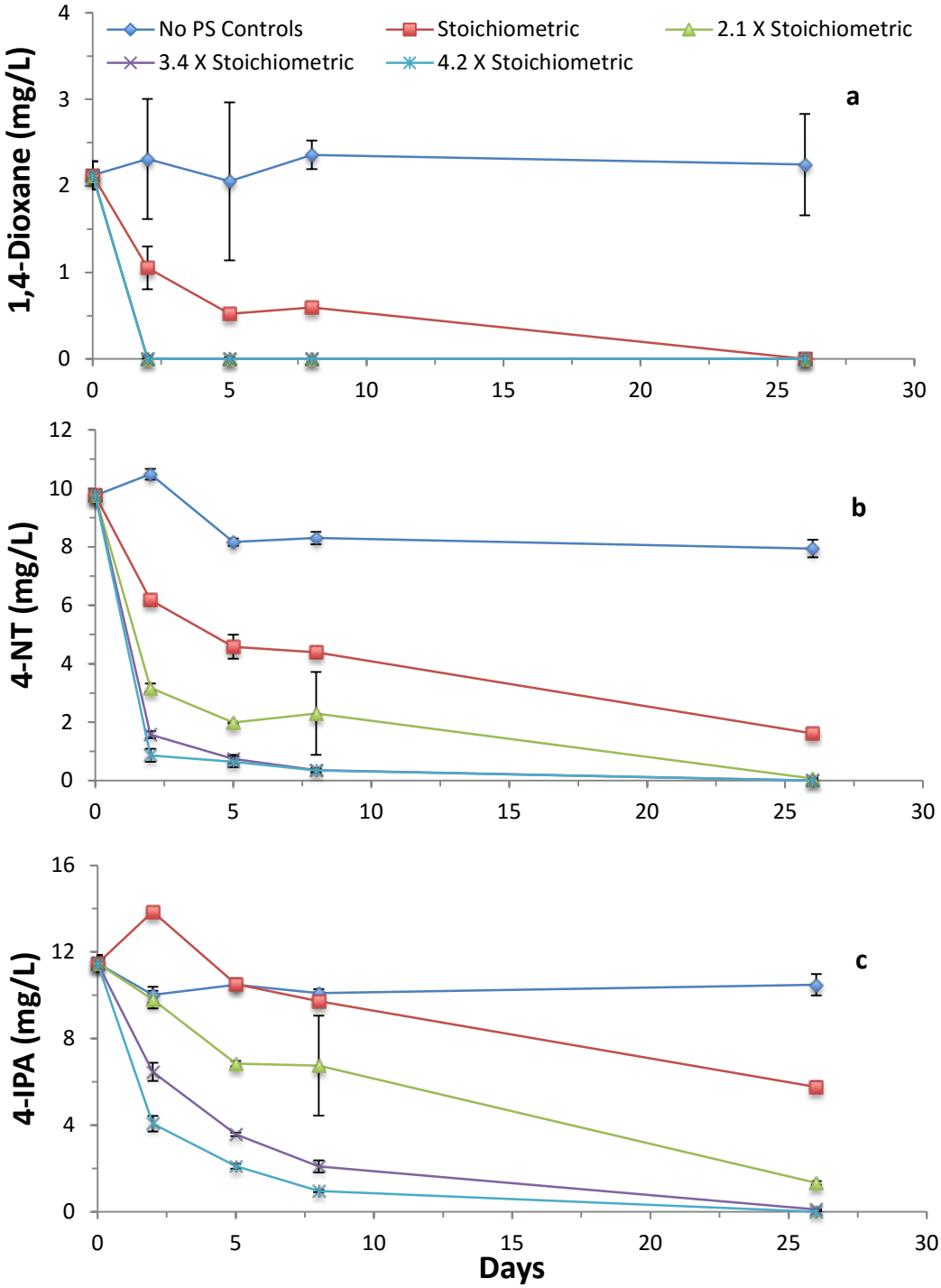

Figure 3.2 Chemical oxidation test results for a) 1,4-dioxane; b) 4-NT; and c) 4-IPA; PS = persulfate. Error bars are one standard deviation for triplicate bottles.

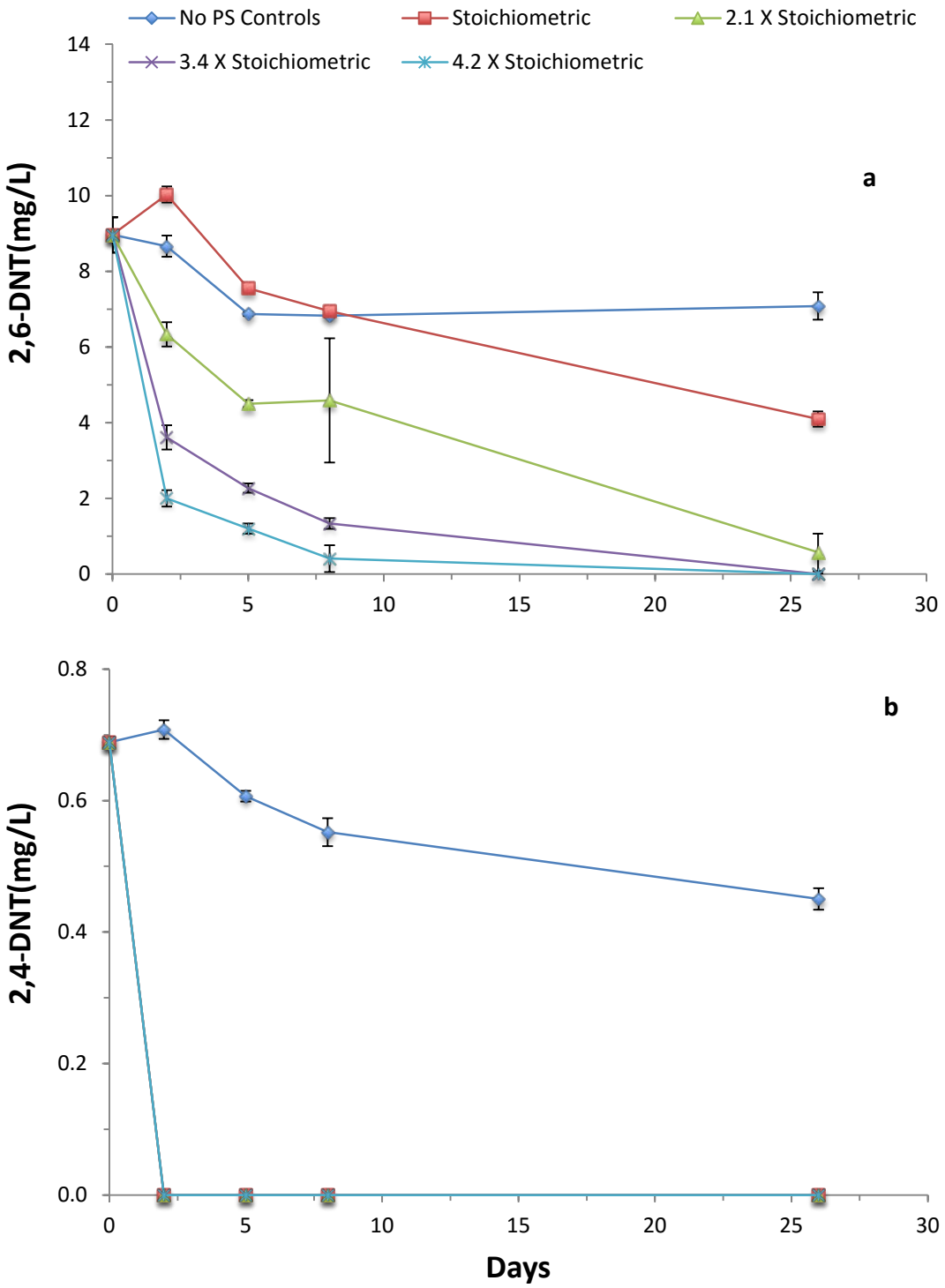

Figure 3.3 Chemical oxidation test results for a) 2,6-DNT; and b) 2,4-DNT. PS = persulfate. Error bars are one standard deviation for triplicate bottles.

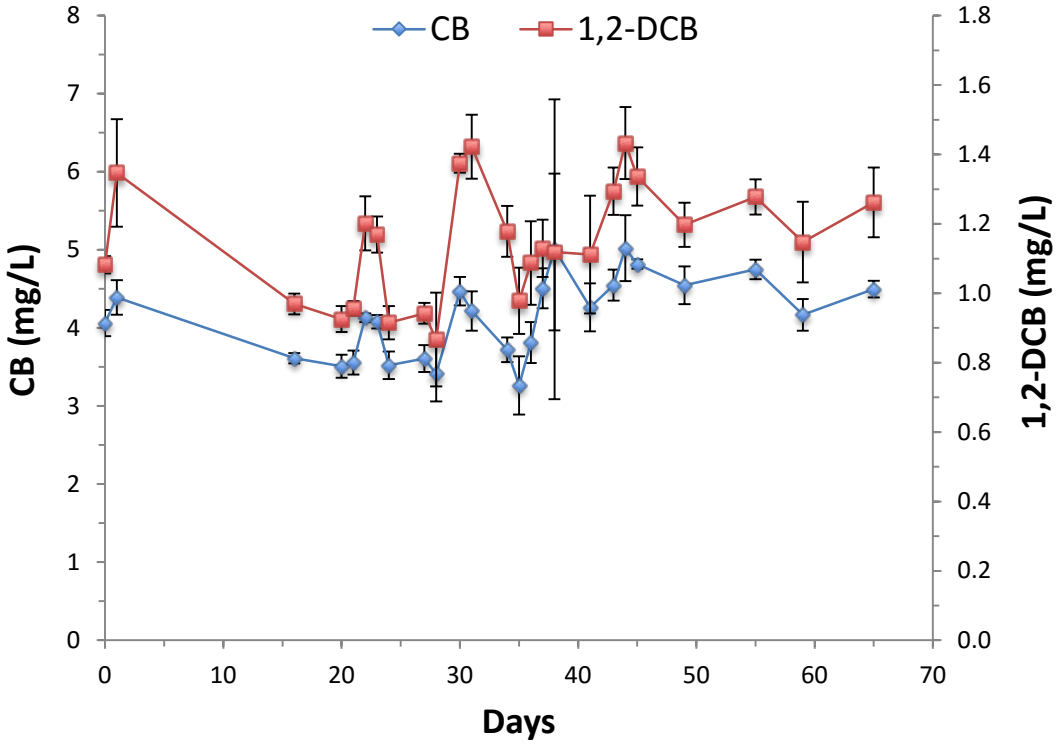

Figure 3.4 Water controls for the initial aerobic microcosms. Error bars represent one standard deviation for triplicate bottles.

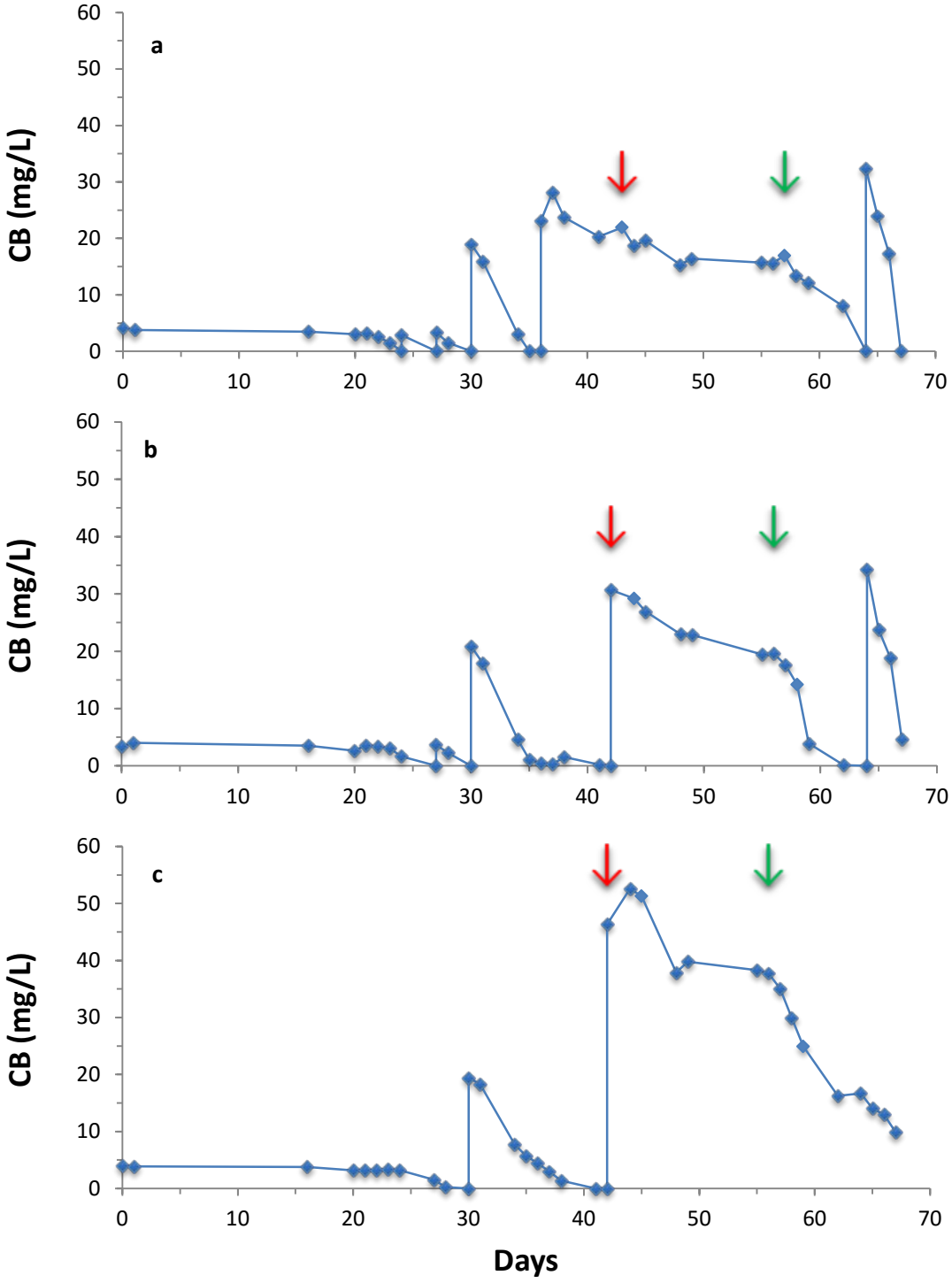

Figure 3.5 Microcosm results for CB in a) bottle #1; b) bottle #2; and c) bottle #3;↓ = pH adjustment; ↓ = addition of BSM.

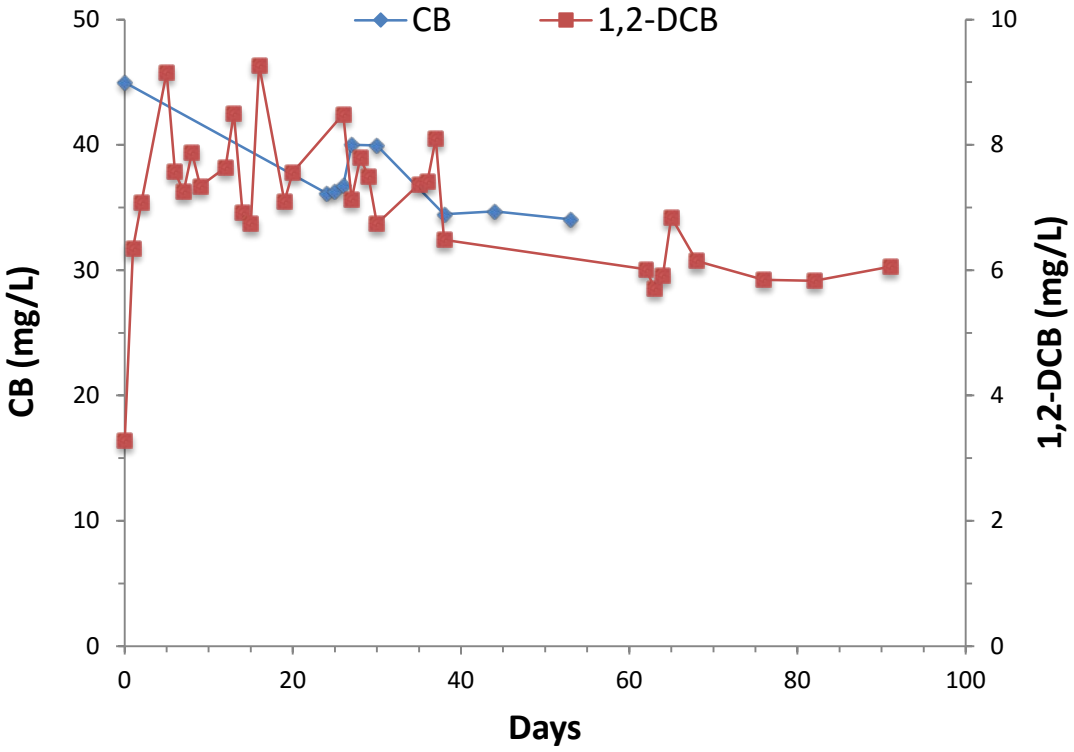

Figure 3.6 CB and 1,2-DCB DDI controls for the development of enrichment cultures.

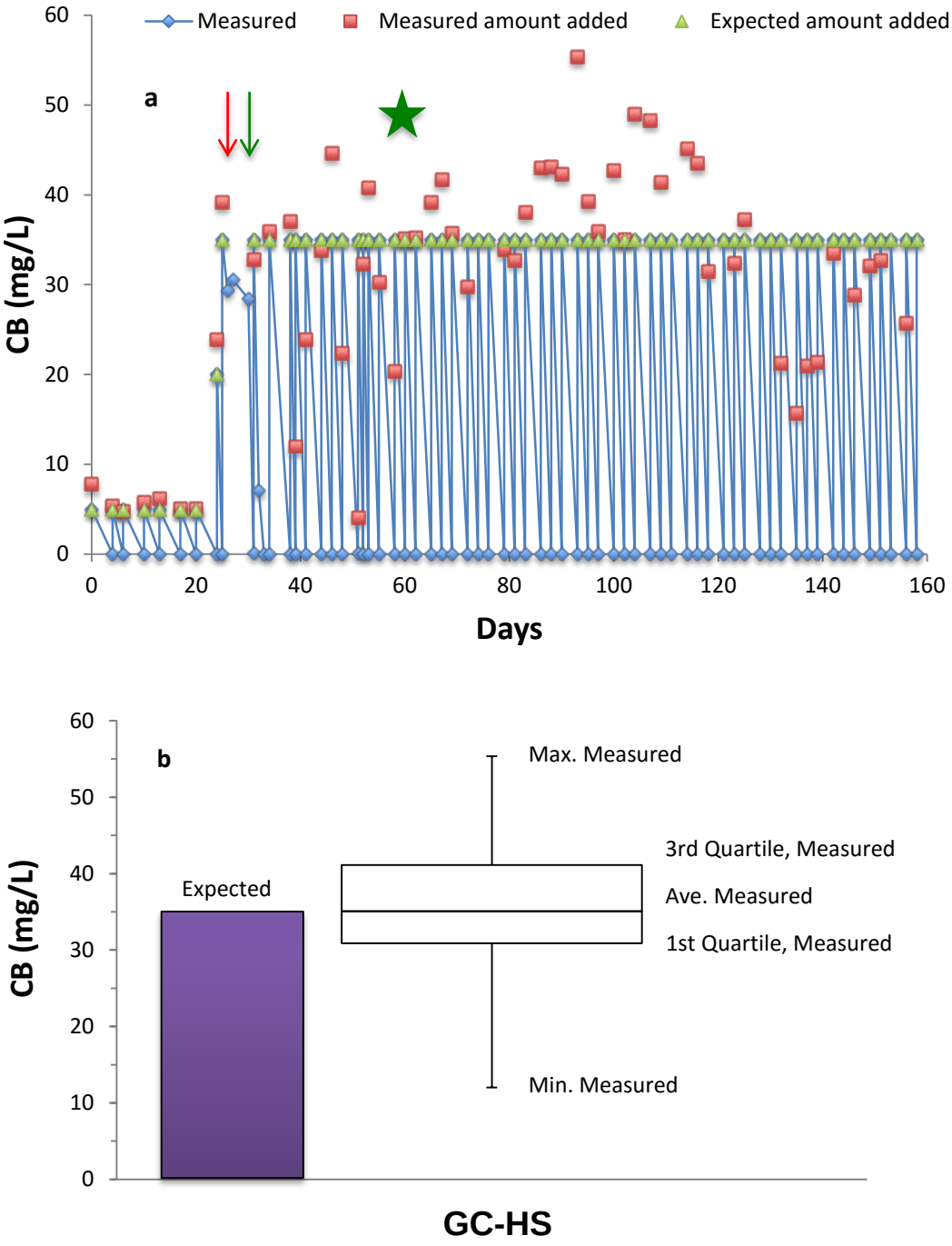

Figure 3.7 Enrichment results for CB for a) measured and expected concentrations based on the volume of saturated water added; and b) box and whisker diagram; ↓ = pH adjustment; ↓ = addition of BSM. ★ = routine additions of CB, O_2, buffer, and BSM.

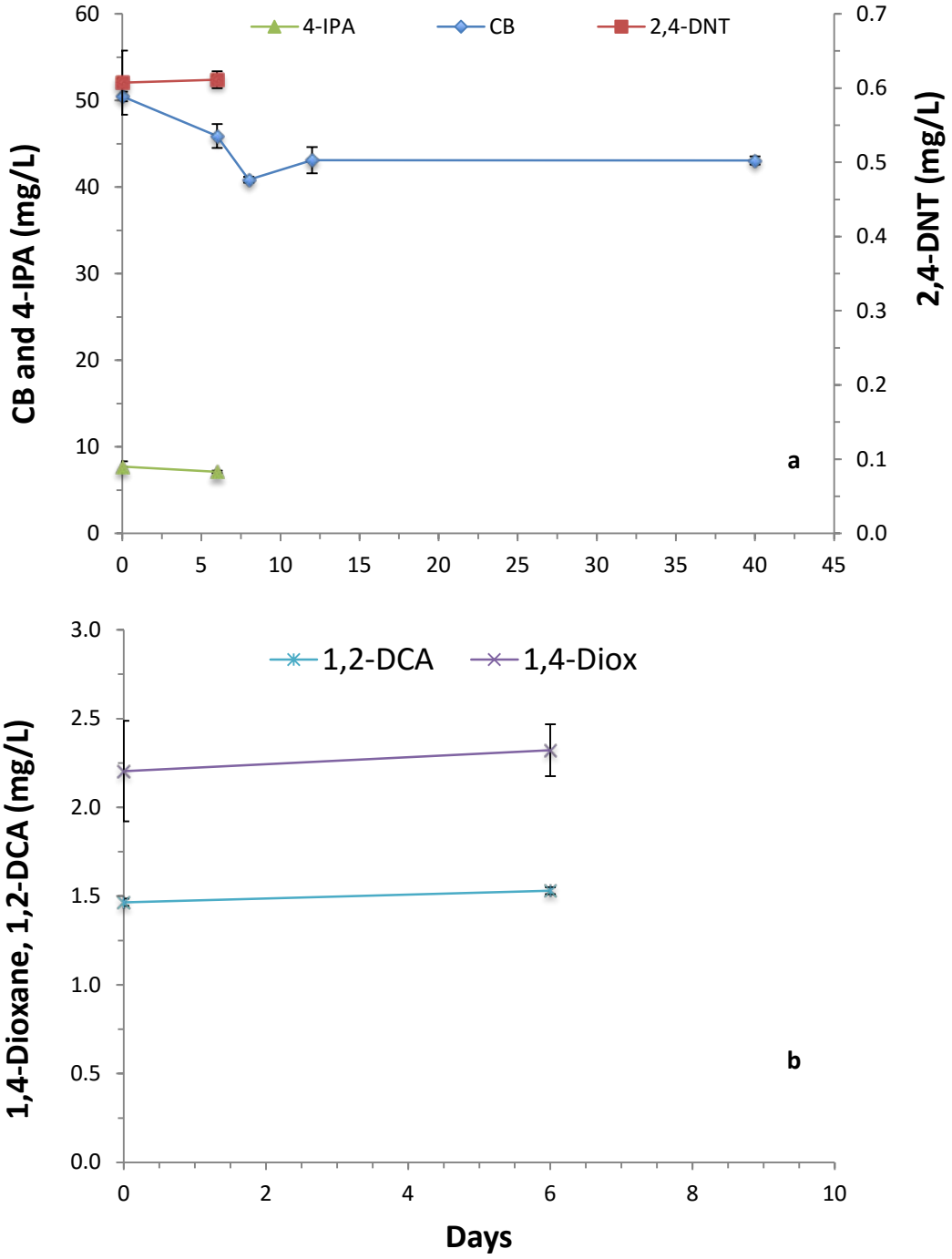

Figure 3.8 Water controls for CB high concentration inhibition experiment #1 for a) CB, 2,4-DNT and 4-IPA; b) 1,4-dioxane and 1,2-DCA. Error bars represent one standard deviation for triplicate bottles.

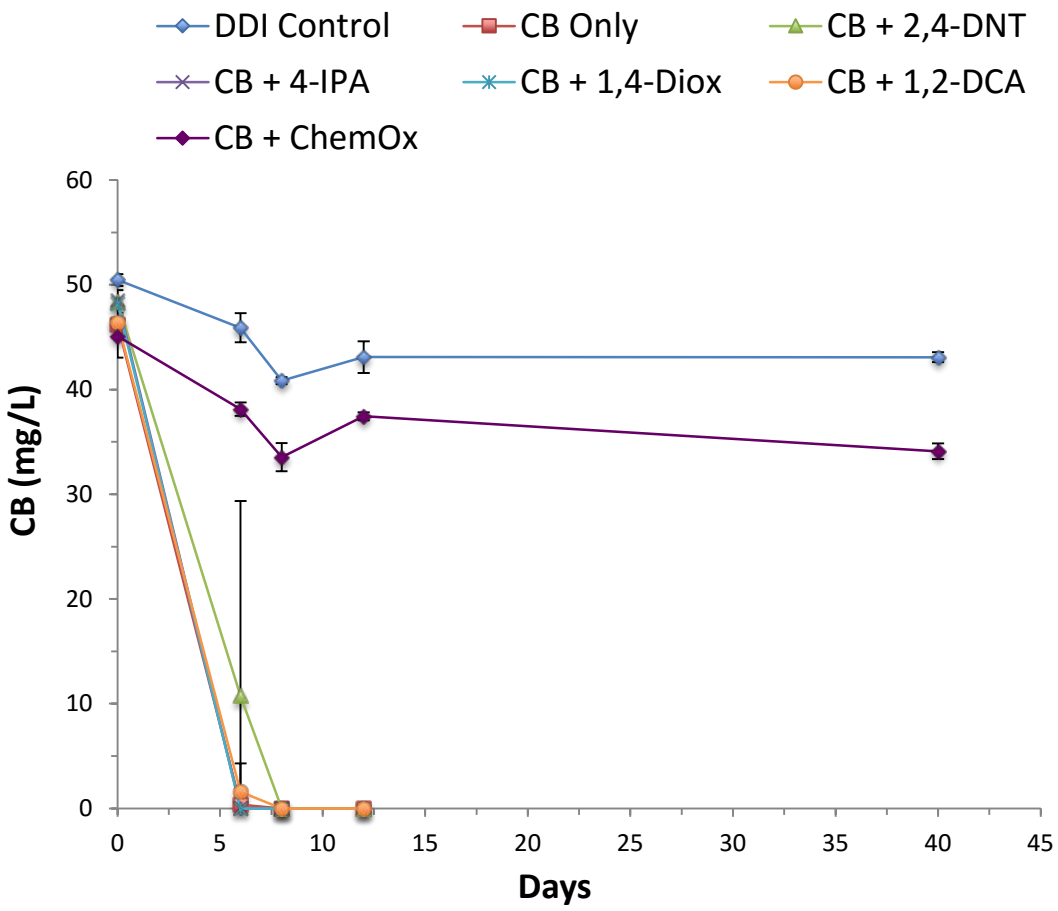

Figure 3.9 Average results for CB high concentration inhibition experiment #1. Error bars represent one standard deviation for triplicate bottles.

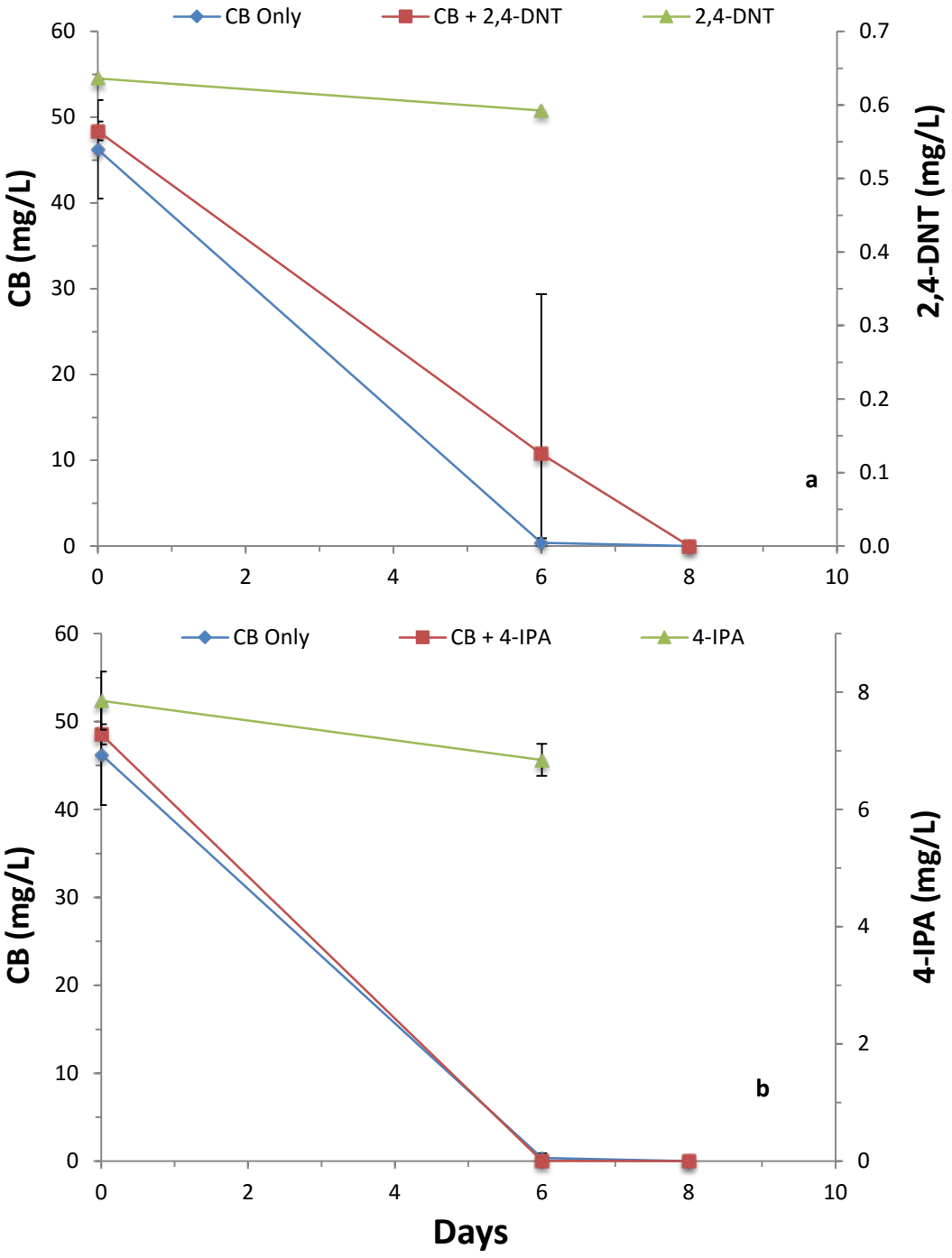

Figure 3.10 CB high concentration inhibition experiment #1 for a) the CB + 2,4-DNT treatment; and b) the CB + 4-IPA treatment. Error bars represent one standard deviation for triplicate bottles.

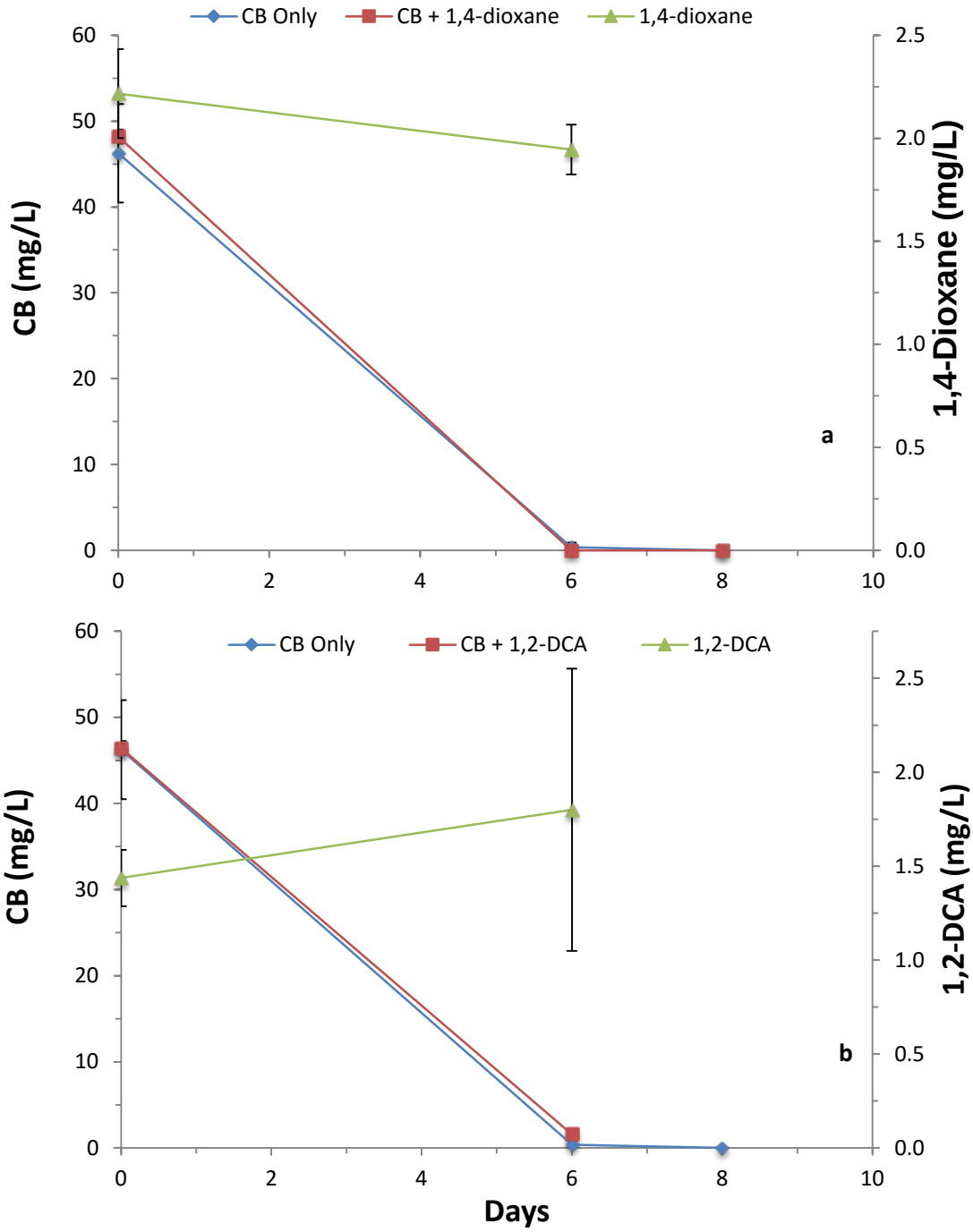

Figure 3.11 CB High concentration inhibition experiment #1 for a) the CB + 1,4-dioxane treatments; and b) the CB + 1,2-DCA treatment. Error bars represent one standard deviation for triplicate bottles.

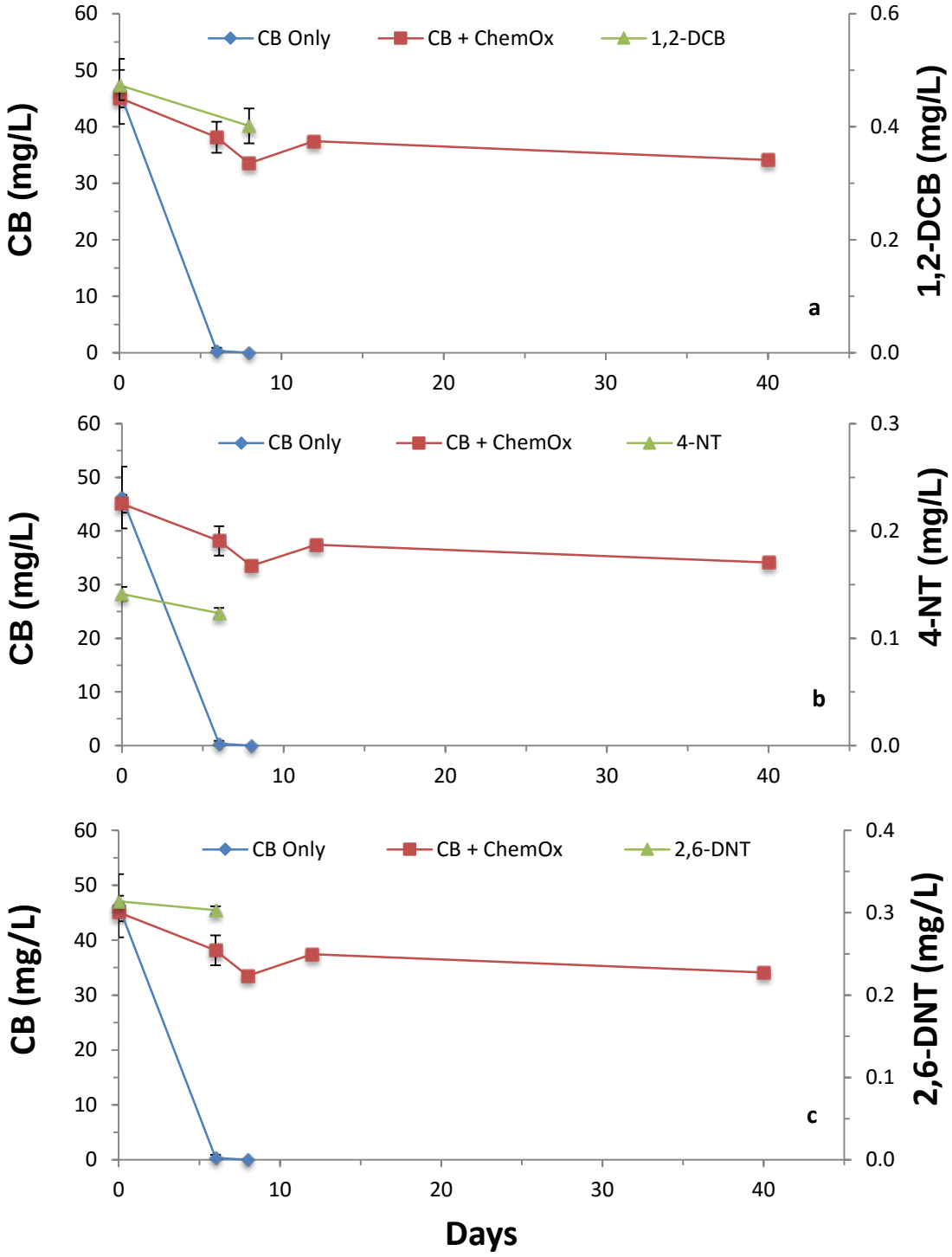

Figure 3.12 CB high concentration inhibition experiment #1for the CB + ChemOx treatment, for a) CB + 1,2-DCB; b) CB + 4-NT; and c) CB + 2,6-DNT. Error bars represent one standard deviation for triplicate bottles.

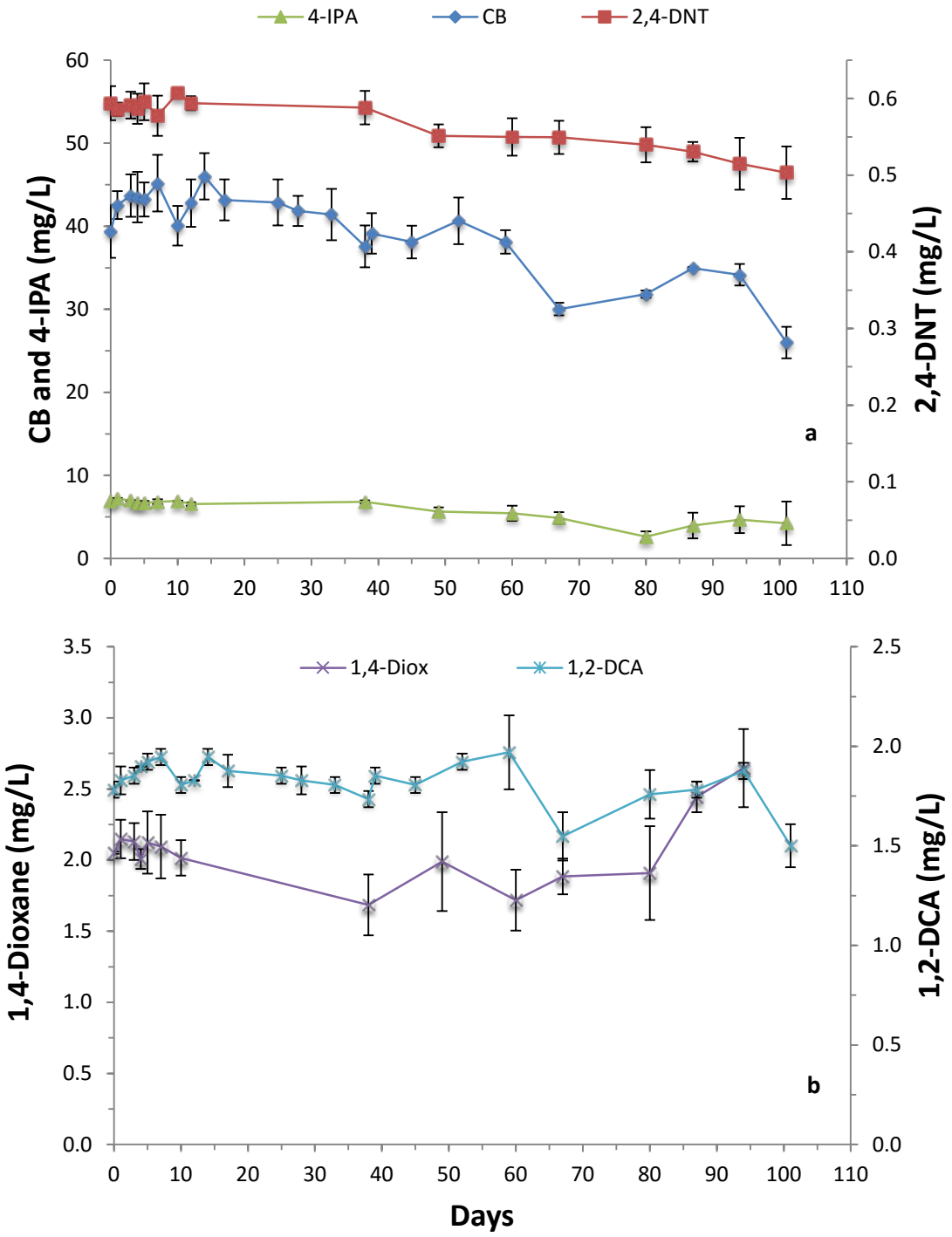

Figure 3.13 Water controls for CB high concentration inhibition experiment #2 for a) CB, 2,4-DNT and 4-IPA; and b) 1,4-dioxane and 1,2-DCA. Error bars represent one standard deviation for triplicate bottles.

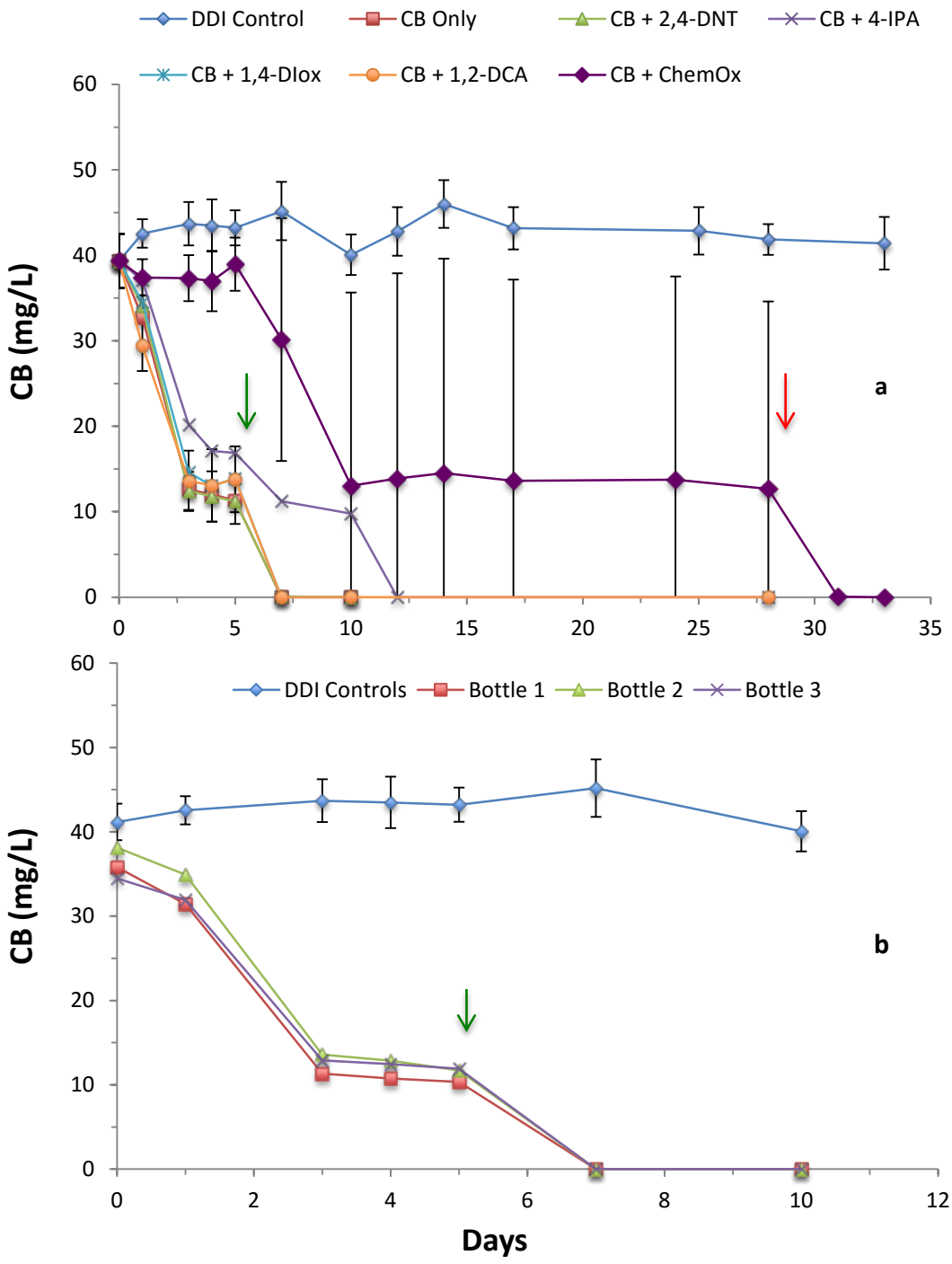

Figure 3.14 Initial results for CB high concentration inhibition experiment #2 for a) CB in all treatments; and b) individual bottles for the CB-only treatment; ↓ = addition of BSM. ↓ = addition of BSM and inoculum. Error bars represent one standard deviation for triplicate bottles.

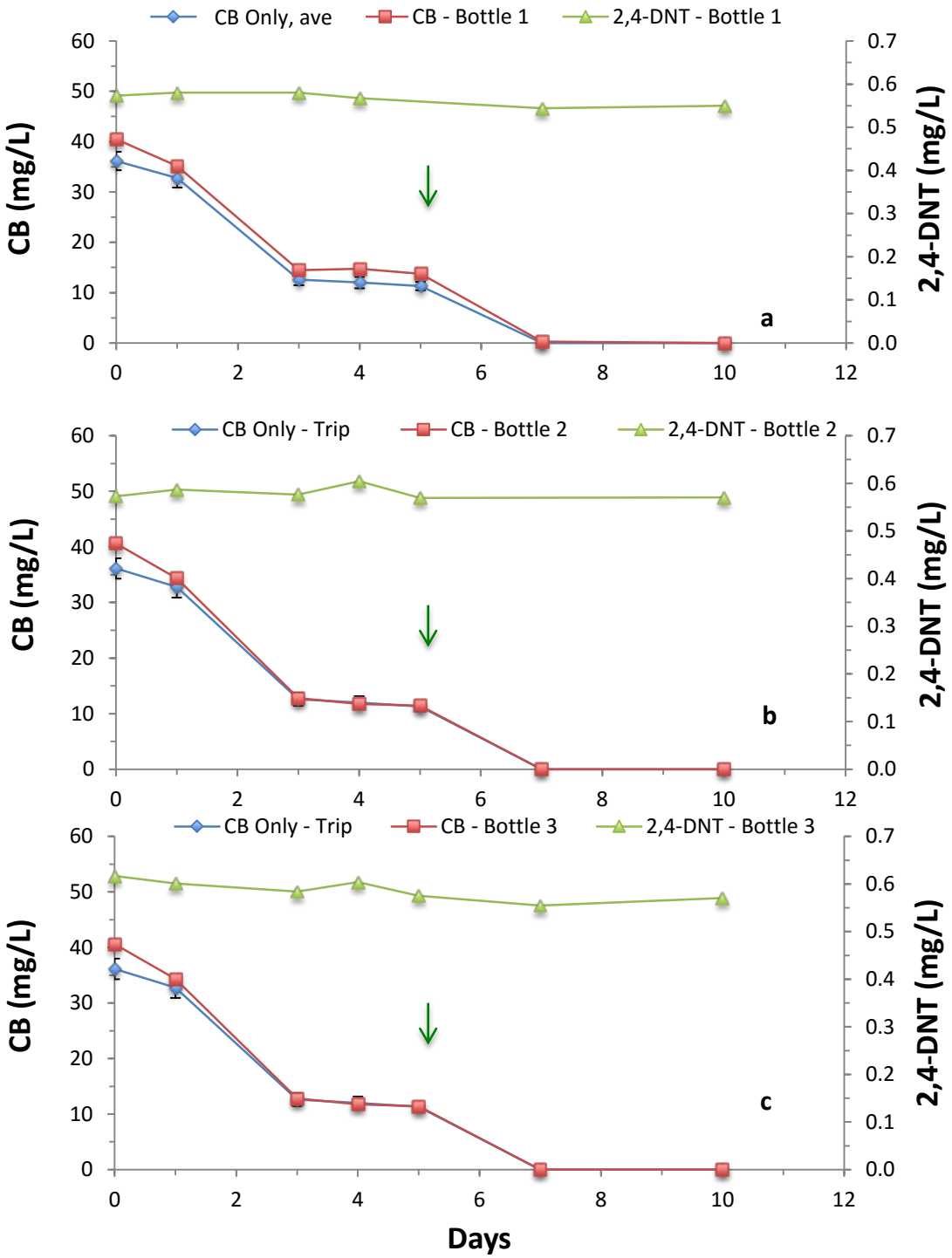

Figure 3.15 Initial results for CB high concentration inhibition experiment #2 for the CB + 2,4-DNT treatment in a) bottle #1; b) bottle #2; and c) bottle #3; ↓ = addition of BSM. Error bars represent one standard deviation for triplicate bottles.

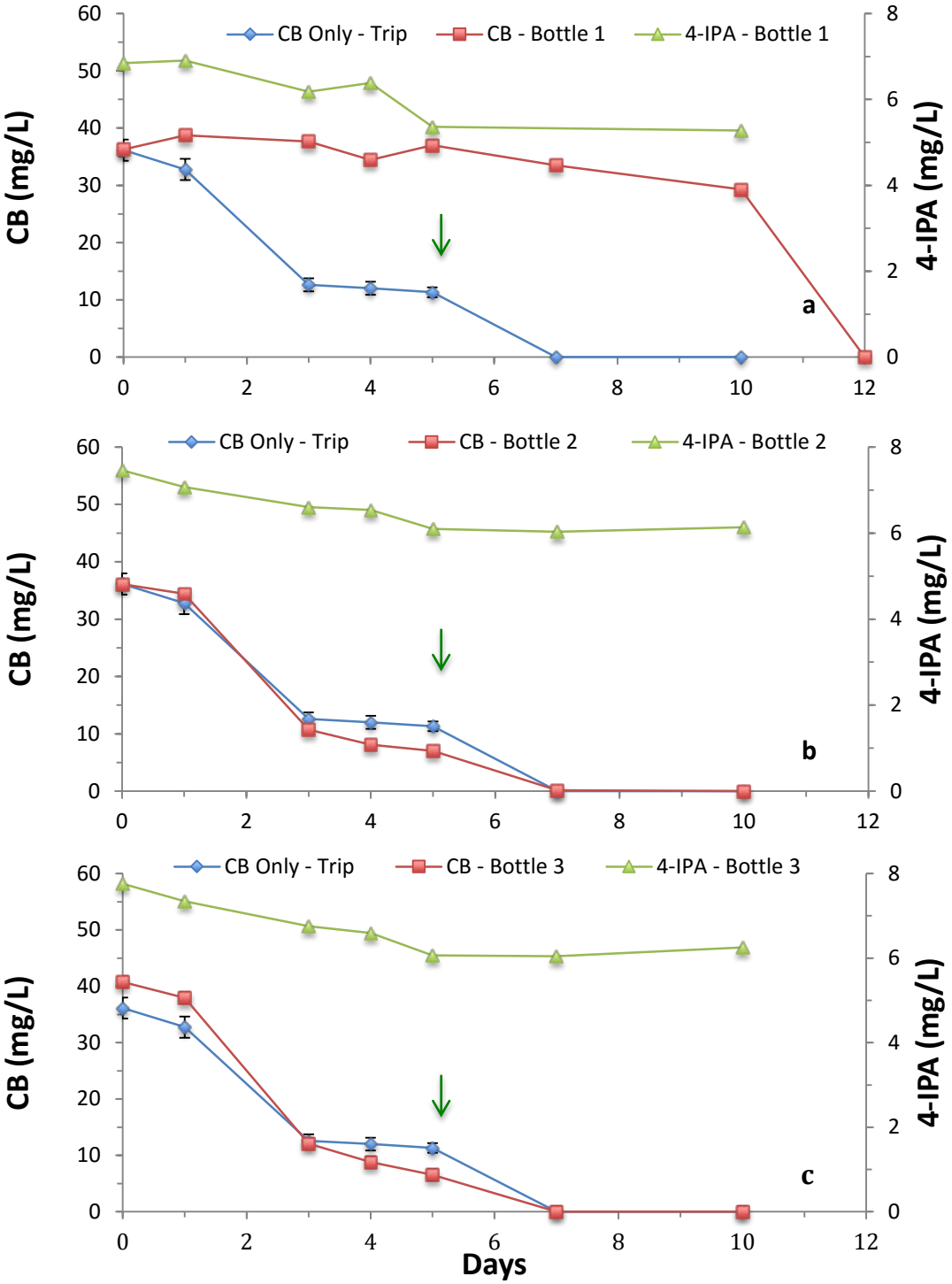

Figure 3.16 Initial results for CB high concentration inhibition experiment #2 for the CB + 4-IPA treatment in a) bottle #1; b) bottle #2; and c) bottle # ↓ = addition of BSM. Error bars represent one standard deviation for triplicate bottles.

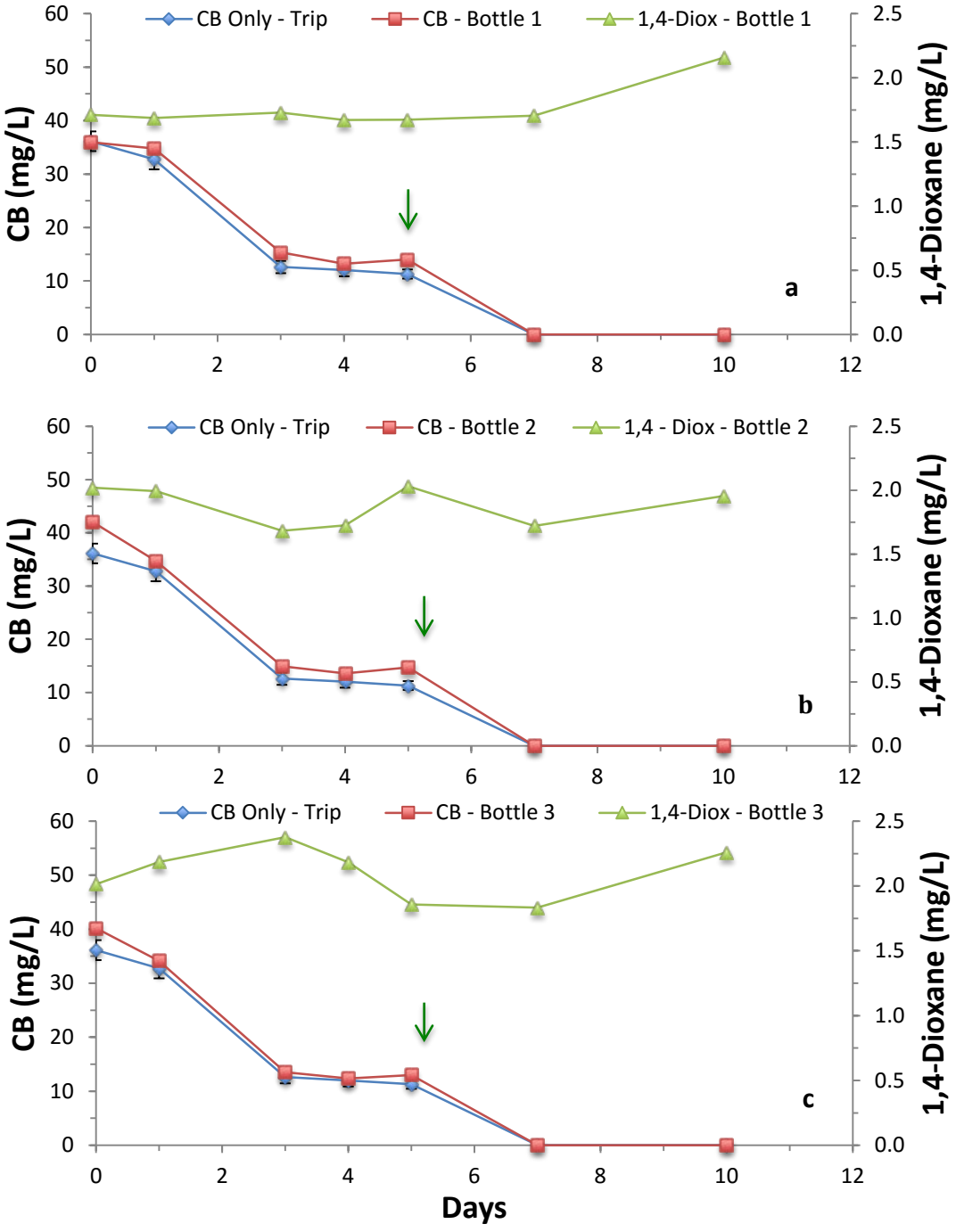

Figure 3.17 Initial results for CB high concentration inhibition experiment #2 for the CB + 1,4-dioxane treatment in a) bottle #1; b) bottle #2; and c) bottle #3;↓ = addition of BSM. Error bars represent one standard deviation for triplicate bottles.

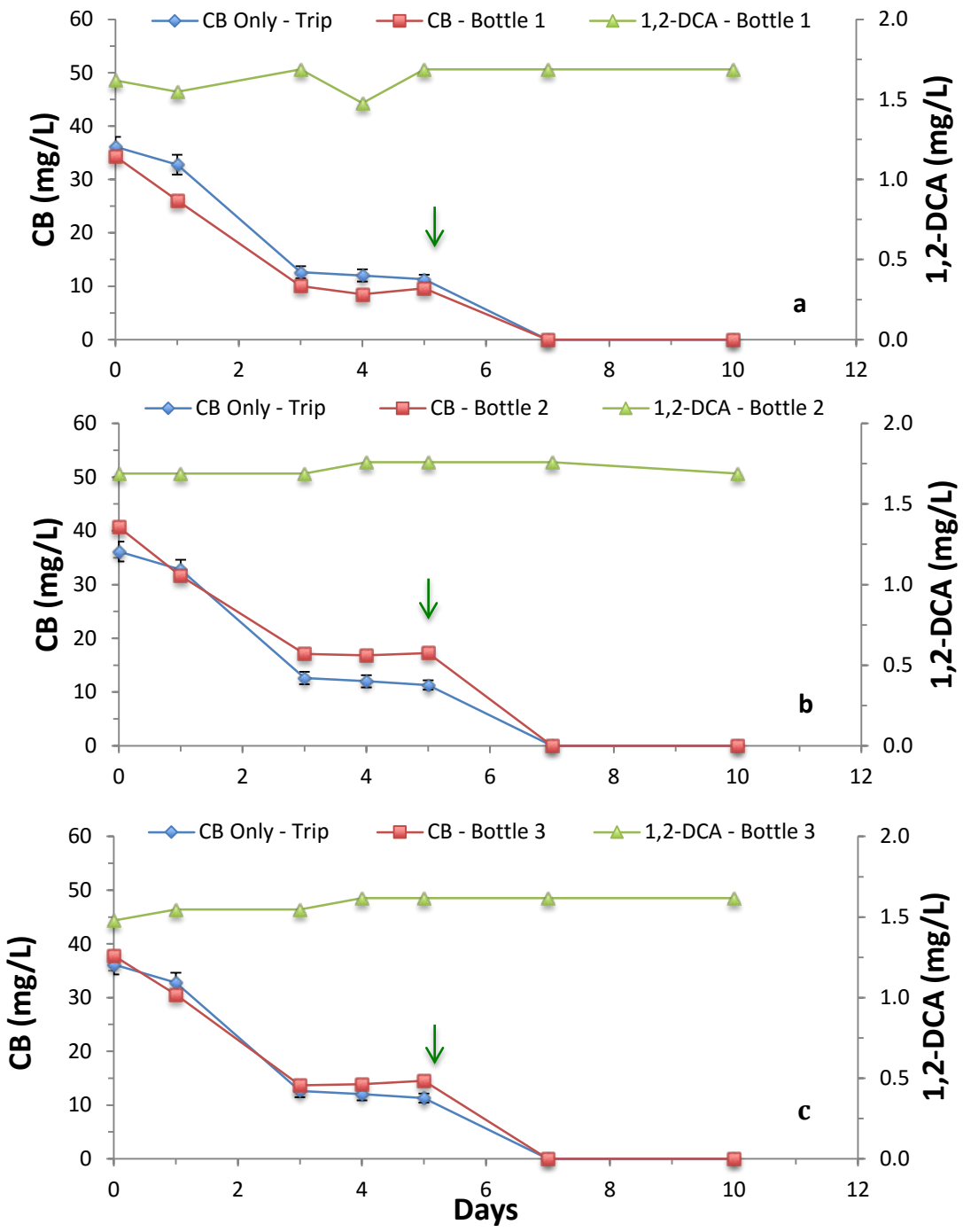

Figure 3.18 Initial results for CB high concentration inhibition experiment #2 for the CB + 1,2-DCA treatment in; a) bottle #1; b) bottle #2; and c) bottle #3; ↓ = addition of BSM. Error bars represent one standard deviation for triplicate bottles.

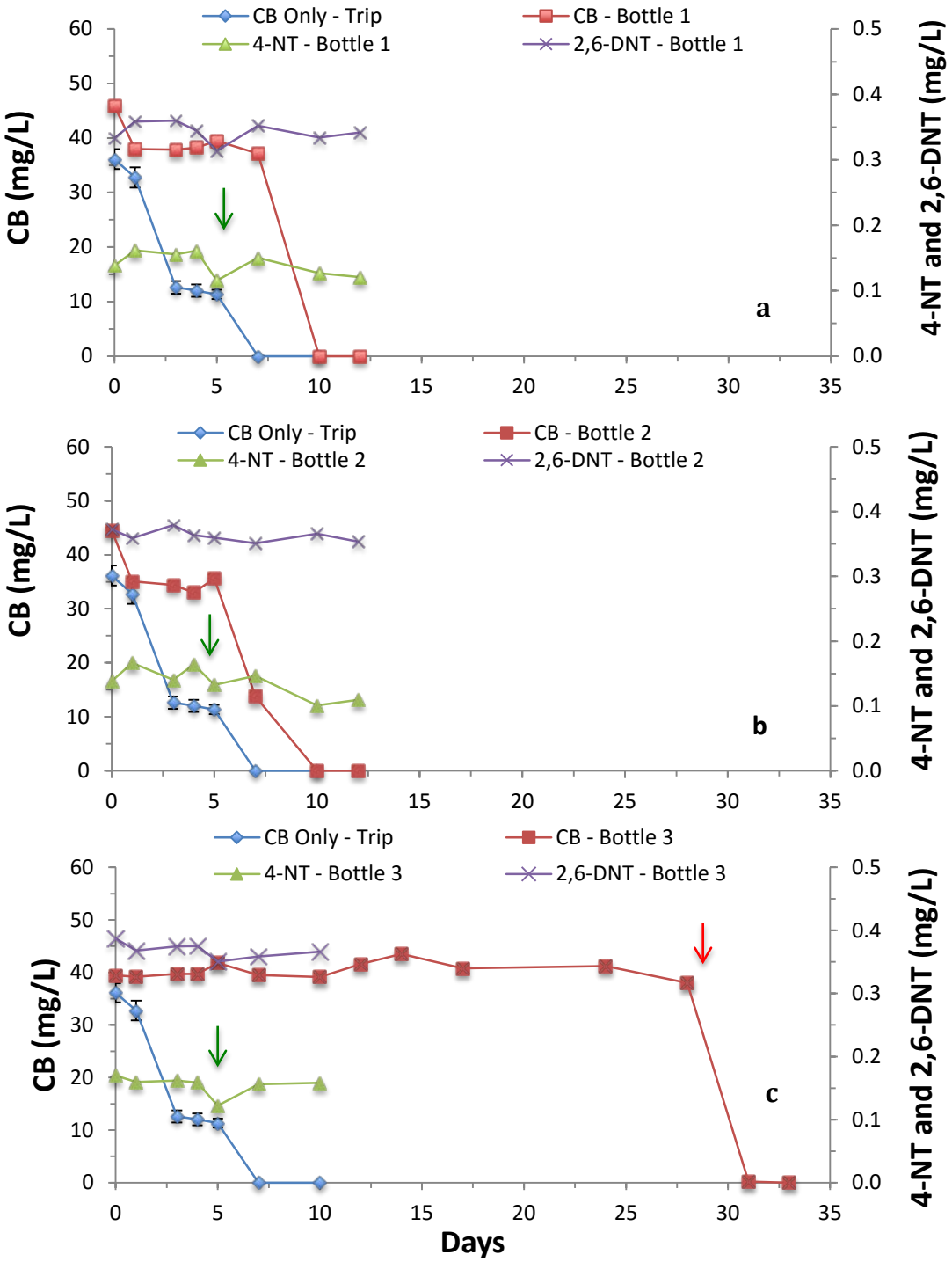

Figure 3.19 Initial results for CB high concentration inhibition experiment #2 for the CB + chemical oxidation GW in; a) bottle #1; b) bottle #2; and c) bottle #3; ↓ = addition of BSM. ↓ = addition of BSM and inoculum. Error bars represent one standard deviation for triplicate bottles.

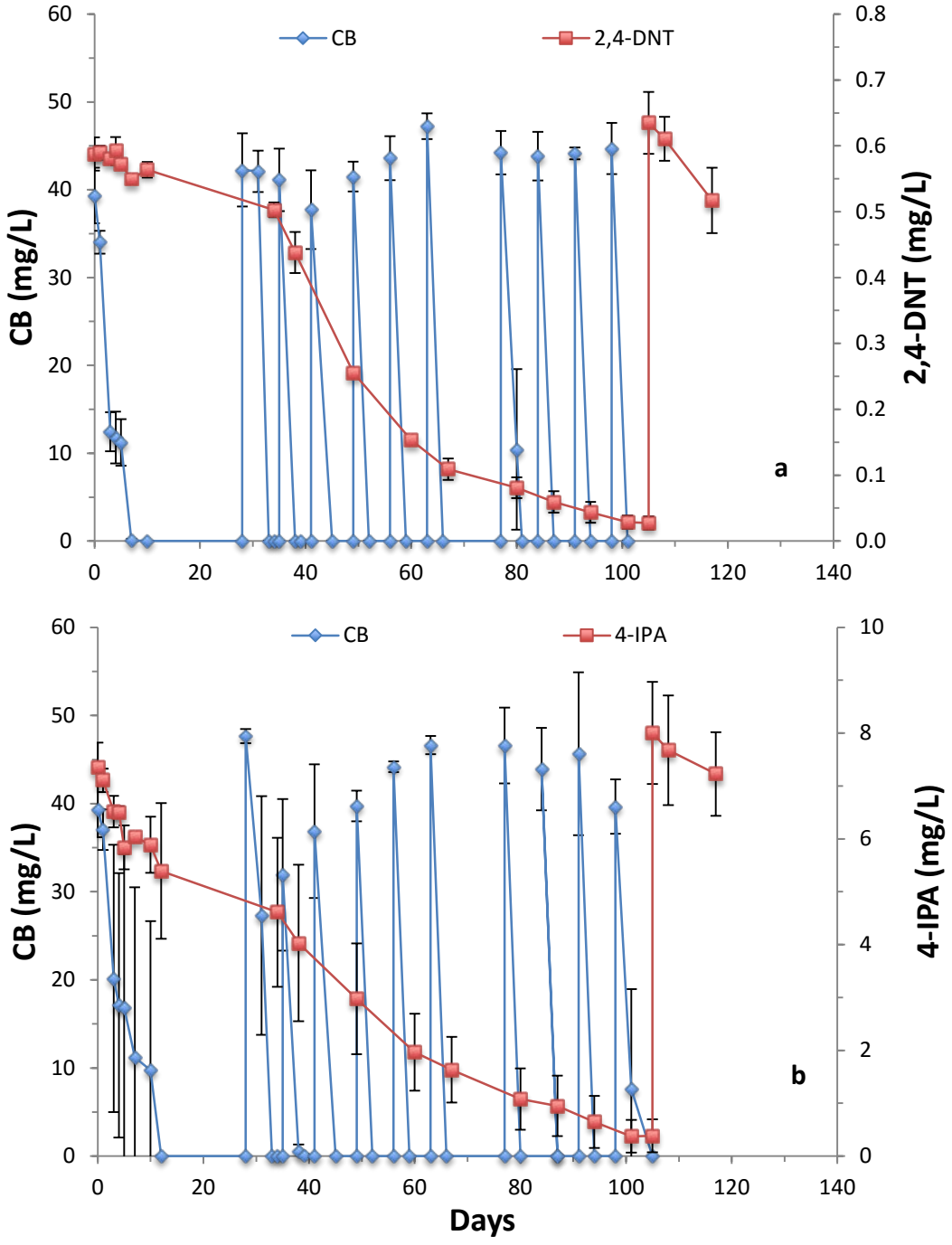

Figure 3.20 Effect of repeated additions of CB during the CB high concentration inhibition experiment #2 for a) the CB + 2,4-DNT treatment; and b) the CB + 4-IPA treatment. Error bars represent one standard deviation for triplicate bottles.

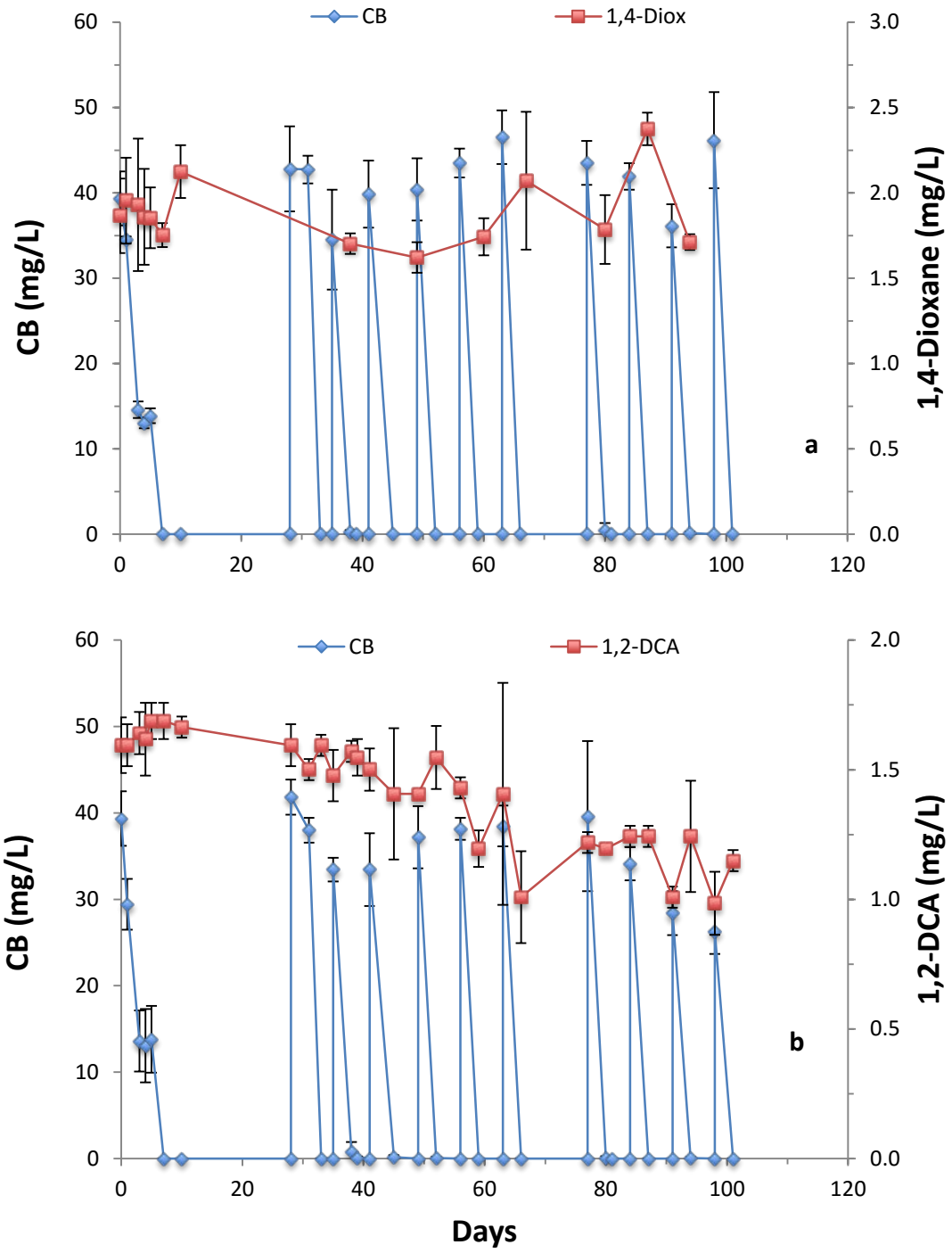

Figure 3.21 Effect of repeated additions of CB during the CB high concentration inhibition experiment #2 for a) the CB + 1,4-dioxane treatment; and b) the CB + 1,2-DCA treatment. Error bars represent one standard deviatios for triplicate bottles.

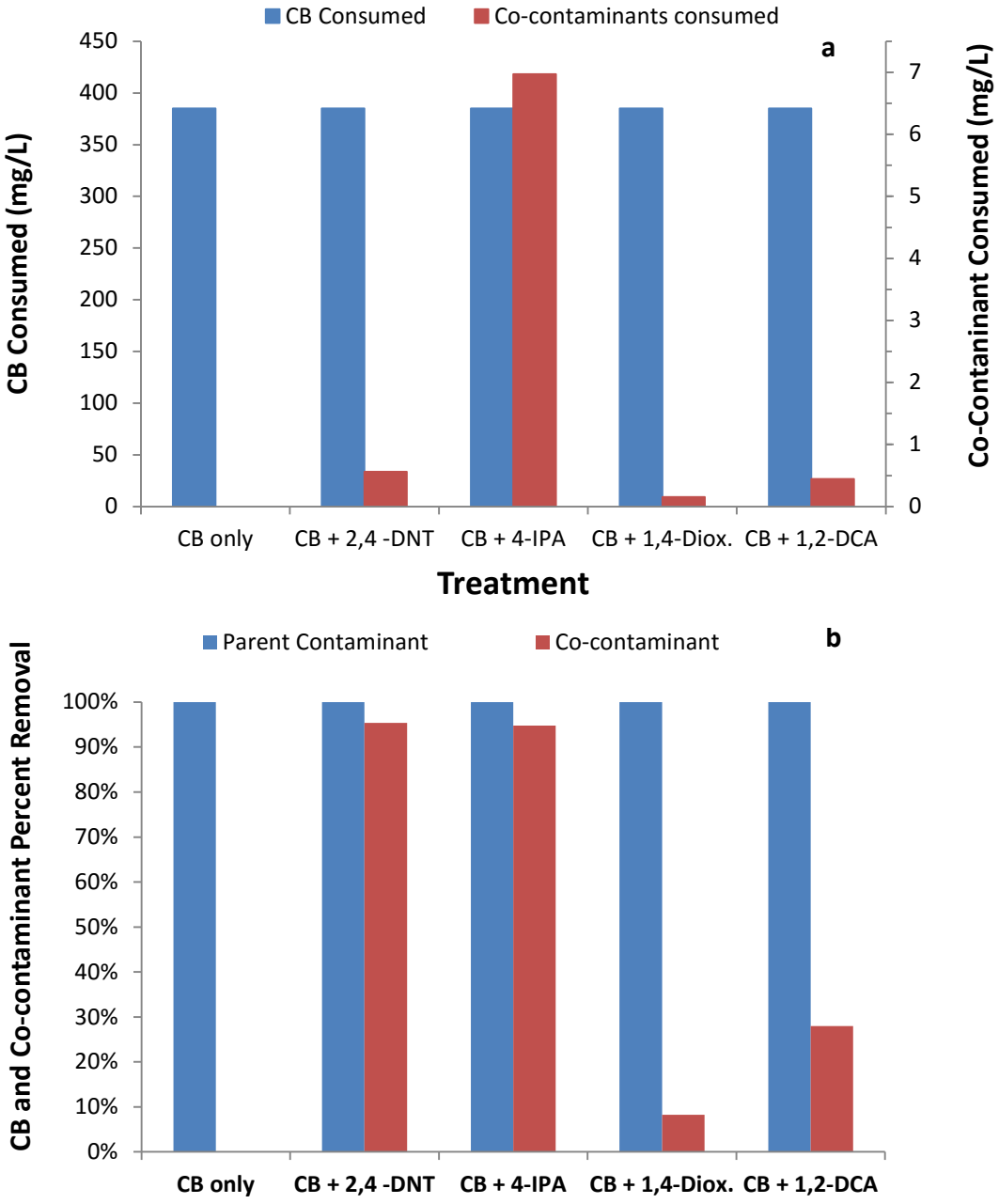

Figure 3.22 Effect of repeated consumption of CB during the CB high concentration inhibition experiment #2 on a) CB and co-contaminant removal (mg/L) in all treatments; and b) CB and co-contaminant percent removal (%) in all treatments.

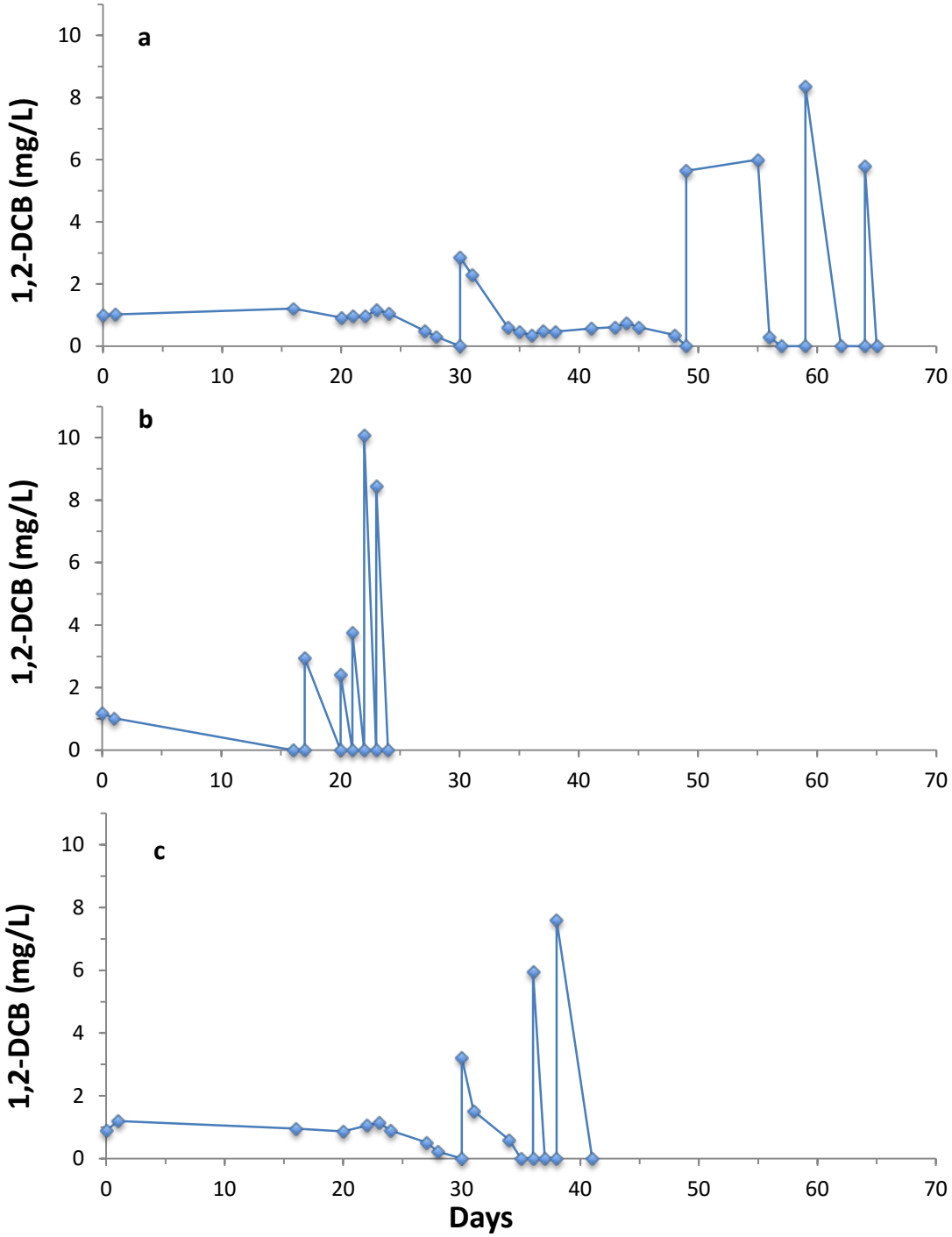

Figure 3.23 Microcosm results for 1,2-DCB in a) bottle #1; b) bottle #2; and c) bottle #3.

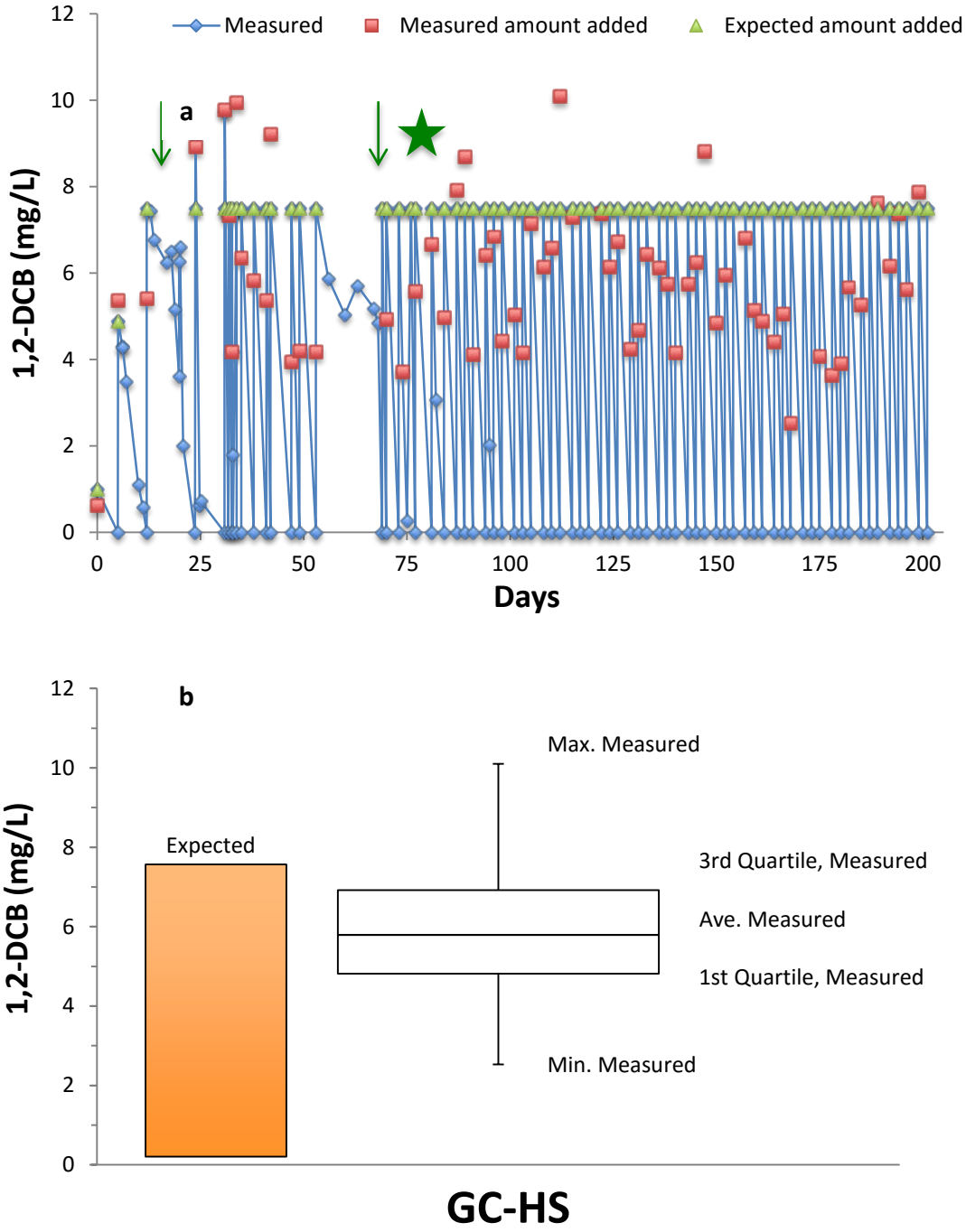

Figure 3.24 Enrichment culture results for 1,2-DCB for a) measured and expected concentrations based on the volume of saturated water added; and b) box and whisker diagram; ↓ = addition of BSM; ★ = routine addition of 1,2-DCB, O_2, buffer, and BSM.

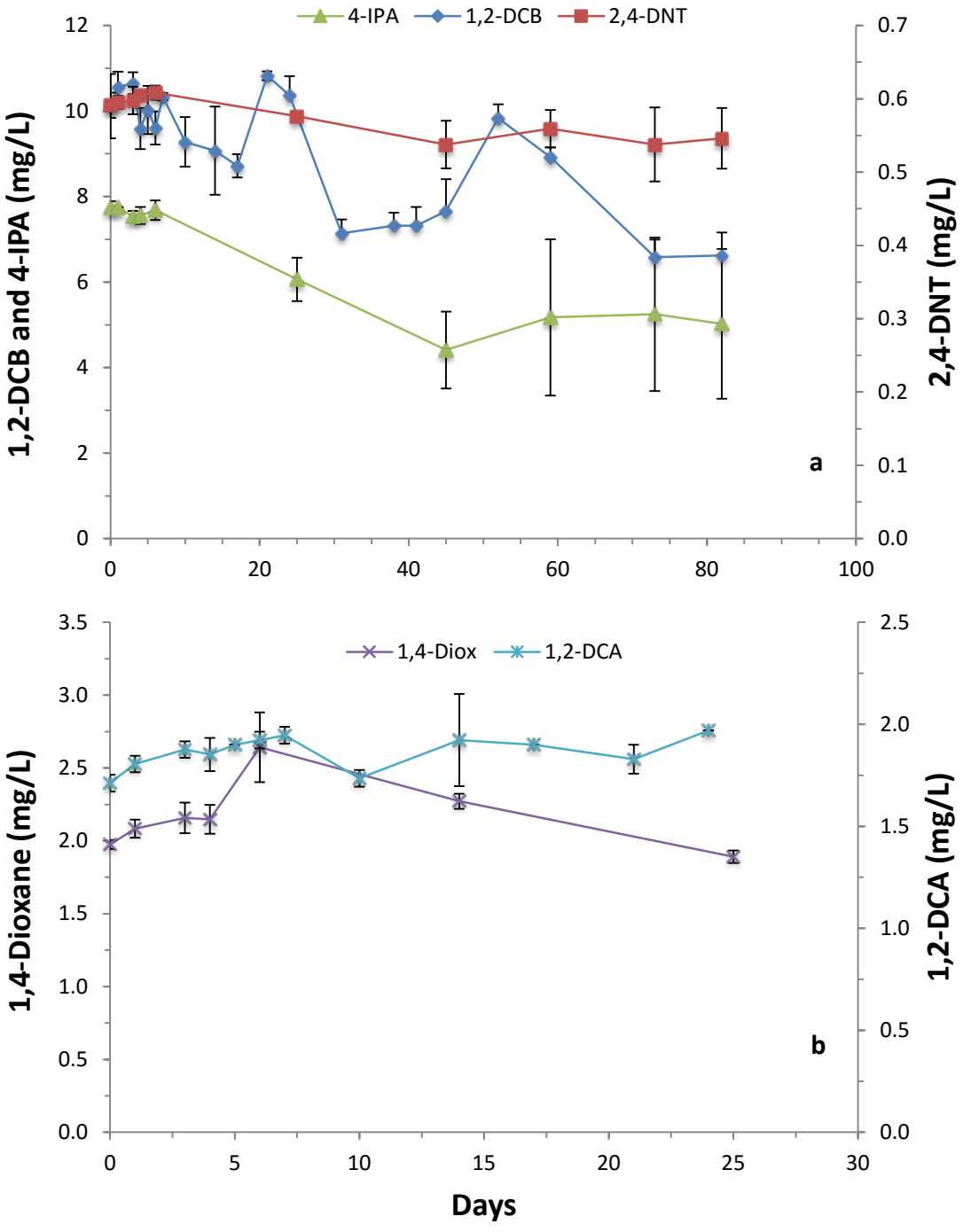

Figure 3.25 DDI water controls for the1,2-DCB high concentration inhibition experiment for a) 1,2-DCB, 4-IPA and 2,4-DNT; and b) 1,4-dioxane and 1,2-DCA.

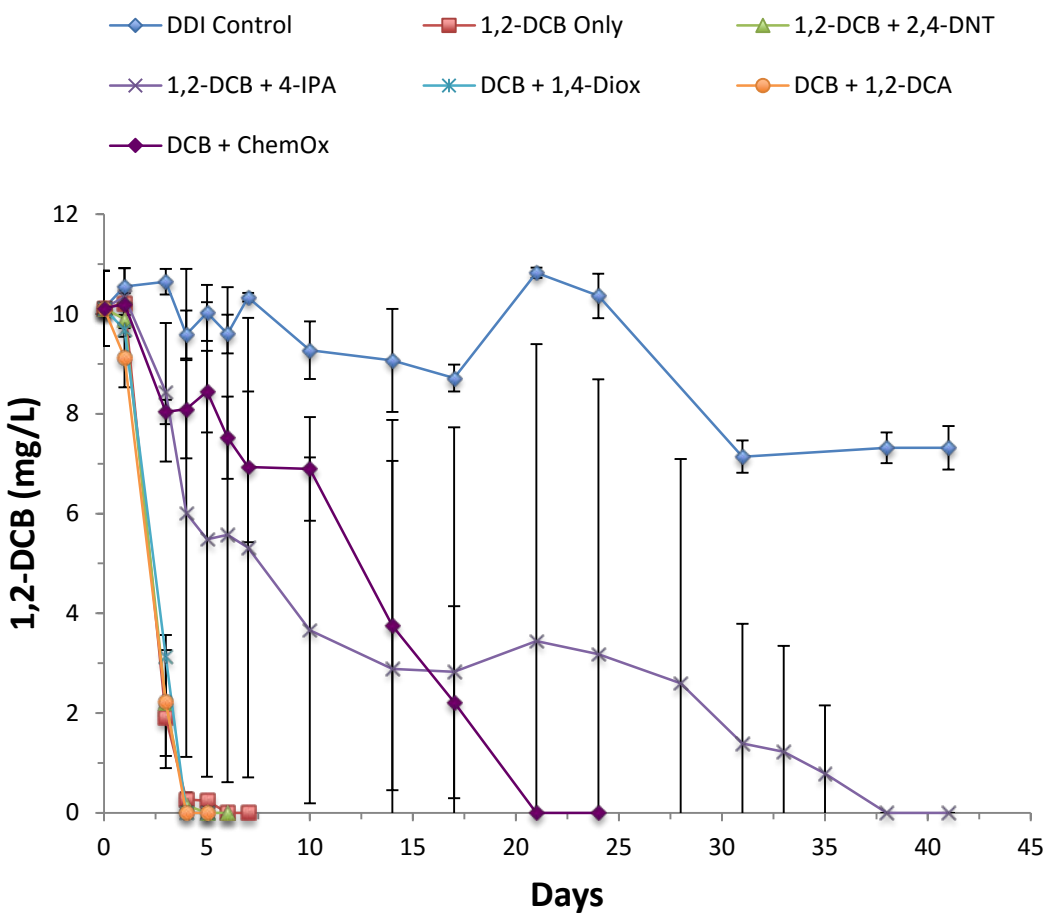

Figure 3.26 Initial results for all treatments evaluated for the 1,2-DCB high concentration inhibition experiment. Error bars represent one standard deviation for triplicate bottles.

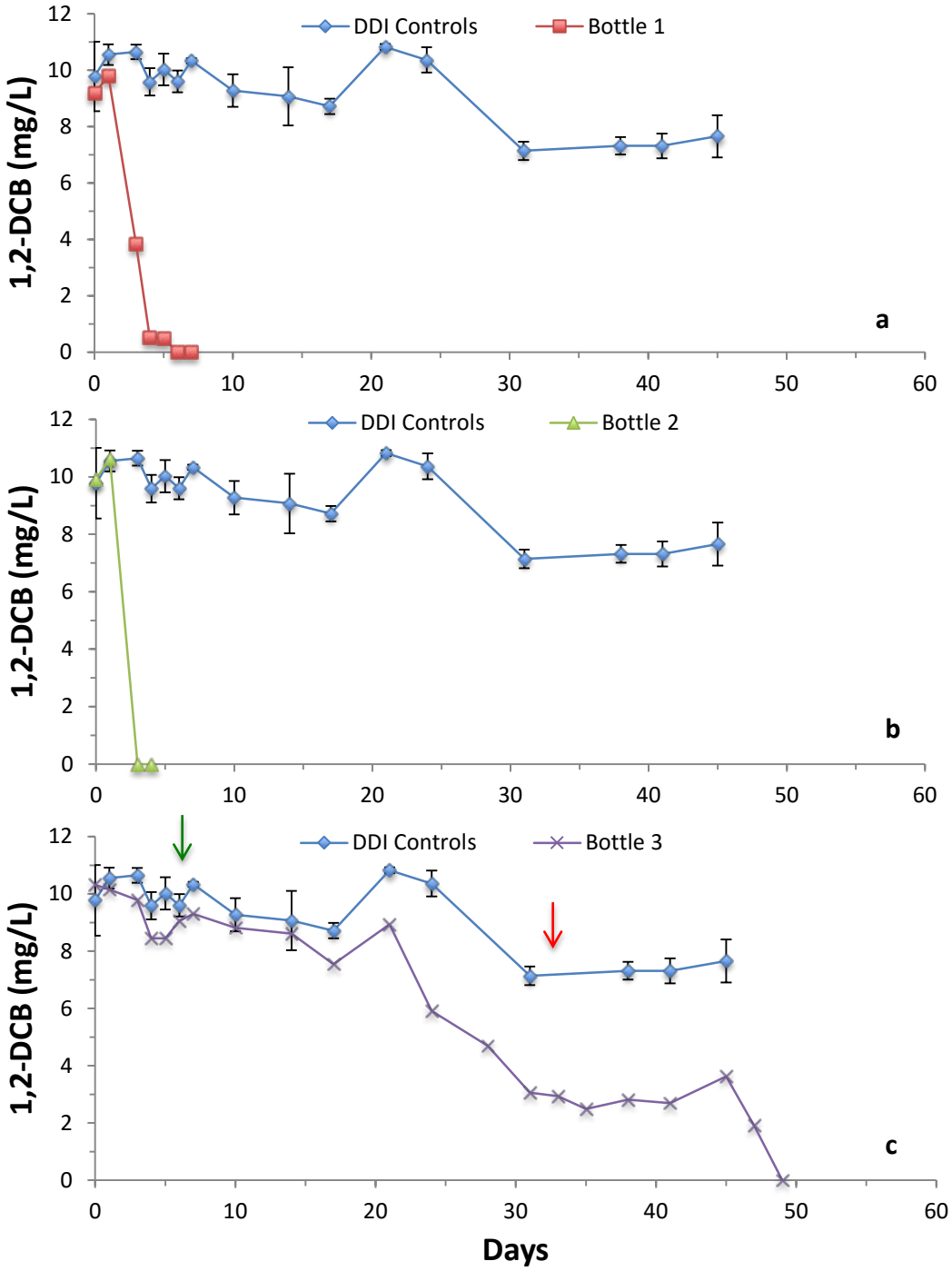

Figure 3.27 Initial results for 1,2-DCB high concentration inhibition experiment #2 for the 1,2-DCB-only treatment in a) bottle #1; b) bottle #2; and c) bottle #3; ↓ = addition of BSM; ↓ = addition of BSM and inoculum.

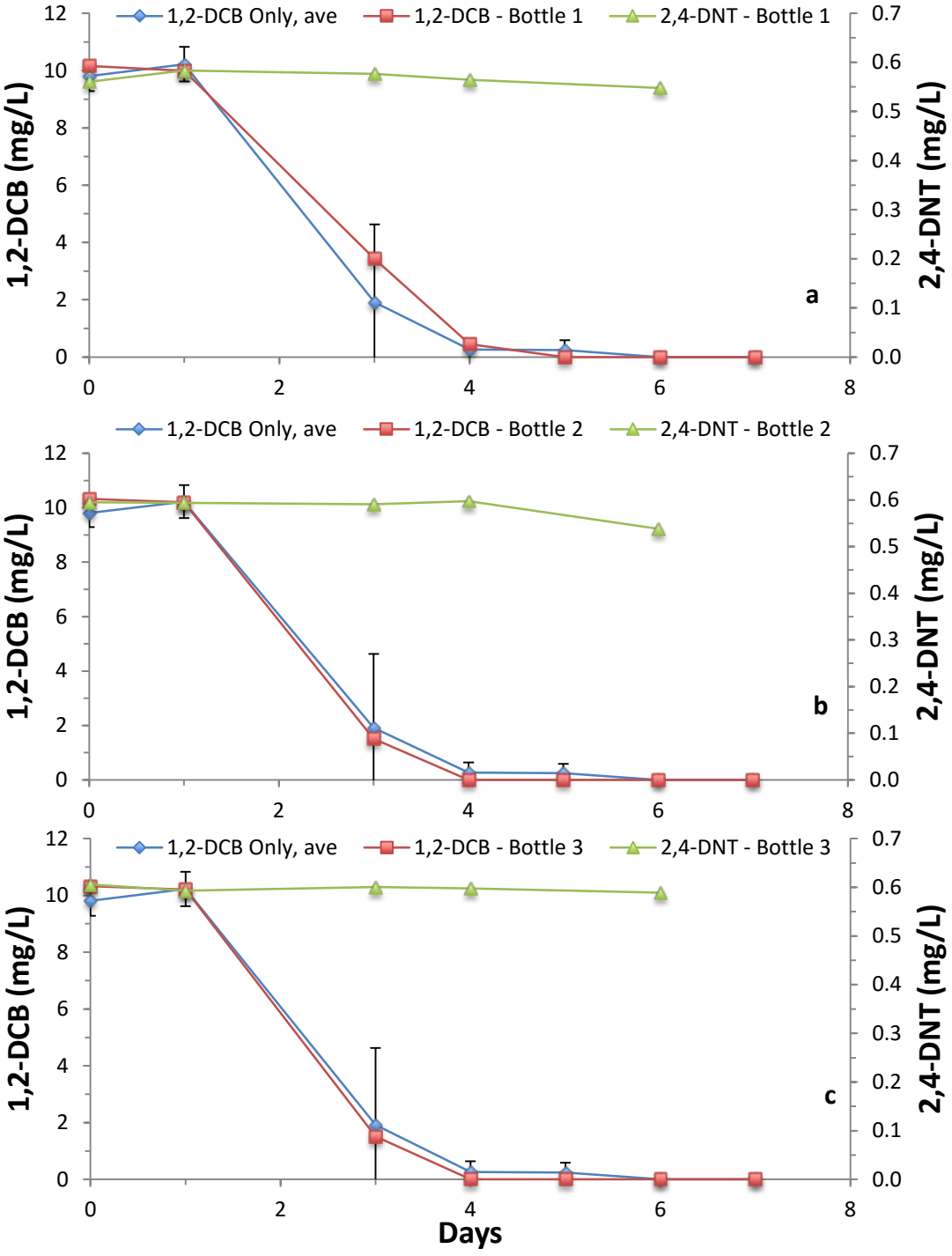

Figure 3.28 Initial results for the 1,2-DCB high concentration inhibition experiment in the 1,2-DCB + 2,4-DNT treatment for a) bottle #1; b) bottle #2; and c) bottle #3. Error bars represent one standard deviation for bottles #1 and #2 of the 1,2-DCB-only treatment.

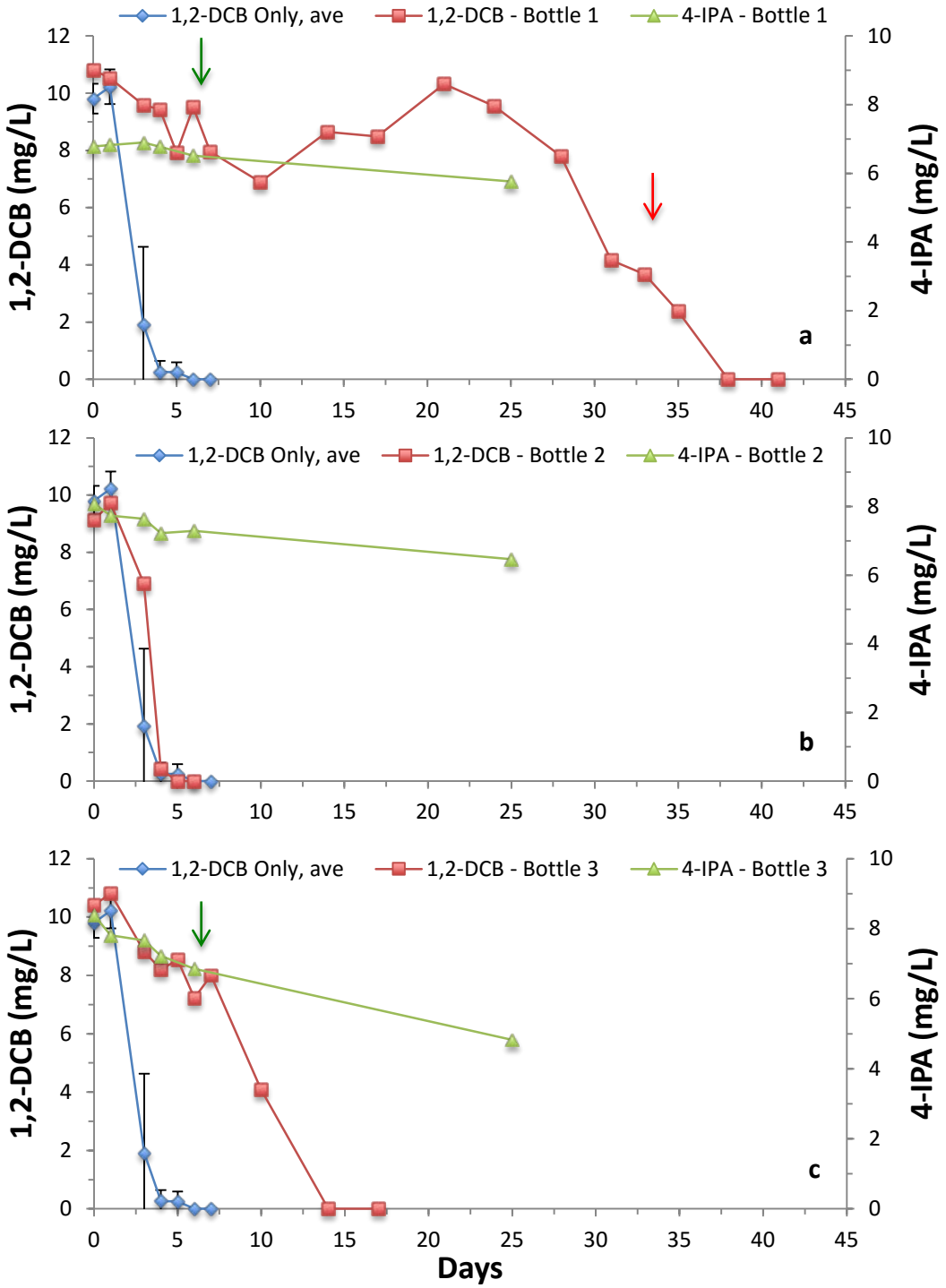

Figure 3.29 Initial results for the 1,2-DCB high concentration inhibition experiment in the 1,2-DCB + 4-IPA treatment for a) bottle #1; b) bottle #2; and c) bottle #3. Error bars represent one standard deviation for bottles #1 and #2 of the 1,2-DCB-only treatment.

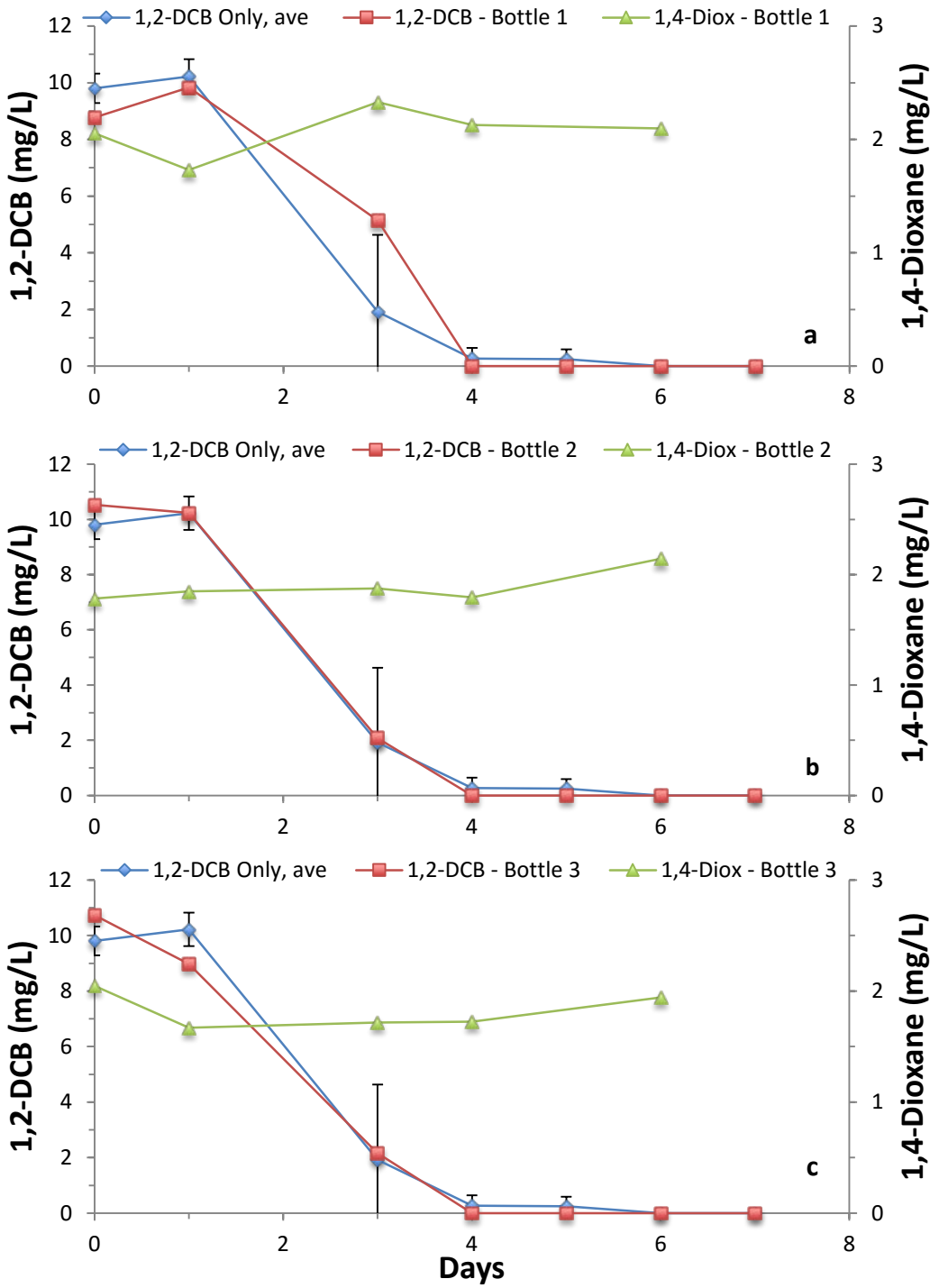

Figure 3.30 Initial results for the 1,2-DCB high concentration inhibition experiment in the 1,2-DCB + 1,4-dioxane treatment for a) bottle #1; b) bottle #2; and c) bottle #3. Error bars represent one standard deviation for bottles #1 and #2 of the 1,2-DCB-only treatment.

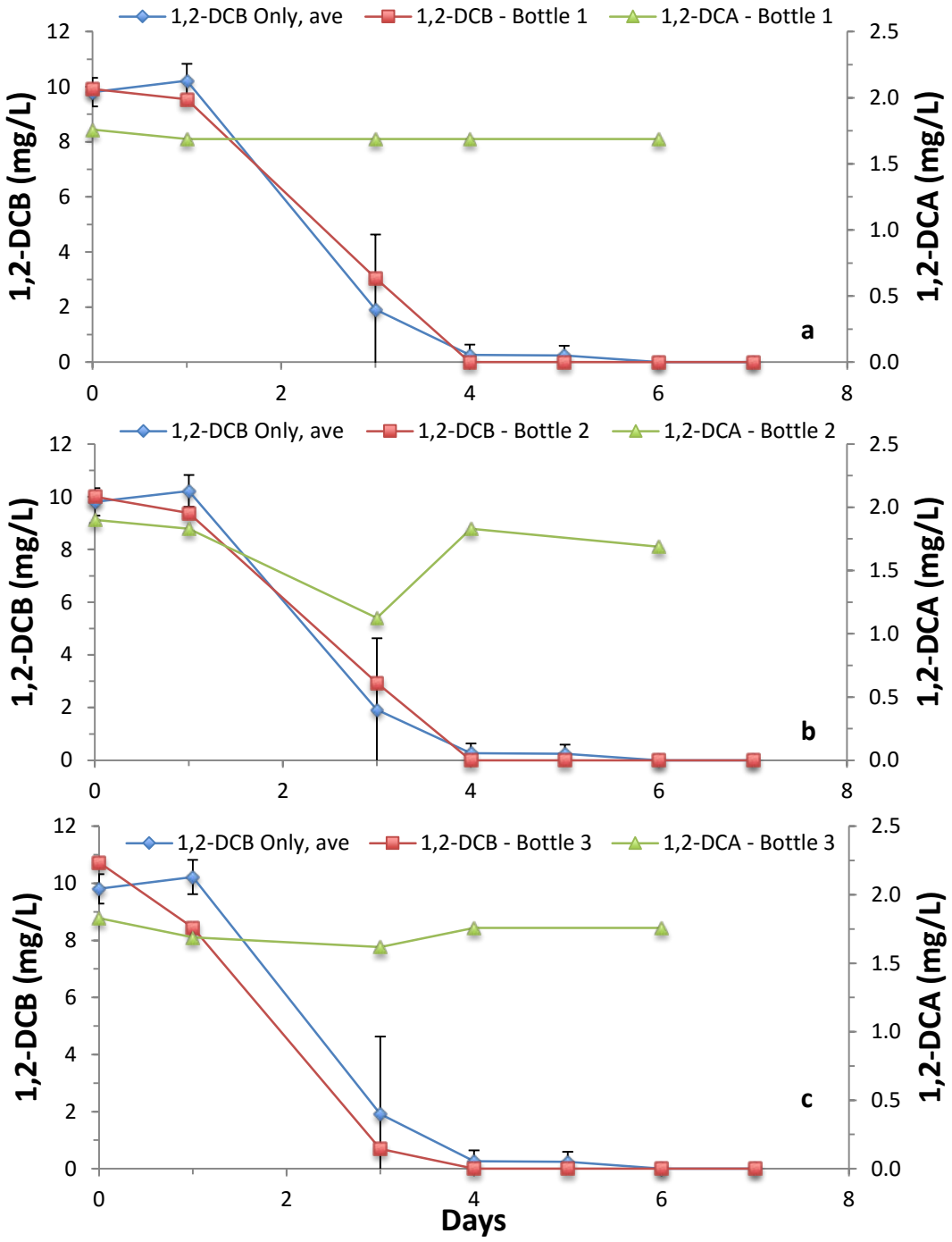

Figure 3.31 Initial results for the 1,2-DCB high concentration inhibition experiment in the 1,2-DCB + 1,2-DCA treatment for a) bottle #1; b) bottle #2; and c) bottle #3. Error bars represent one standard deviation for bottles #1 and #2 of the 1,2-DCB-only treatment.

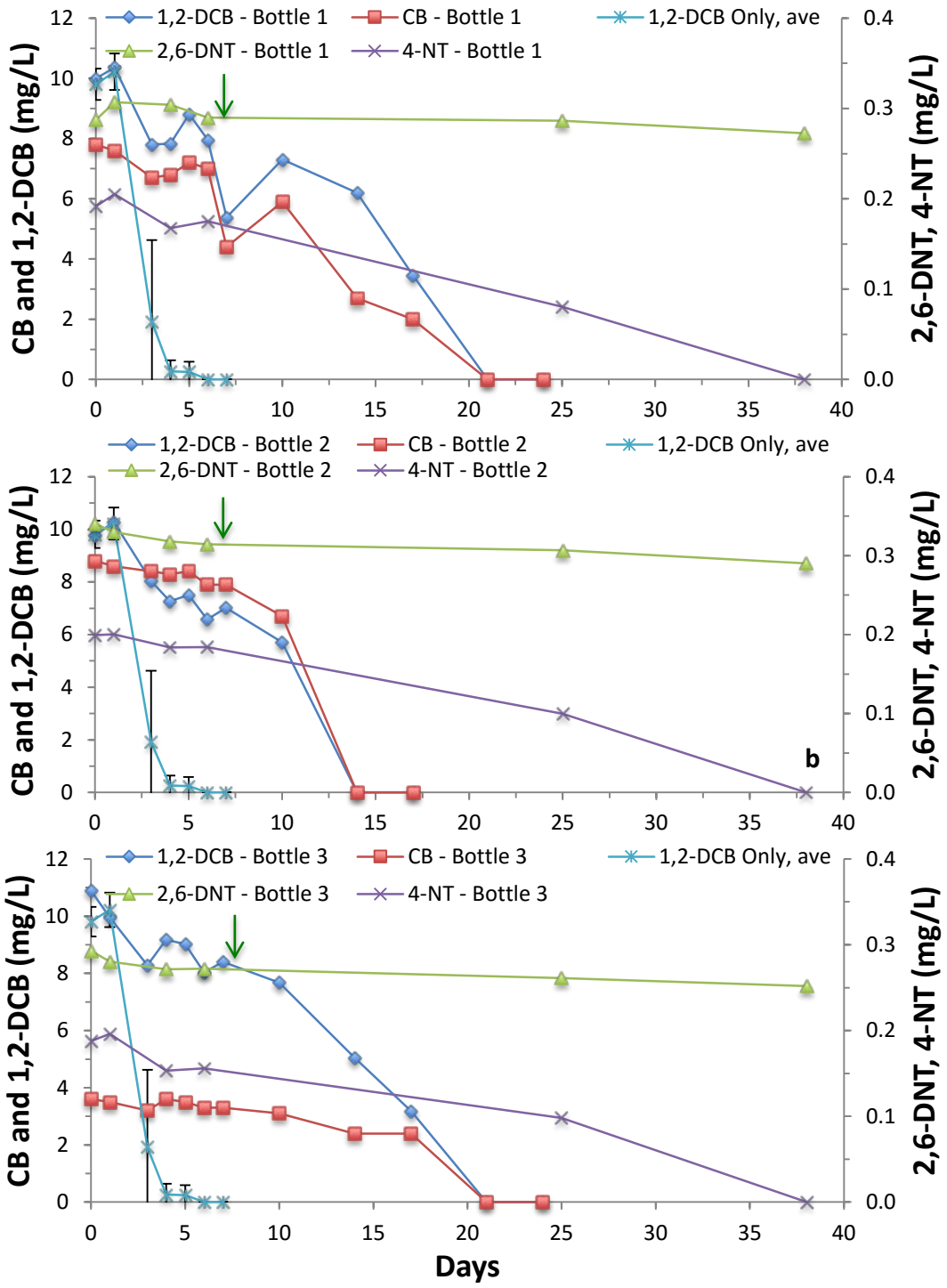

Figure 3.32 Initial results for the 1,2-DCB high concentration inhibition experiment in the 1,2-DCB + chemical oxidation treatment for a) bottle #1; b) bottle #2; and c) bottle #3; ↓ = addition of BSM. Error bars represent one standard deviation for bottles #1 and #2 of the 1,2-DCB-only treatment.

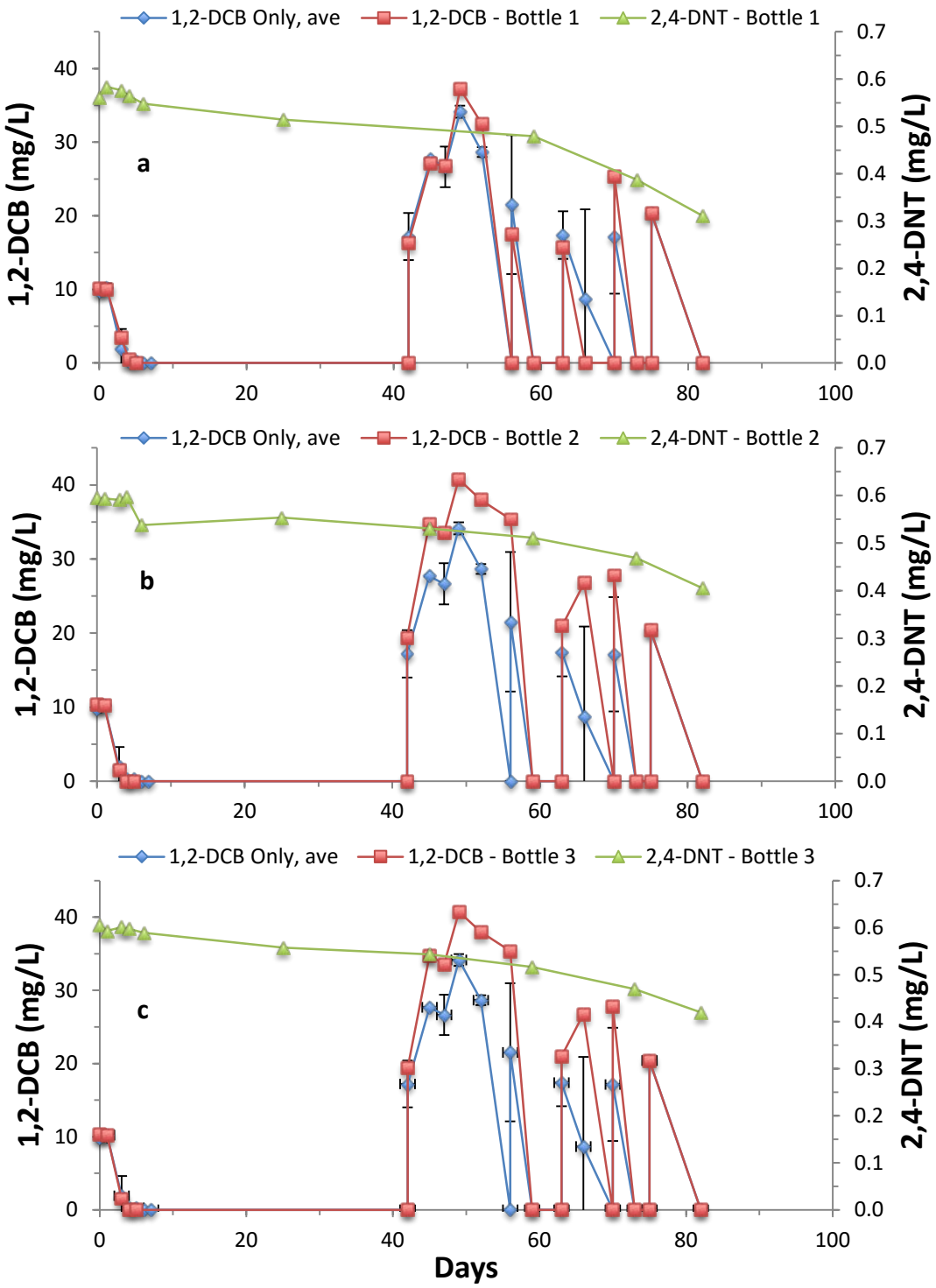

Figure 3.33 Effect of repeated additions of 1,2-DCB during 1,2-DCB high concentration inhibition experiment #2 in the treatment with 2,4-DNT for a) bottle #1; b) bottle #2; and c) bottle #3.

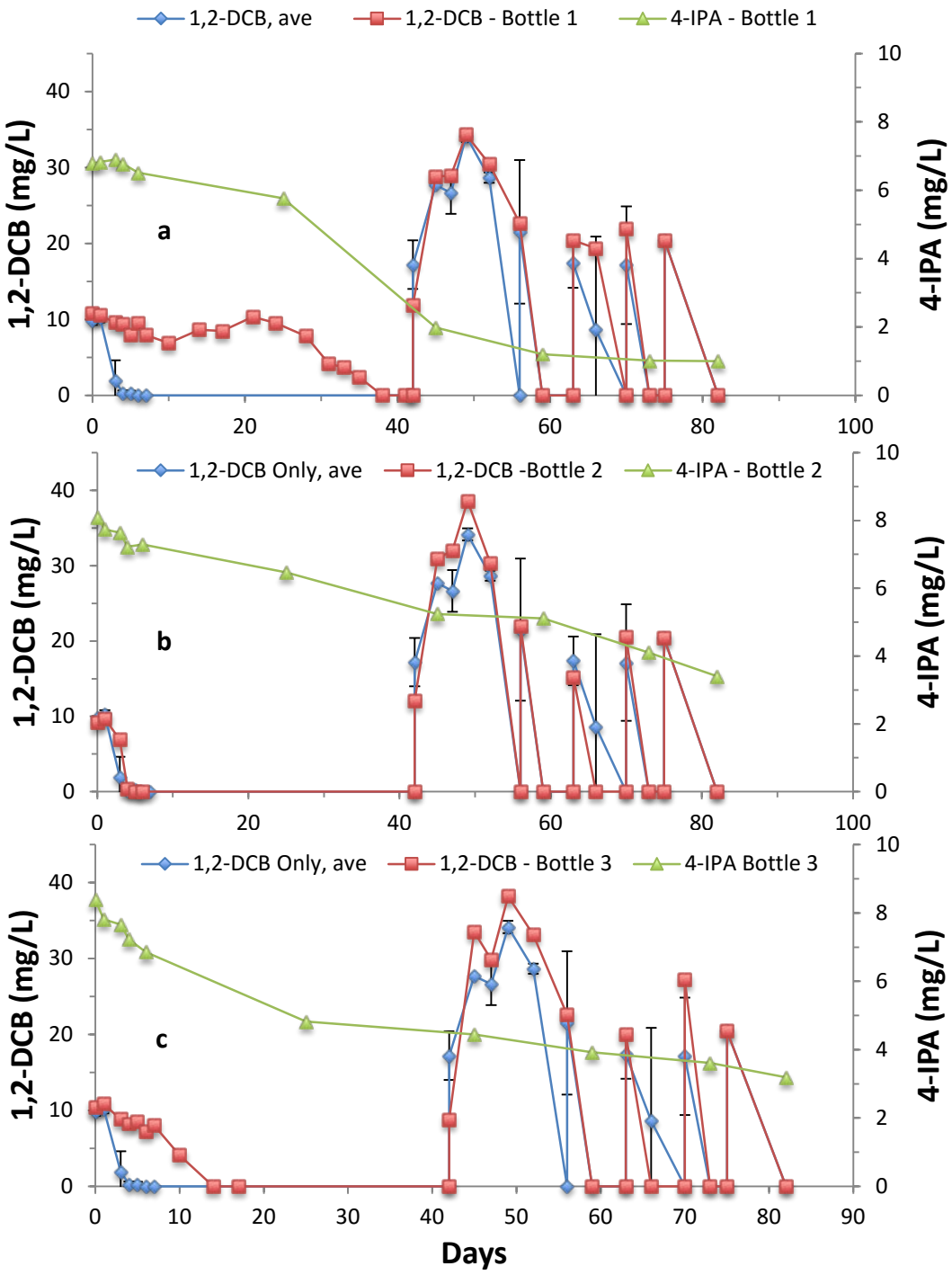

Figure 3.34 Effect of repeated additions of 1,2-DCB during 1,2-DCB high concentration inhibition experiment #2 in the treatment with 4-IPA for a) bottle #1; b) bottle #2; and c) bottle #3.

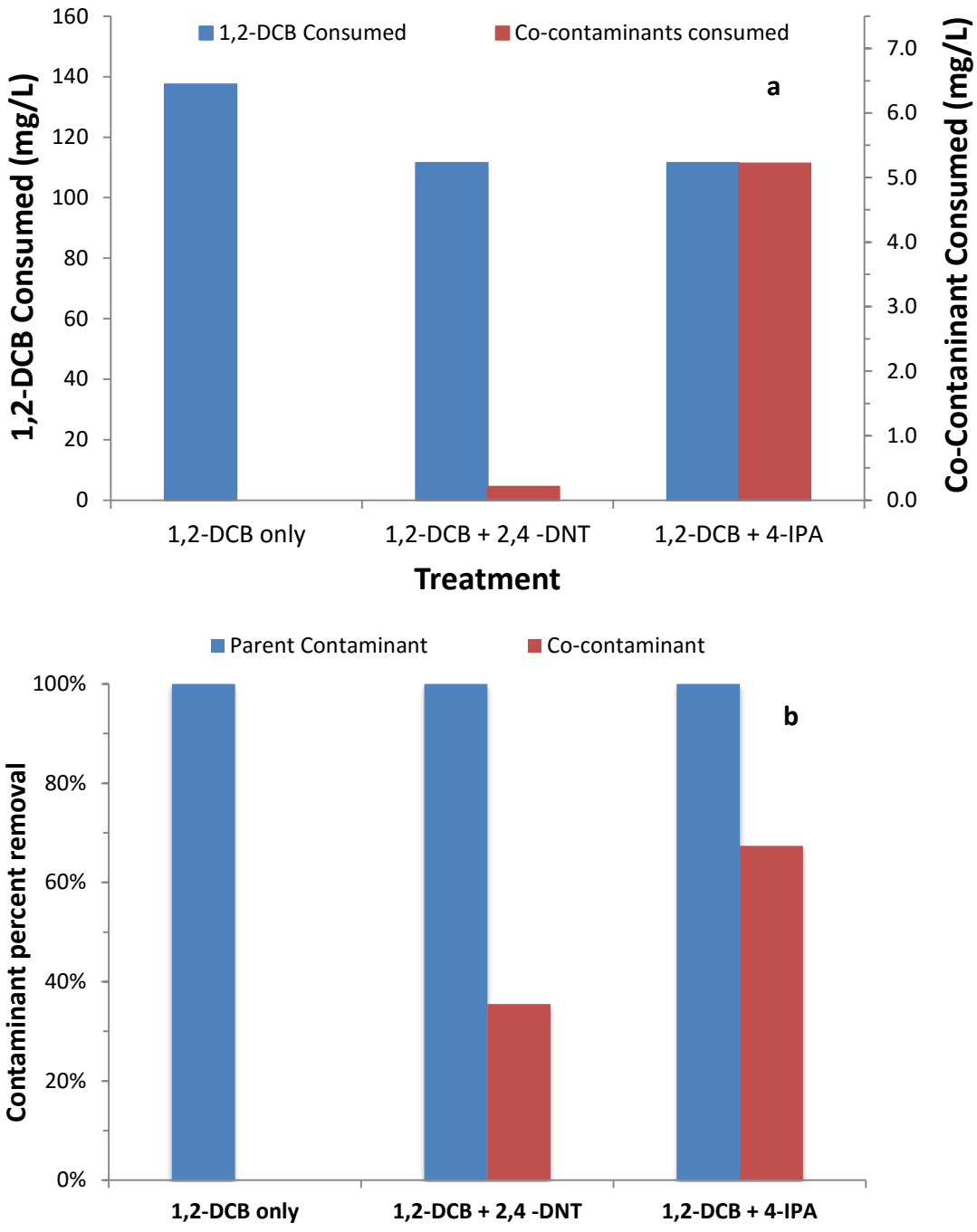

Figure 3.35 Effect of repeated consumption of 1,2-DCB during the 1,2-DCB high concentration inhibition experiment #2 on a) 1,2-DCB and co-contaminant removal (mg/L) in all treatments; and b) CB and co-contaminant percent removal (%) in all treatments.

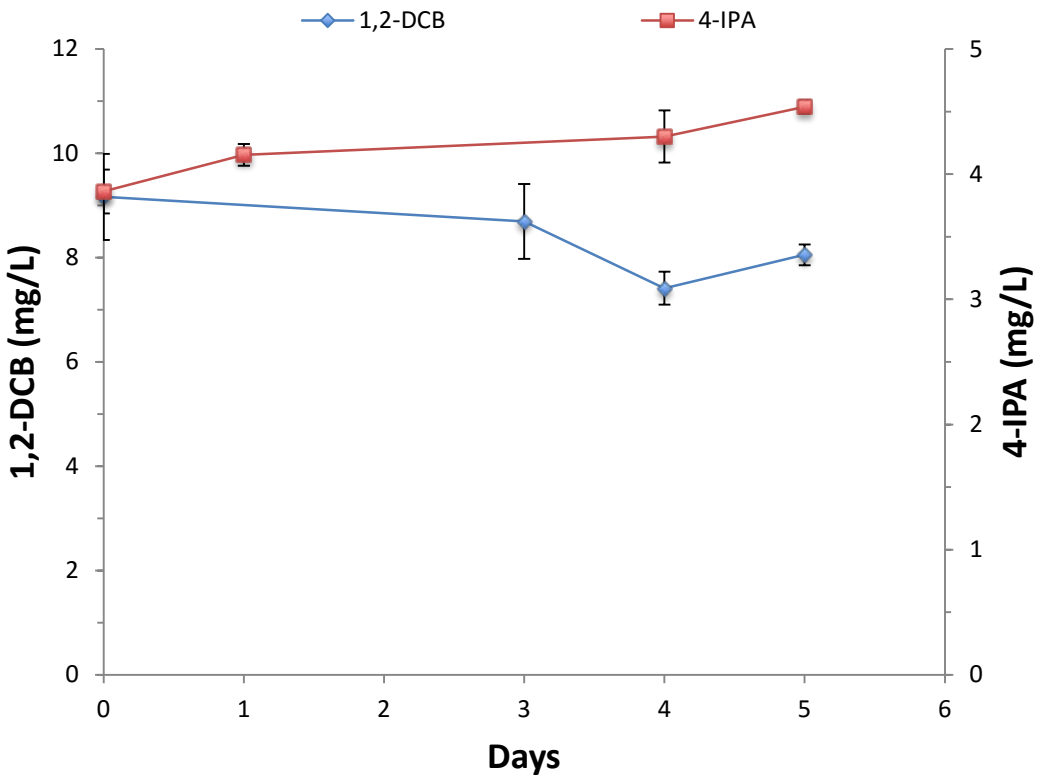

Figure 3.36 DDI water controls for the 1,2-DCB + 4-IPA low concentration inhibition experiment.

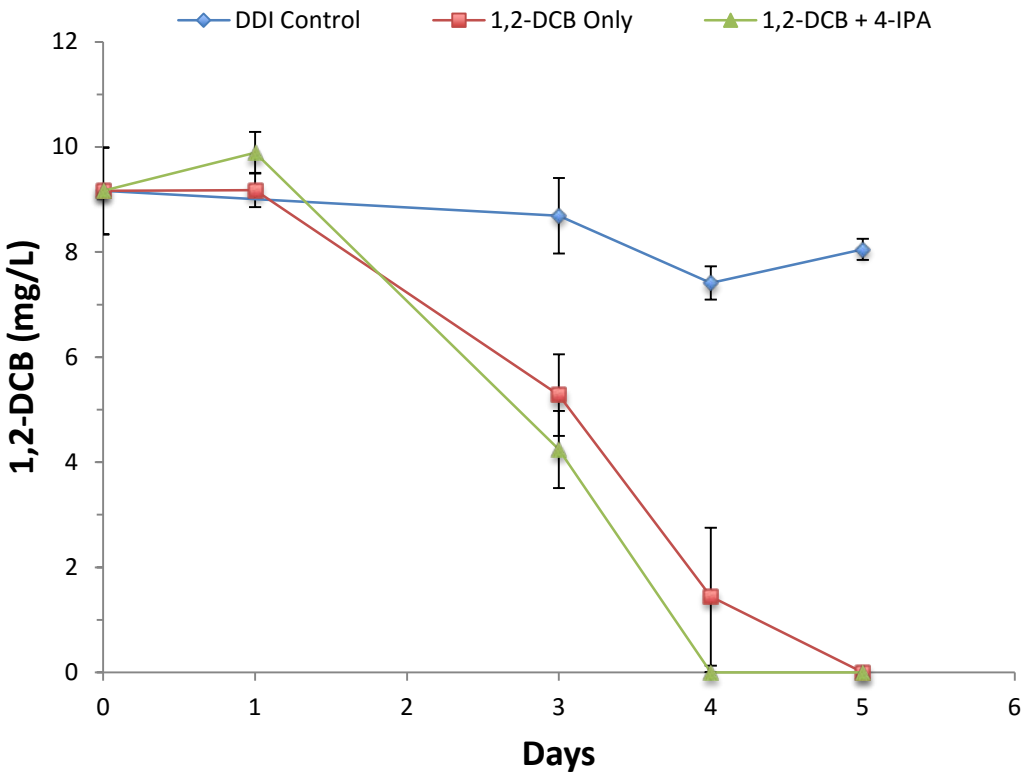

Figure 3.37 Average results for the 1,2-DCB low concentration inhibition experiment evaluating the effect of 4-IPA. Error bars represent one standard deviation of triplicate bottles.

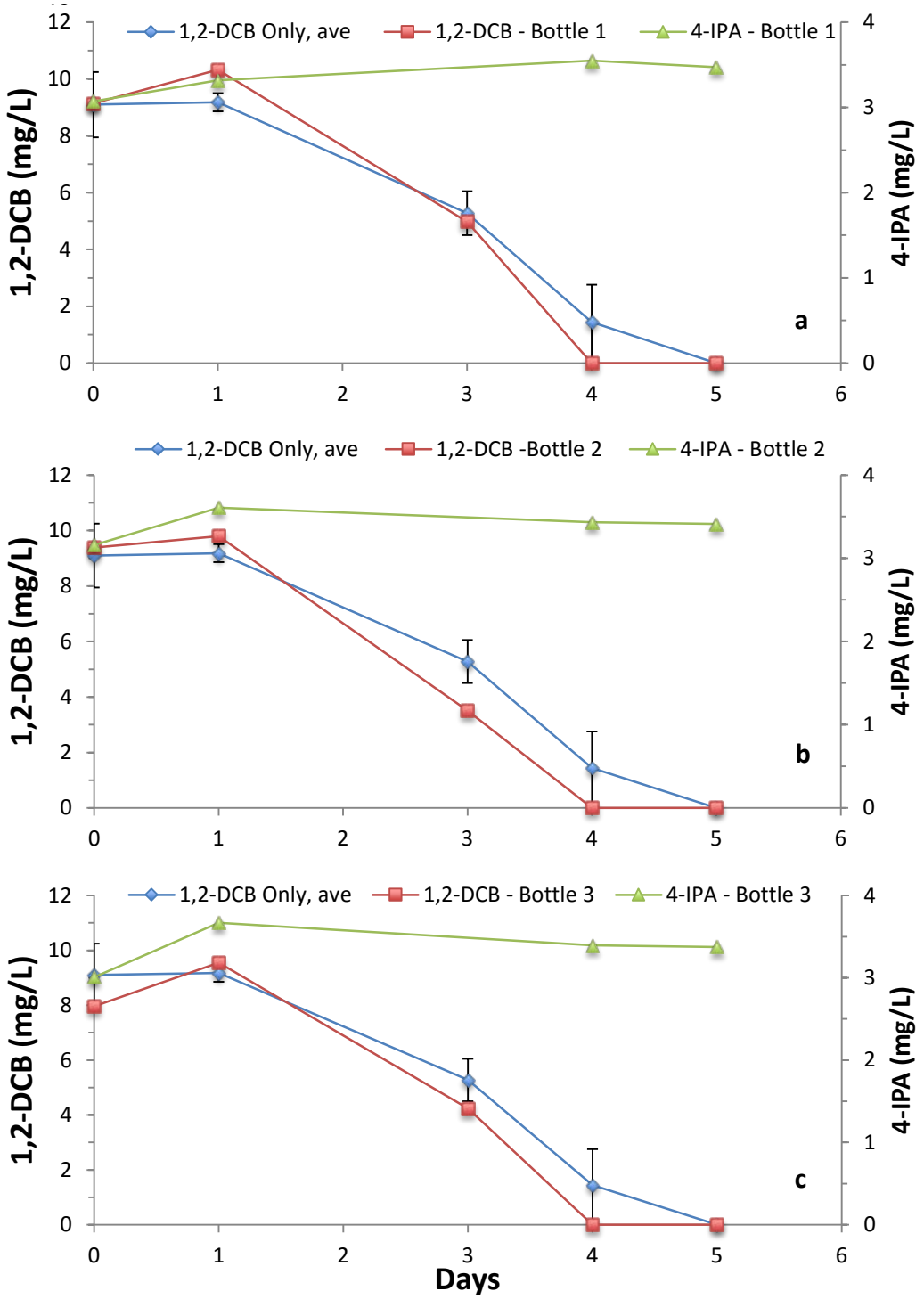

Figure 3.38 Results for 1,2-DCB and 4-IPA in the low concentration inhibition experiment for a) bottle #1; b) bottle #2; and c) bottle #3. Error bars represent one standard deviation of triplicate bottles.

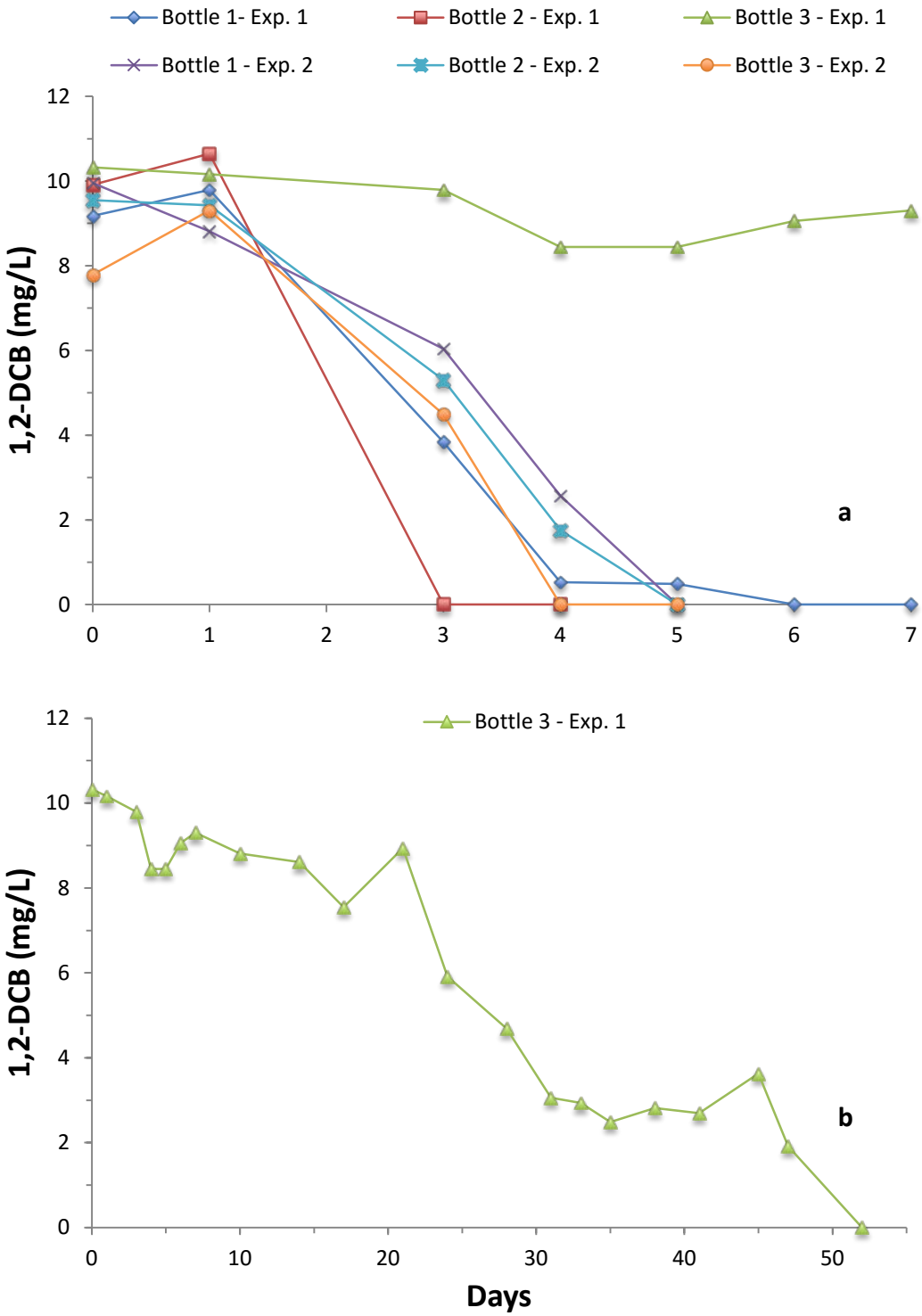

Figure 3.39 Comparison of the 1,2-DCB results between the high and low concentration inhibition experiment with 4-IPA for the a) all bottles; b) bottle #3, 1,2-DCB high concentration experiment.

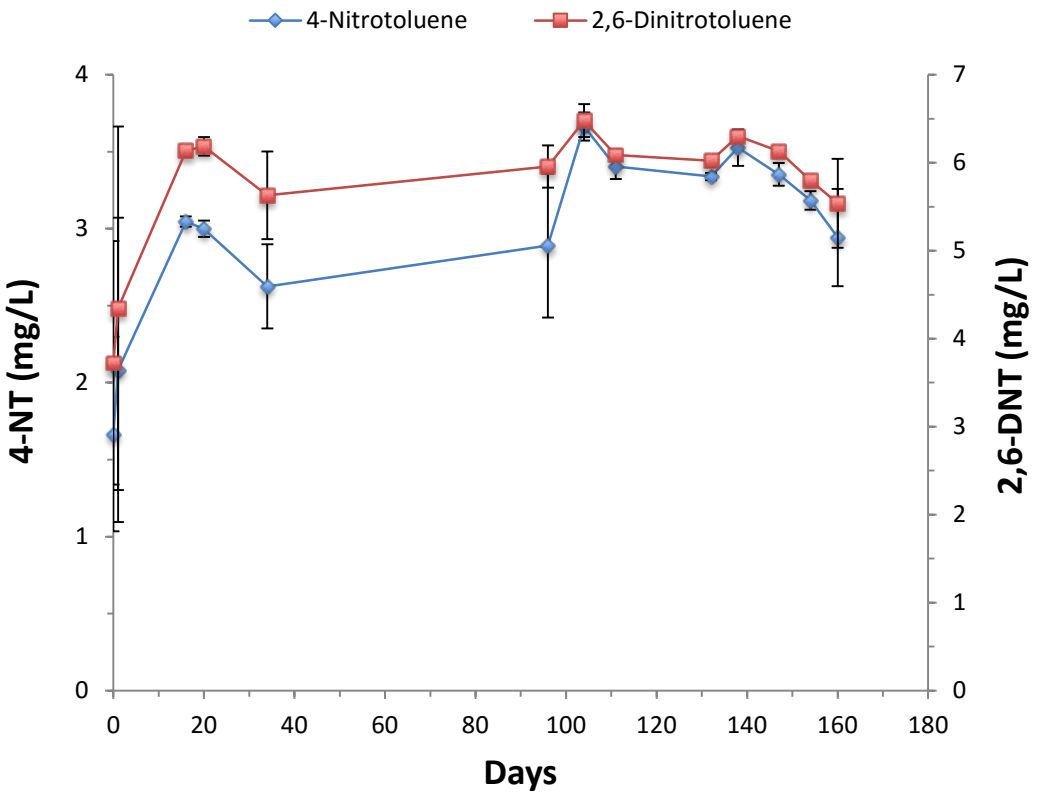

Figure 3.40 4-NT and 2,6-DNT in the DDI water controls for the microcosms experiment. Error bars represent one standard deviation for triplicate bottles.

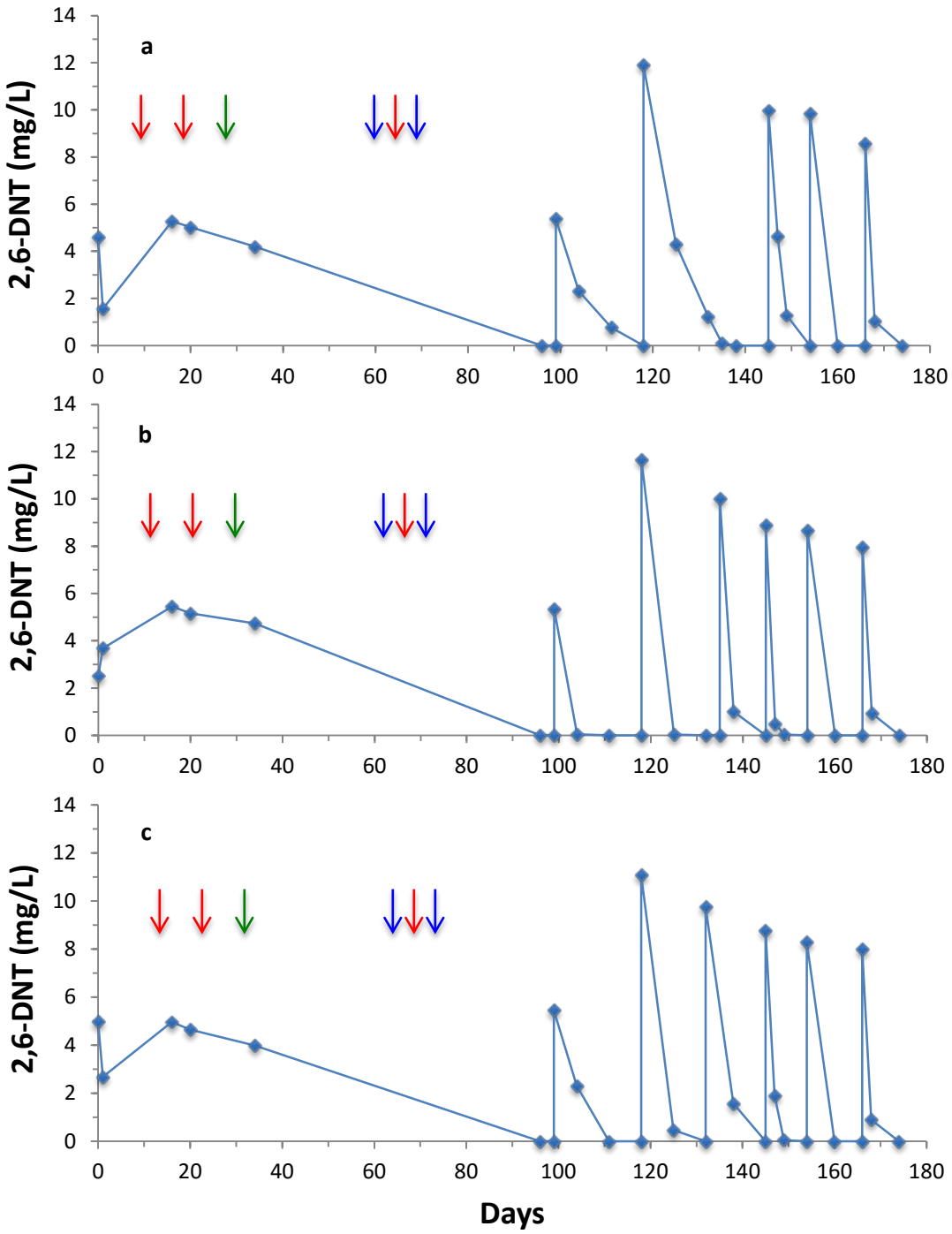

Figure 3.41 Microcosm results for 2,6-DNT in a) bottle #1; b) bottle #2; and c) bottle #3; ↓ = addition of lactate; ↓ = addition of sulfate; ↓ addition of AASM.

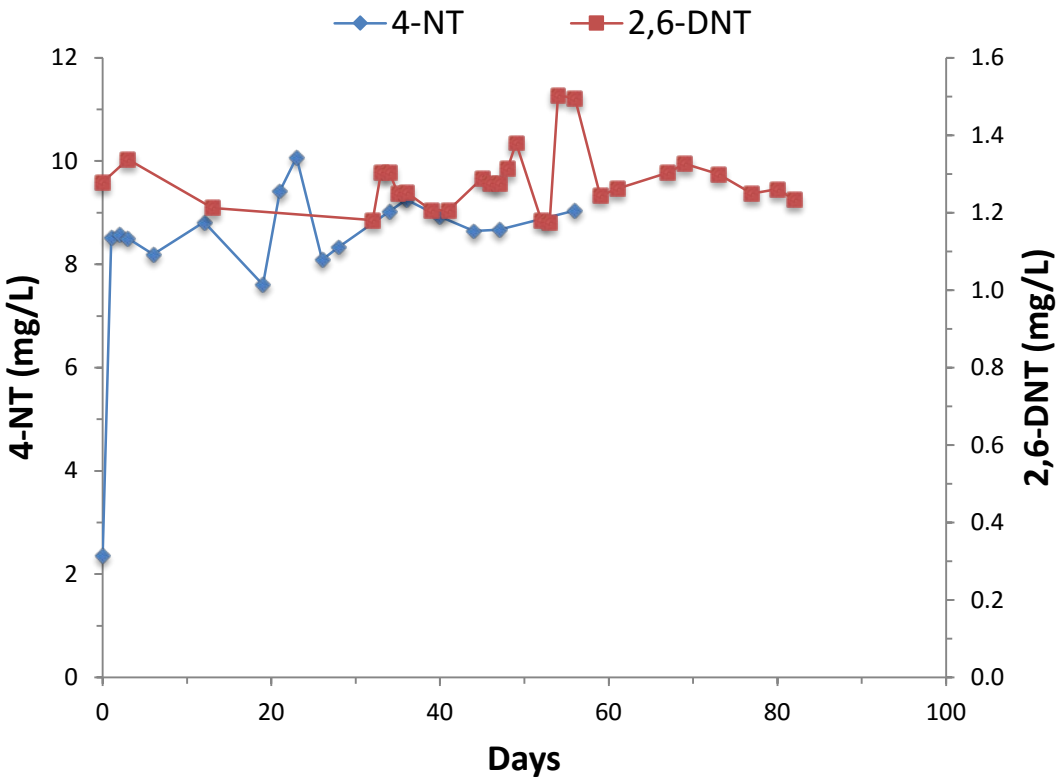

Figure 3.42 DDI water controls for comparison to the 4-NT and 2,6-DNT enrichment cultures.

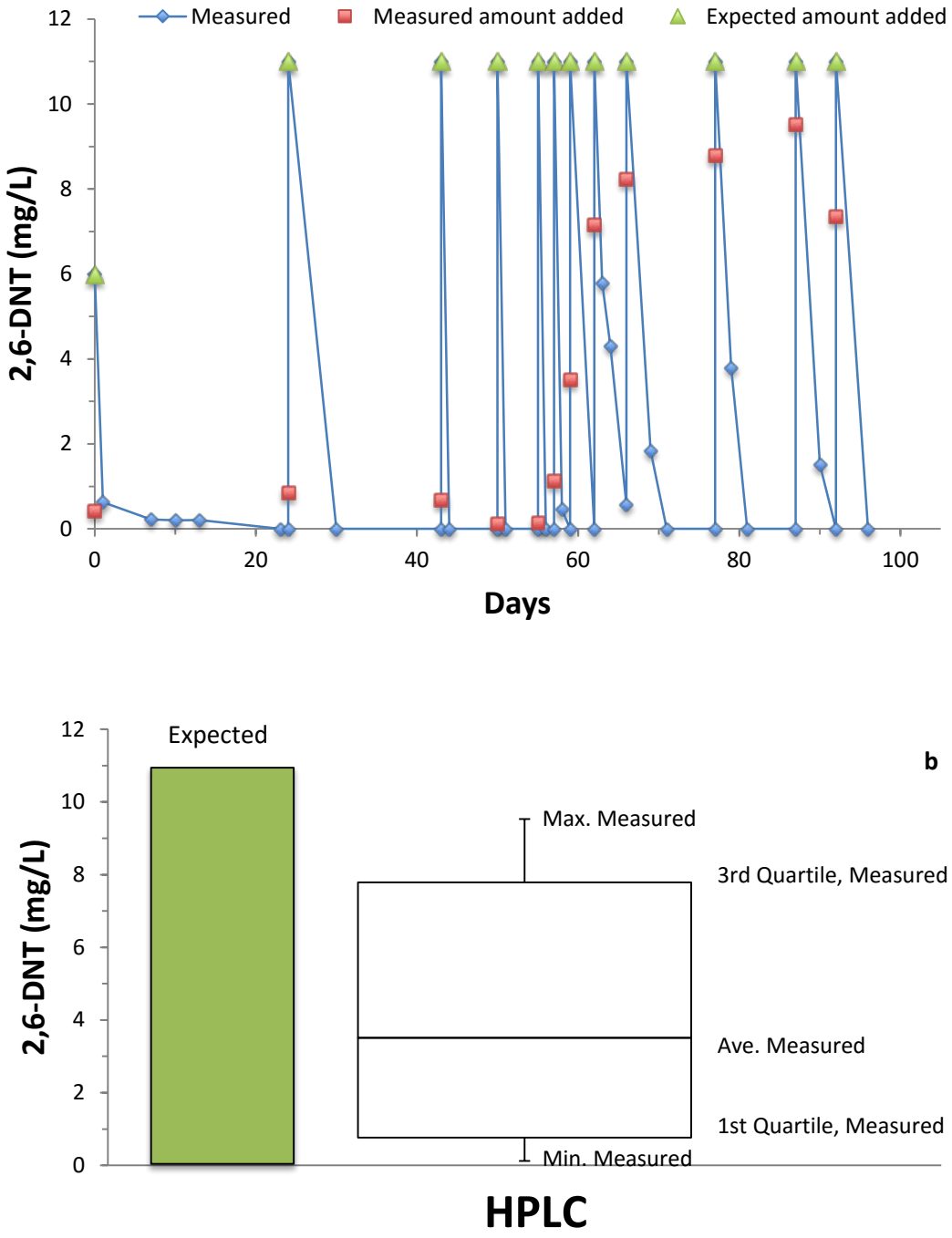

Figure 3.43 Enrichment culture results for 2,6-DNT for a) measured and expected concentrations based on the mass of neat compound added; and b) box and whisker diagram.

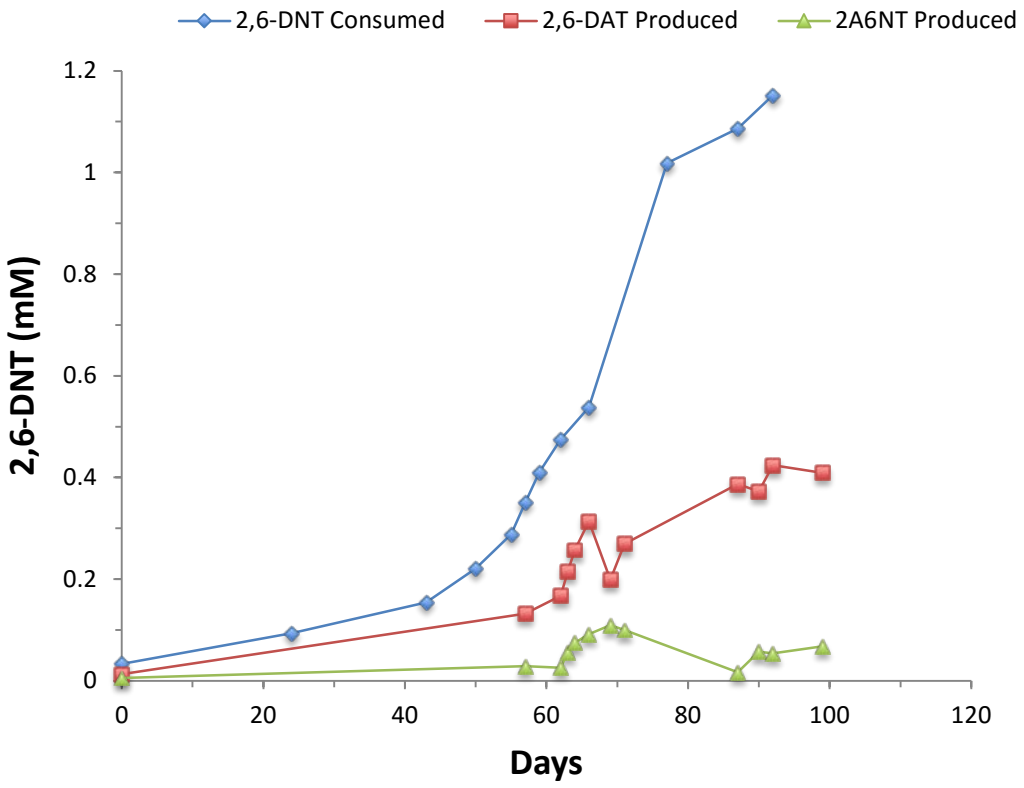

Figure 3.44 Cumulative levels of 2,6-DNT, 2,6-DAT and 2A6NT in the 2,6-DNT enrichment culture.

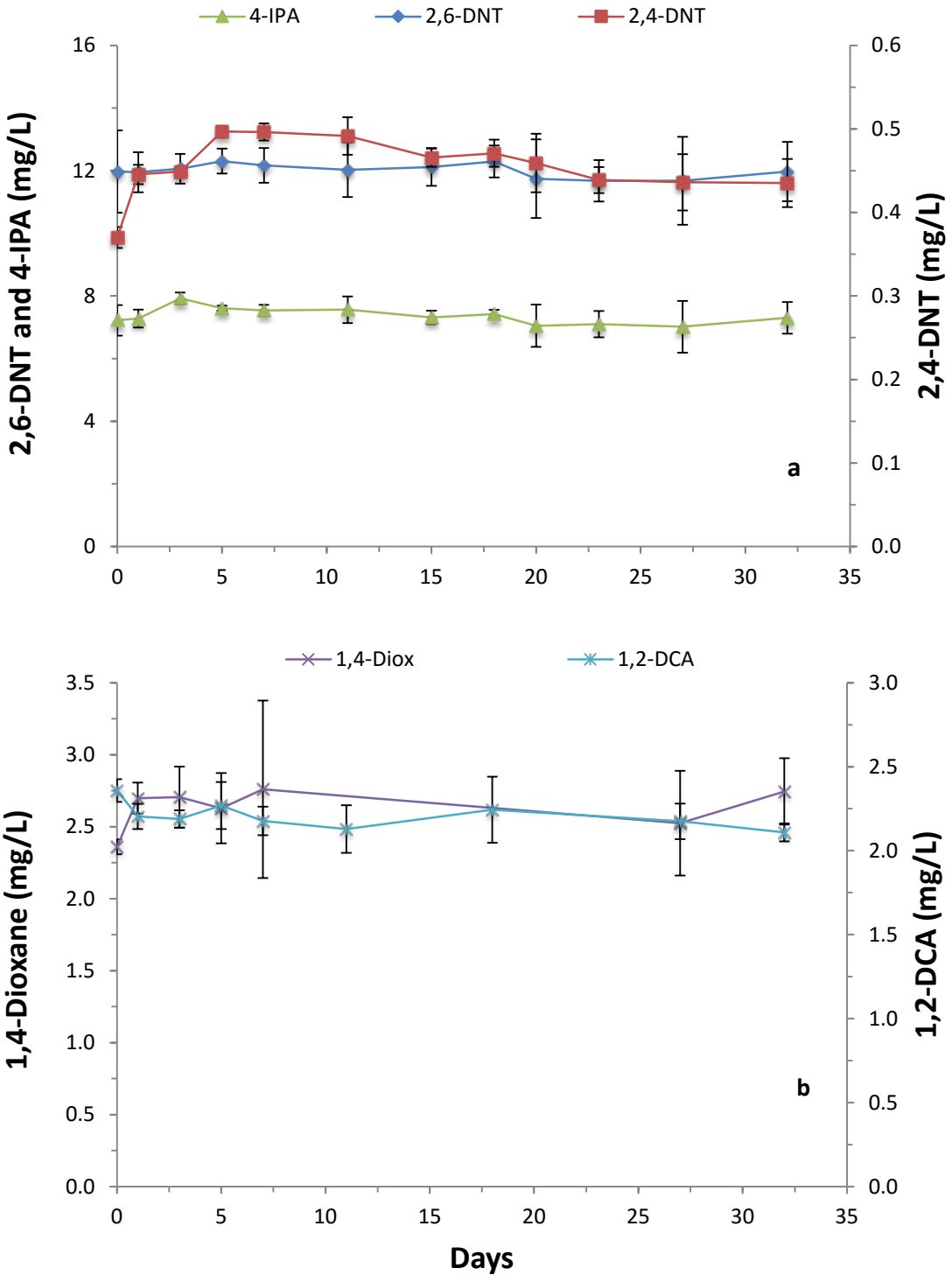

Figure 3.45 DDI water controls for the 2,6-DNT high concentration inhibition experiment for a) 2,6-DNT, 4-IPA and 2,4-DNT; and b) 1,4-dioxane and 1,2-DCA.

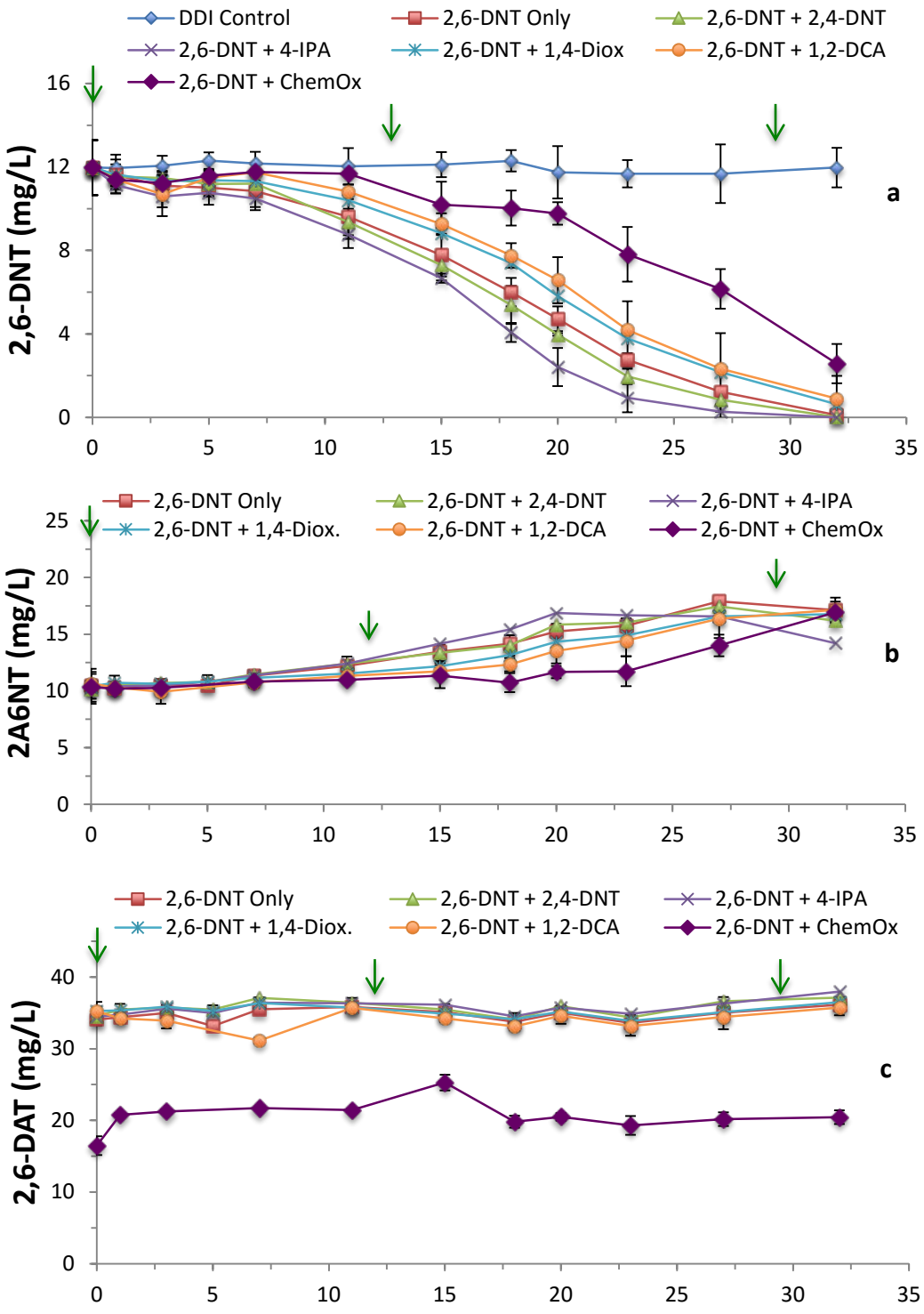

Figure 3.46 2,6-DNT high concentration inhibition experiment for a) 2,6-DNT in all treatments; b) 2A6NT in all treatments; and c) 2,6-DAT in all treatments; ↓ = addition of lactate. Error bars represent one standard deviation for triplicate bottles.

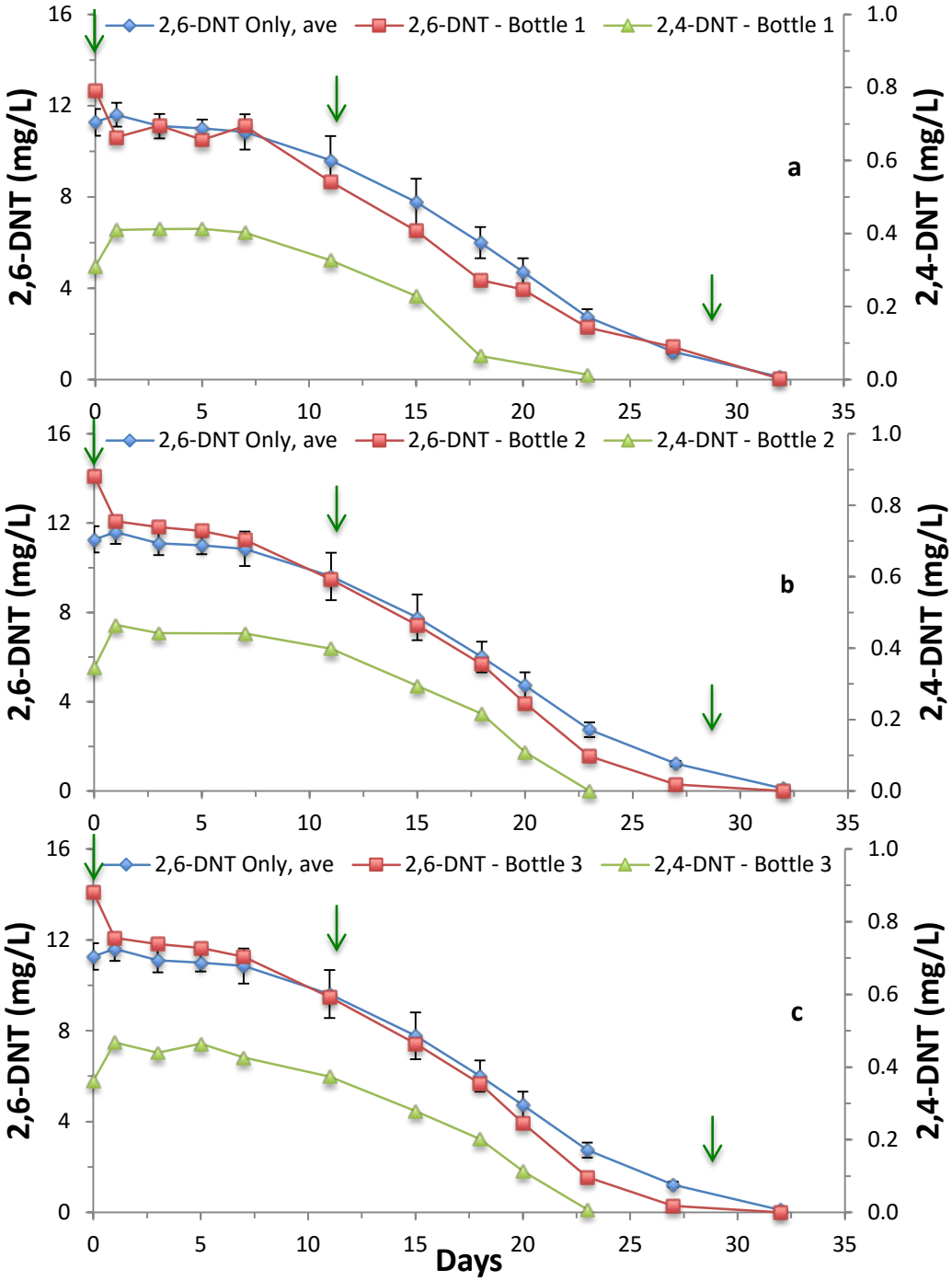

Figure 3.47 2,6-DNT high concentration inhibition experiment for the treatment with 2,6-DNT + 2,4-DNT for a) bottle #1; b) bottle #2; and c) bottle #3; ↓ = addition of lactate. Error bars represent one standard deviation for triplicate bottles.

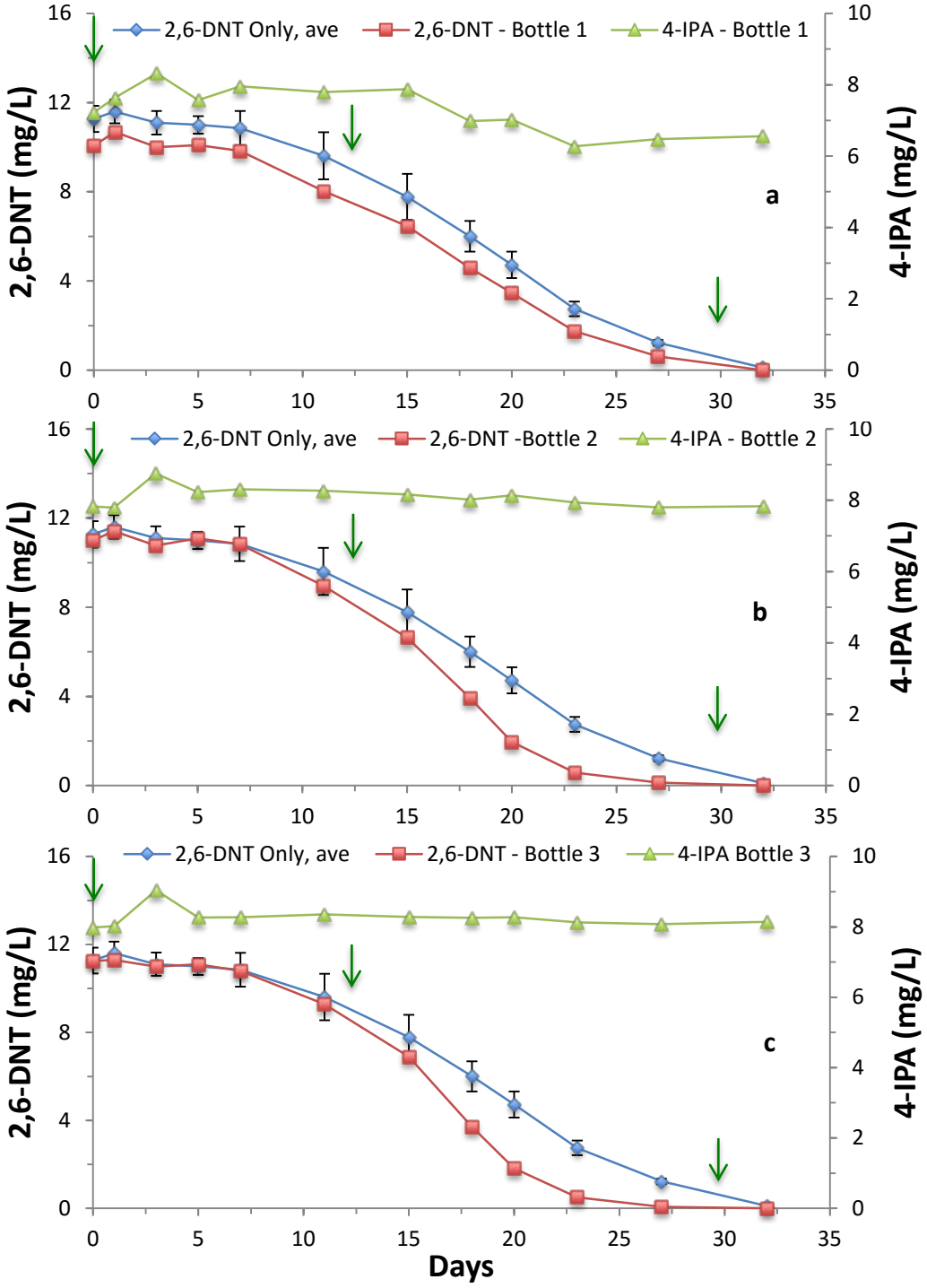

Figure 3.48 2,6-DNT high concentration inhibition experiment for the treatment with 2,6-DNT + 4-IPA for a) bottle #1; b) bottle #2; and c) bottle #3; ↓ = addition of lactate. Error bars represent one standard deviation for triplicate bottles.

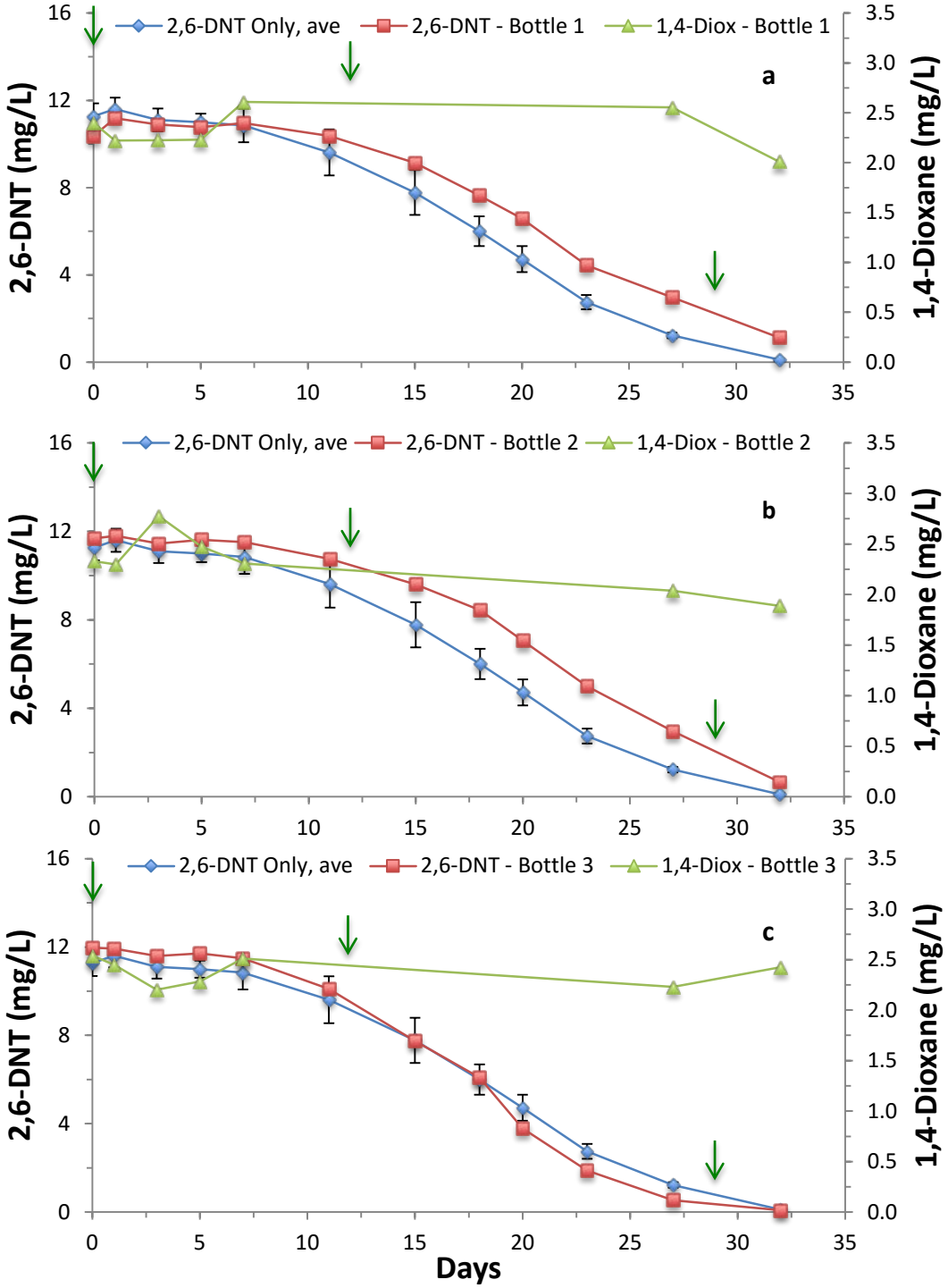

Figure 3.49 2,6-DNT high concentration inhibition experiment for the treatment with 2,6-DNT + 1,4-dioxane for a) bottle #1; b) bottle #2; and c) bottle #3; ↓ = addition of lactate. Error bars represent one standard deviation for triplicate bottles.

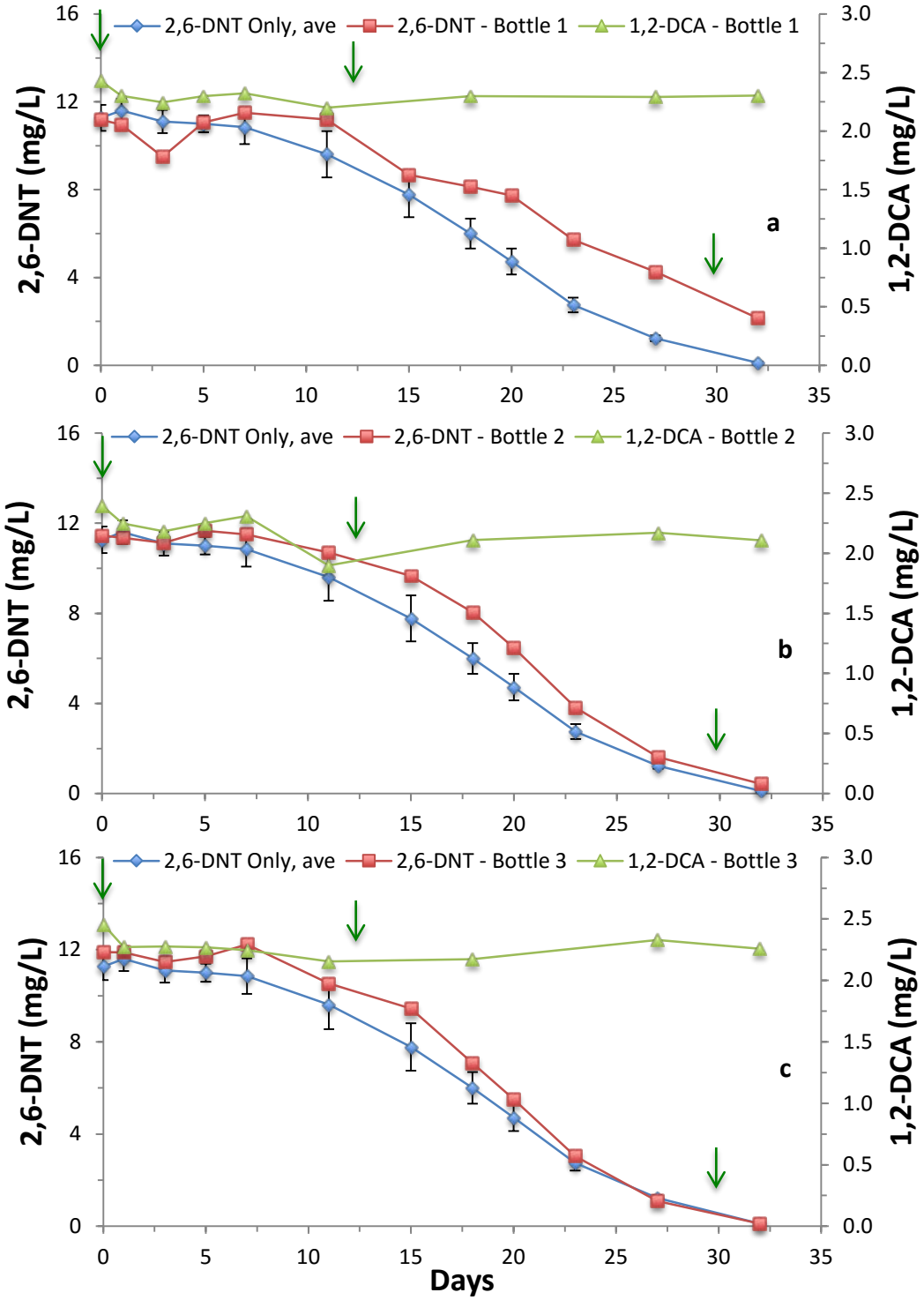

Figure 3.50 2,6-DNT high concentration inhibition experiment for the treatment with 2,6-DNT + 1,2-DCA for a) bottle #1; b) bottle #2; and c) bottle #3; ↓ = addition of lactate. Error bars represent one standard deviation for triplicate bottles.

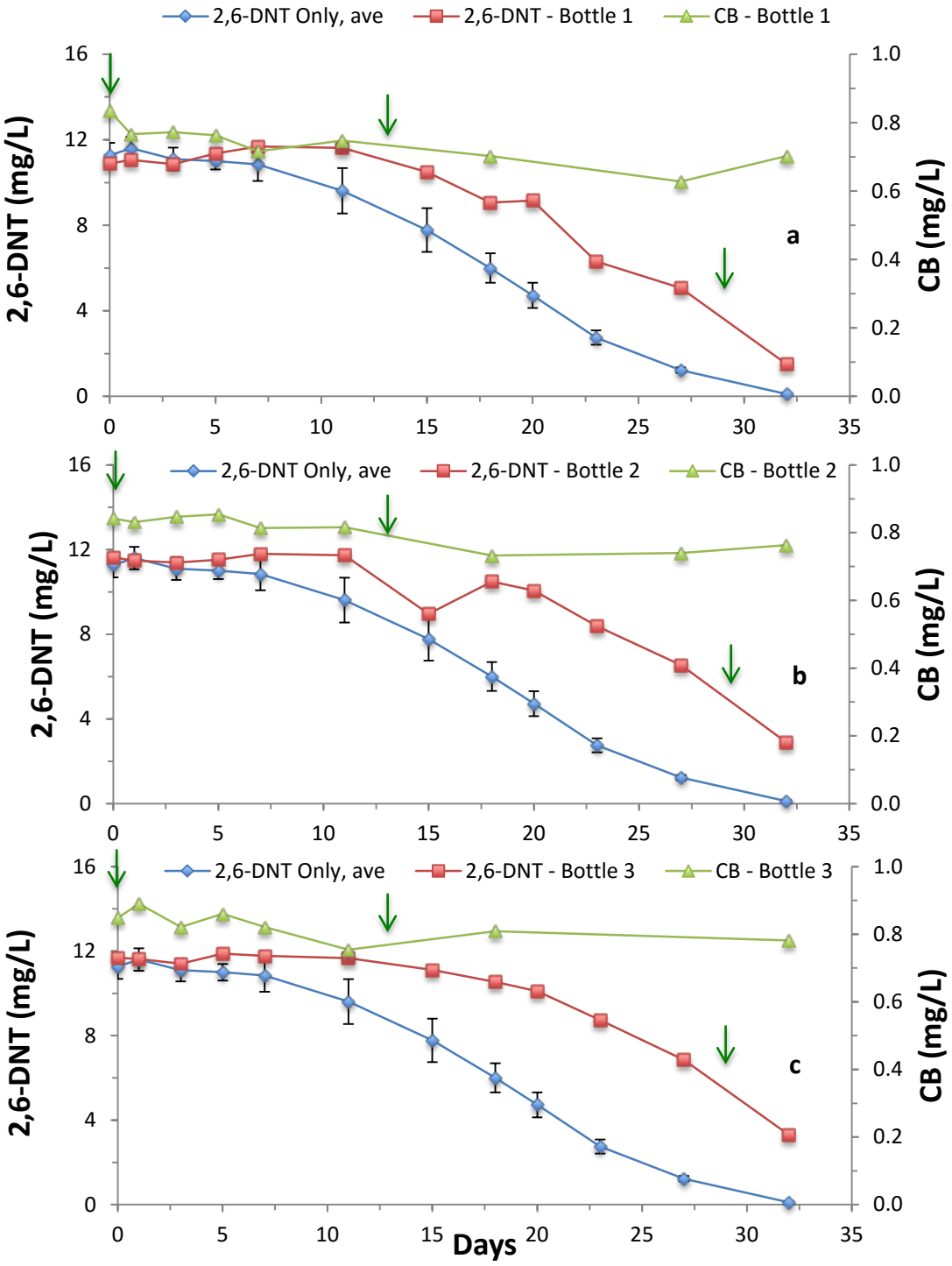

Figure 3.51 2,6-DNT high concentration inhibition experiment for the treatment with 2,6-DNT + chemical oxidation water for a) bottle #1; b) bottle #2; and c) bottle #3; ↓ = addition of lactate. Error bars represent one standard deviation for triplicate bottles.

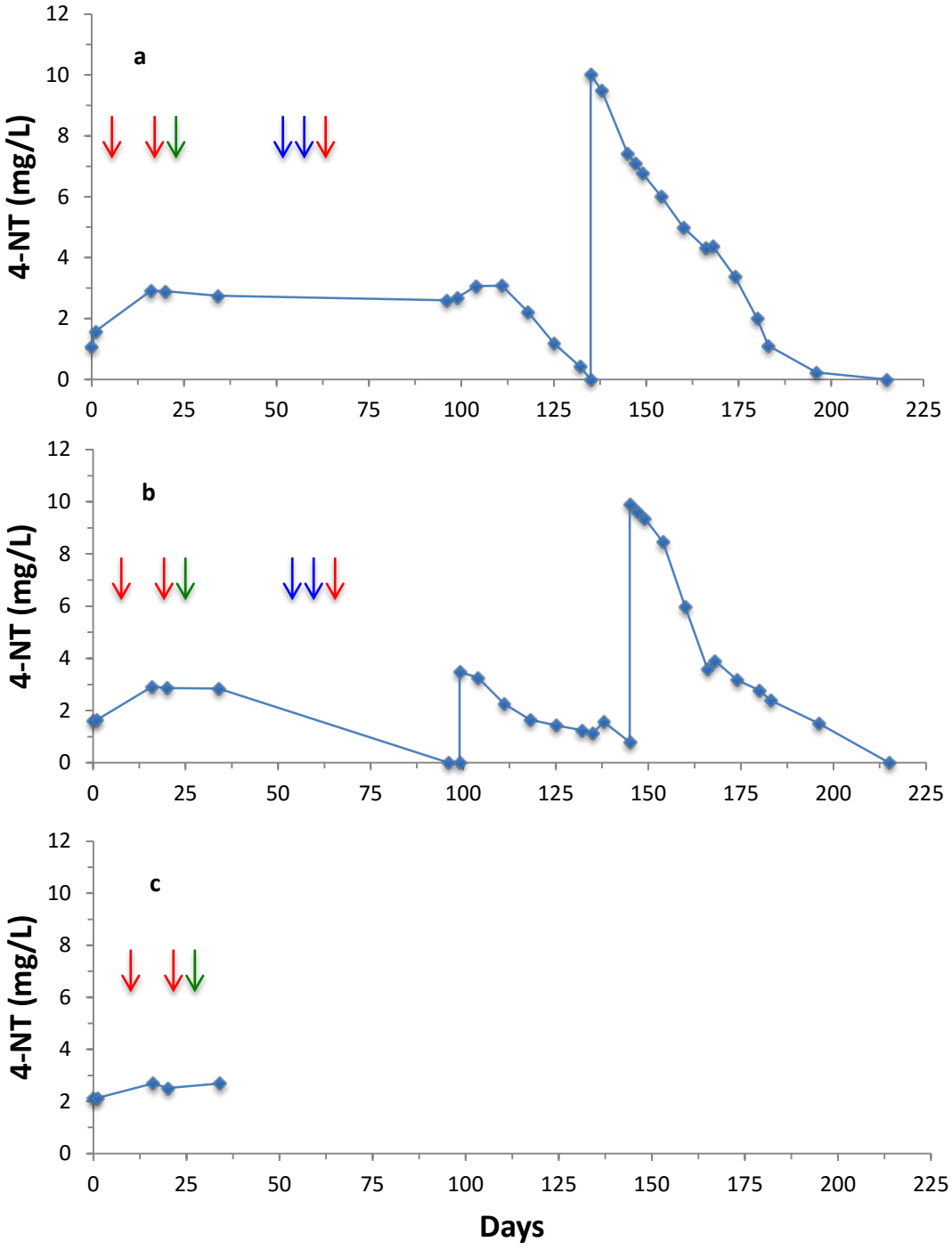

Figure 3.52 Microcosm results for 4-NT in a) bottle #1; b) bottle #2; and c) bottle #3. ↓ = addition of Lactate; ↓ = addition of sulfate; ↓ = addition of 5 mL AASM.

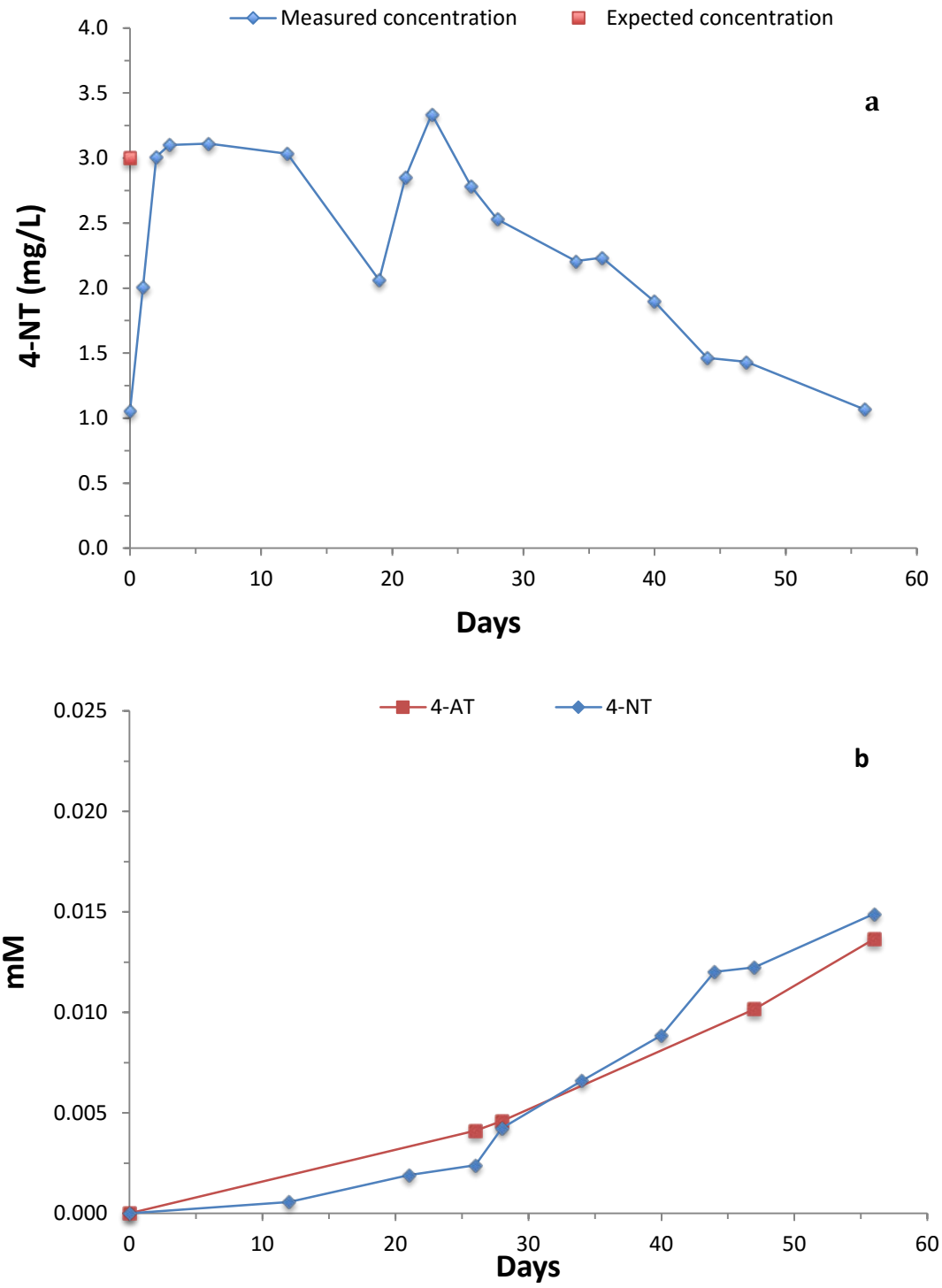

Figure 3.53 Enrichment culture results for 4-NT; a) measured and expected concentrations based on the mass of neat compound added; b) Cumulative levels of 4-NT, and 4-NT in the 2,6-DNT enrichment culture.

APPENDICES

Appendix A: Media Preparation

Aerobic MSM: Basal Salt Media

Stock solutions prepared for medium preparation:

- **BSM A**

In a 1000 mL volumetric flask add the following components, then fill the volumetric flask up to the to 1000 mL mark with DDI water

Table A.1 Chemicals and amounts required for the preparation of BSM A

Chemical	Formula	Mass Added (g)
Potassium Phosphate	K_2HPO_4-$3H_2O$	85.0
Sodium Phosphate	NaH_2PO_4-H_2O	20.0
Ammonium Chloride	NH4Cl	40.0

- **BSM B**

In a 1000 mL volumetric flask add the following components, then fill the volumetric flask up to the to 1000 mL mark with DDI water. The water pH was adjusted to 4-5 with HCL prior to adding the BSM B chemicals

Table A.2 Chemicals and amounts required for the preparation of BSM B

Chemical	Formula	Mass Added (g)
Nitrillotriacetic acid	$N(CH_2CO_2Na)_3$-H_2O	2.46
Magnesium Sulfate	$MgSO_4$-$7H_2O$	4.00
Ferrous Sulfate	$FeSO_4$-$7H_2O$	0.24
Manganese Sulfate	$MnSO_4$-H_2O	0.06

Zinc Sulfate	$ZnSO_4$-$7H_2O$	0.06
Cobalt Chloride	$CoCl_2$-$6H_2O$	0.02

- **BSM Solution**

To make 1 L of BSM solution the following volumes of BSM A and BSM B were added to a 1000 mL volumetric flask, and DDI was added until reaching the target mark

50 mL BSM A

50 mL BSM B

900 mL distilled water

Anaerobic MSM

- **Trace metals solution**

In a 100 mL volumetric flask add the following components, then fill the volumetric flask up to the 100 mL mark with DDI water

Table A.3 Chemicals and amounts required for the preparation of the trace metals solution

Chemical	**Formula**	**Amount Added**
Boric Acid	H_3BO_3	0.03 g
Zinc sulfate Heptahydrate	$ZnSO_4 \cdot 7H_2O$	0.0211 g
Nickel (II) Chloride Hexahydrate	$NiCl_2 \cdot 6H_2O$	0.075 g
Manganese(II) Chloride Tetrahydrate	$MnCl_2 \cdot 4H_2O$	0.1 g
Copper(II) Chloride Dihydrate	$CuCl_2 \cdot 2H_2O$	0.01 g

Cobalt(II) Chloride Hexahydrate	$CoCl_2 \cdot 6H_2O$	0.15 g
Sodium Selenite Pentahydrate	Na_2SeO_3	0.002 g
Aluminum Sulfate	$Al_2(SO_4)_3 \cdot 16H_2O$	0.01 g
Hydrochloric Acid		1 mL (37%)

- **AASM**

In a 1000 mL volumetric flask add the following components, then fill the volumetric flask up to the 1000 mL mark with DDI water

Table A.4 Chemicals and amounts required for the preparation of AASM

Chemical	Formula	Amount Added
Ammonium Chloride	NH_4Cl	0.2000 g
Dipotassium Phosphate	K_2HPO_4	0.0763 g
Potassium Phosphate Monobasic	$KH_2:PO_4$	0.0550 g
Magnesium Chloride Hexahydrate	$MgCl_2:6H_2O$	0.2000 g
Sodium Bicarbonate	$NaHCO_3$	5.0000 g
Yeast Extract	NA	0.0500 g
Trace Metal Solution	NA	10.0000 mL

Appendix B: Initial Concentrations in Soil

Table B.1 Summary of initial concentrations for the mixed soil used to prepare the aerobic microcosms.

Equipment and Method	Compound	Retention Time (min)	Mixed Soil from Aerobic Locations					
			Bottle 1		Bottle 2		Bottle 3	
			Peak Area Units	(mg/L)	Peak Area Units	(mg/L)	Peak Area Units	(mg/L)
GC-114 DXELIQ3	1,4-Dioxane	10.0	0.0	0.0	0.0	0.0	0.0	0.0
GC-Ethel 112 Gosset	1,2-DCA	8.2	0.0	0.0	0.0	0.0	0.0	0.0
GC-114 CB013114	CB	11.0	0.0	0.0	0.0	0.0	0.0	0.0
	1,2-DCB	21.5	0.0	0.0	0.0	0.0	0.0	0.0
	GC Unknown 1	19.7	3.7	-	3.9	-	4.5	-
HPLC 55% MeOH	4-IPA (240nm)	7.5	0.0	0.0	0.0	0.0	0.0	0.0
	4-NT (268 nm)	8.1	0.0	0.0	0.0	0.0	0.0	0.0
	2,4-DNT (250nm)	23.3	0.0	0.0	0.0	0.0	0.0	0.0
	2,6-DNT (240nm)	24.9	0.0	0.0	0.0	0.0	0.0	0.0
	HPLC Unknown 1 (268nm)	3.40	0.0	-	0.0	-	0.0	-

Table B.2 Summary of initial concentrations for the mixed soil used to prepare the anaerobic microcosms.

Equipment and Method	Compound	Retention Time (min)	Mixed Soil from Anaerobic Locations					
			Bottle 1		Bottle 2		Bottle 3	
			Peak Area Units	(mg/L)	Peak Area Units	(mg/L)	Peak Area Units	(mg/L)
GC-114 DXELIQ3	1,4-Dioxane	10.00	0.0	0.0	0.0	0.0	0.0	0.0
GC-Ethel 112 Gosset	1,2 DCA	8.20	0.0	0.0	0.0	0.0	0.0	0.0
GC-114 CB013114	CB	11.00	0.0	0.0	0.0	0.0	0.0	0.0
	1,2 DCB	21.50	0.0	0.0	0.0	0.0	0.0	0.0
	GC Unknown 1	19.70	7.8	-	3.5	-	4.4	-
	GC Unknown 2	23.10	6.4	-	0.0	-	0.0	-
	GC Unknown 3	6.10	59.3	-	0.0	-	0.0	-
HPLC 55% MeOH	4-IPA (240nm)	7.50	0.0	0.0	0.0	0.0	0.0	0.0
	4NT (268 nm)	8.10	0.0	0.0	0.0	0.0	0.0	0.0
	2,4 DNT (250nm)	23.30	0.0	0.0	0.0	0.0	0.0	0.0
	2,6 DNT (250nm)	24.90	0.0	0.0	0.0	0.0	0.0	0.0
	HPLC Unknown 1 (268nm)	3.40	0.0	-	0.0	-	0.0	-
	HPLC Unknown 2 (240nm)	2.5-2.7	0.2	-	0.7	-	0.6	-
	HPLC Unknown 3 (240nm)	3.90	0.0	-	0.3	-	0.0	-
	HPLC Unknown 4 (240nm)	4.38	0.0	-	0.2	-	0.0	-
	HPLC Unknown 5 (240nm)	4.63	0.0	-	0.3	-	0.0	-
	HPLC Unknown 6 (240nm)	5.02	0.0	-	0.4	-	0.0	-
	HPLC Unknown 7 (240nm)	5.53	0.0	-	0.4	-	0.0	-

Appendix C: Response Factors

Table C.1 Oxygen response factors.

Date	Response Factor (% O_2/Peak Area Unit)
26-Oct-15	2.70%
27-Oct-15	2.62%
29-Oct-15	2.43%
5-Nov-15	3.22%
11-Nov-15	3.33%
13-Nov-15	2.87%
17-Nov-15	2.83%
23-Nov-15	2.87%
3-Dec-15	2.81%
10-Dec-15	2.95%
5-Jan-16	2.74%
11-Jan-16	2.83%
12-Jan-16	2.83%
13-Jan-16	2.86%
14-Jan-16	2.82%
19-Jan-16	2.78%
26-Jan-16	2.88%
29-Jan-16	2.88%
1-Feb-16	2.88%
3-Feb-16	2.84%
5-Feb-16	2.71%
8-Feb-16	2.76%
10-Feb-16	2.68%
12-Feb-16	2.67%
15-Feb-16	2.63%
17-Feb-16	2.67%
22-Feb-16	2.75%
26-Feb-16	2.68%
7-Mar-16	2.87%
11-Mar-16	2.84%
14-Mar-16	2.92%
21-Mar-16	2.87%
23-Mar-16	2.87%

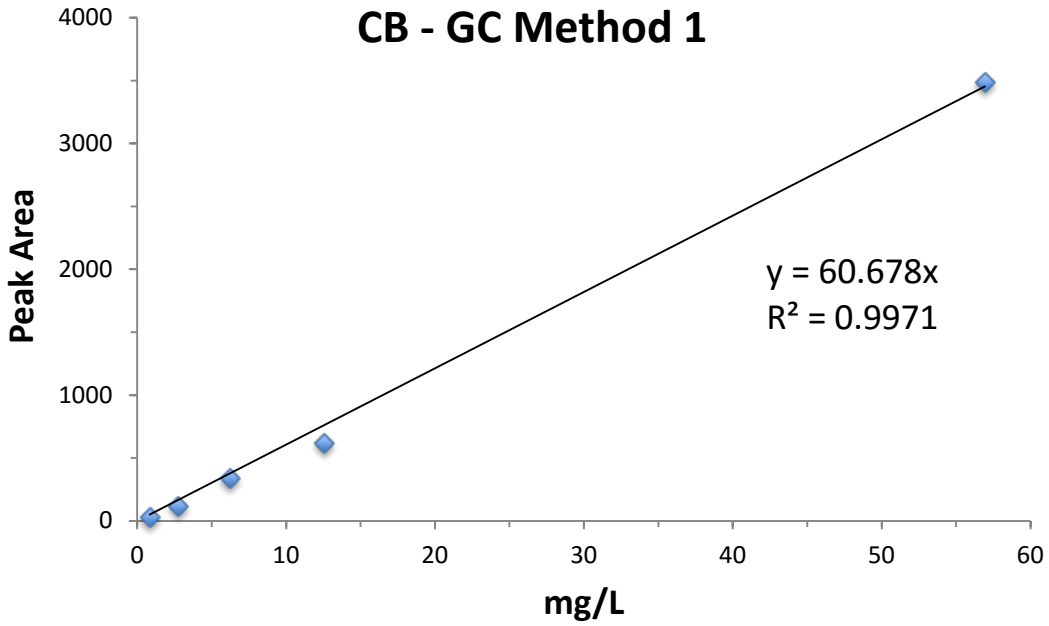

Figure C.1 Calibration curve for CB for Method 1 in the HP 6890 Series II GC.

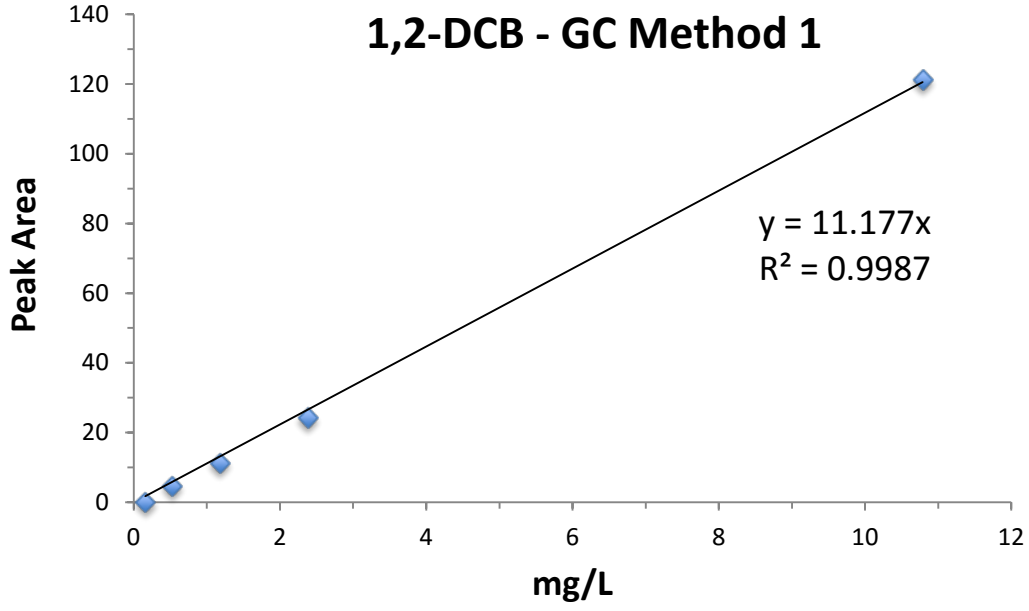

Figure C.2 Calibration curve for 1,2-DCB for Method 1 in the HP 6890 Series II GC.

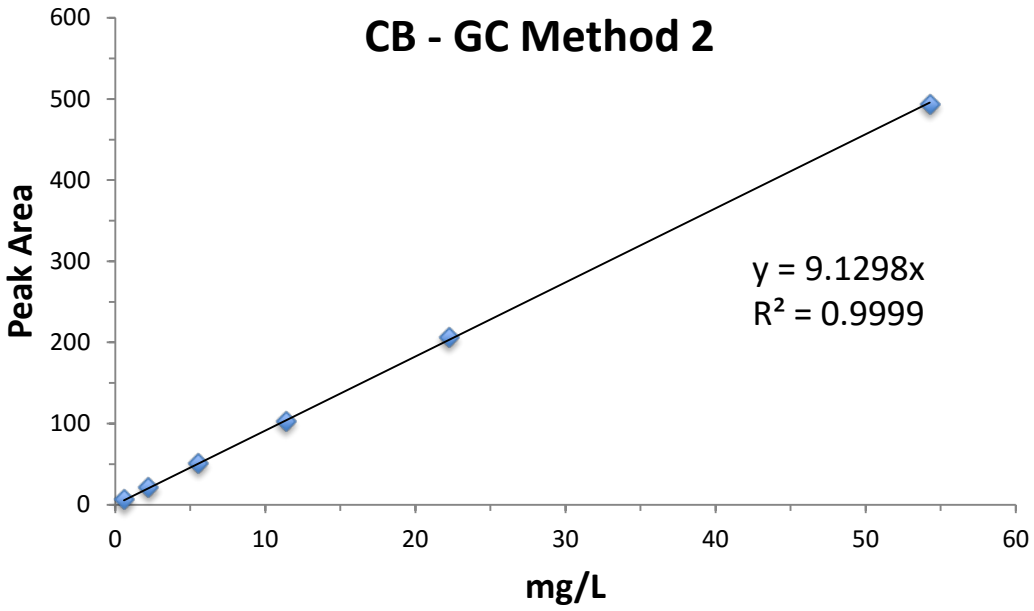

Figure C.3 Calibration curve for CB for Method 2 in the HP 6890 Series II GC.

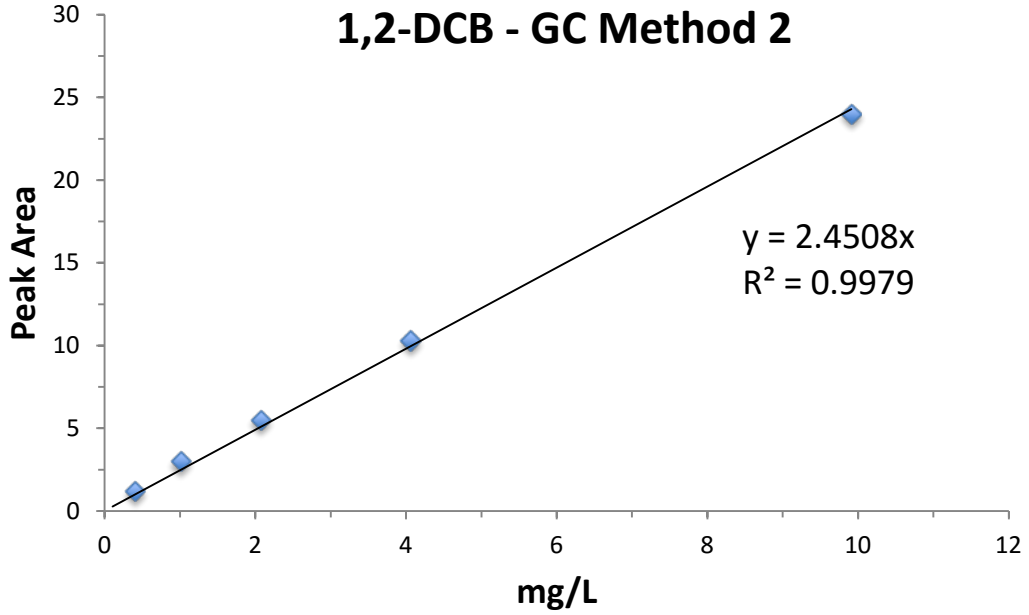

Figure C.4 Calibration curve for 1,2-CB for Method 2 in the HP 6890 Series II GC.

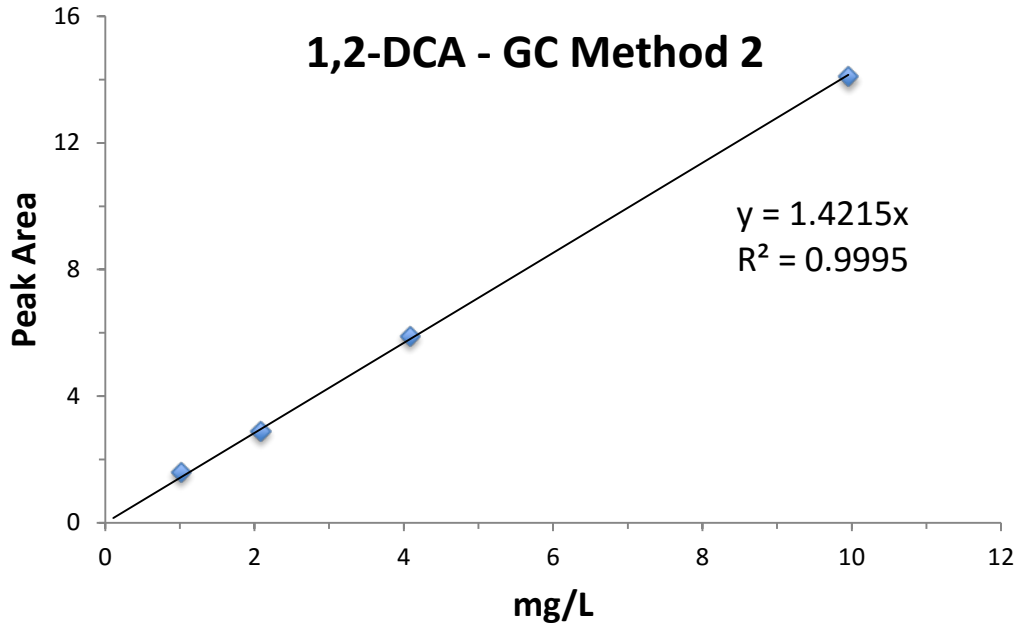

Figure C.5 Calibration curve for 1,2-CB for Method 2 in the HP 6890 Series II GC.

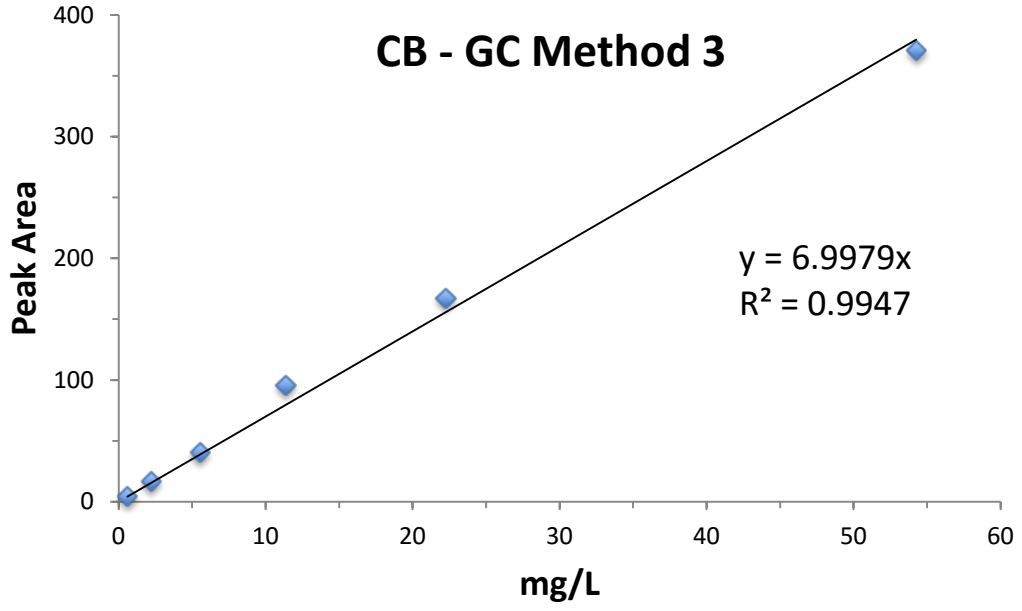

Figure C.6 Calibration curve for CB for Method 3 in the HP 5890 Series II GC.

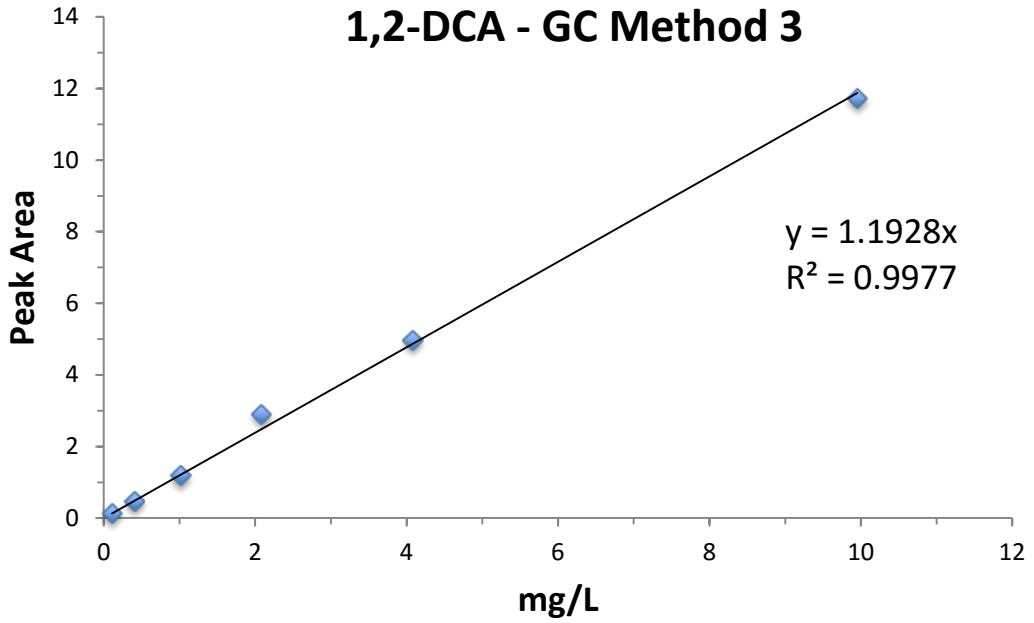

Figure C.7 Calibration curve for 1,2-DCA for Method 3 in the HP 5890 Series II GC.

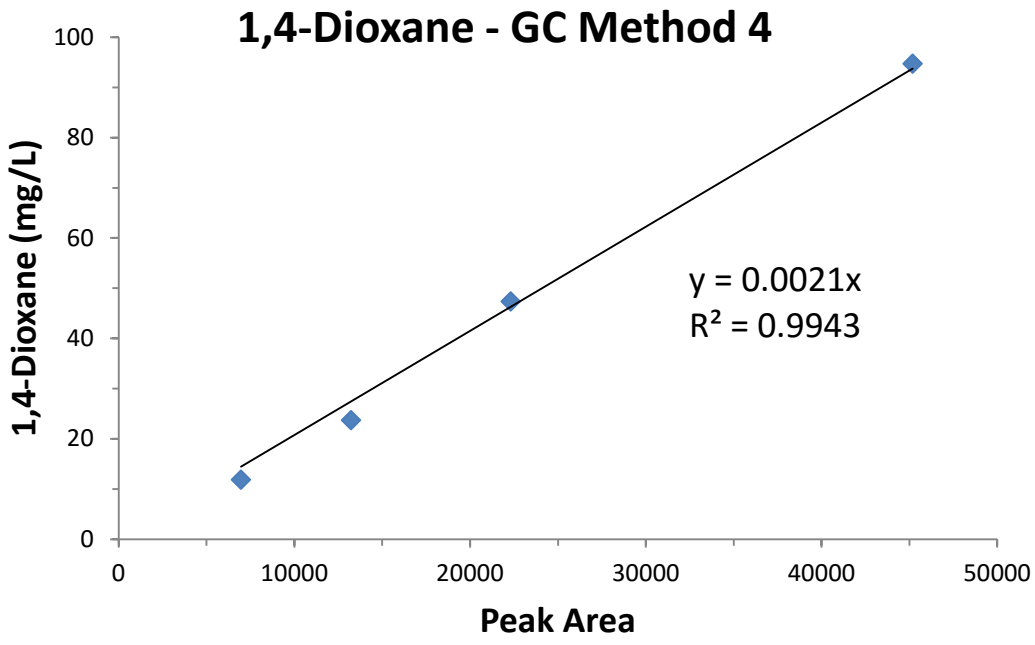

Figure C.8 Calibration curve for 1,4-Dioxane for Method 4 in the HP 5890 Series II GC.

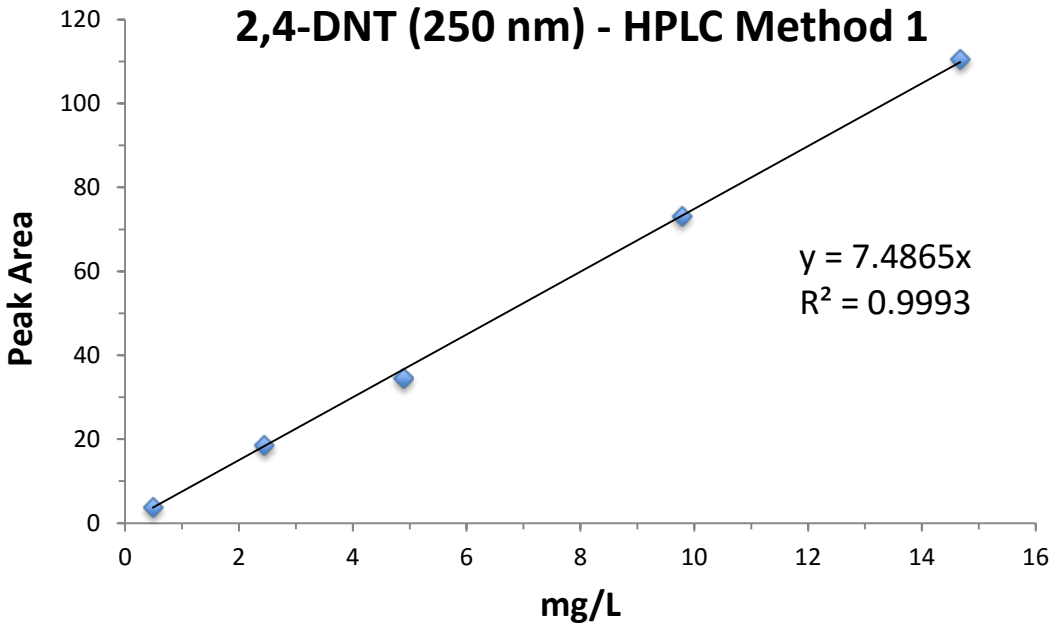

Figure C.9 Calibration curve for 2,4-DNT for Method 1 in the HPLC Dionex 3000 Ultimate Series.

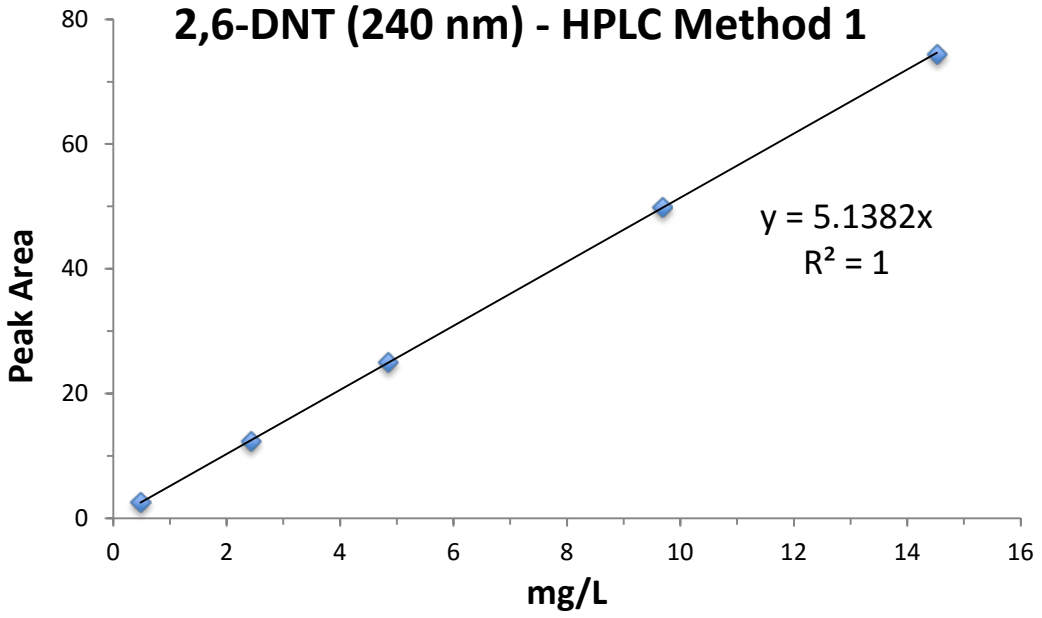

Figure C.10 Calibration curve for 2,6-DNT for Method 1 in the HPLC Dionex 3000 Ultimate Series.

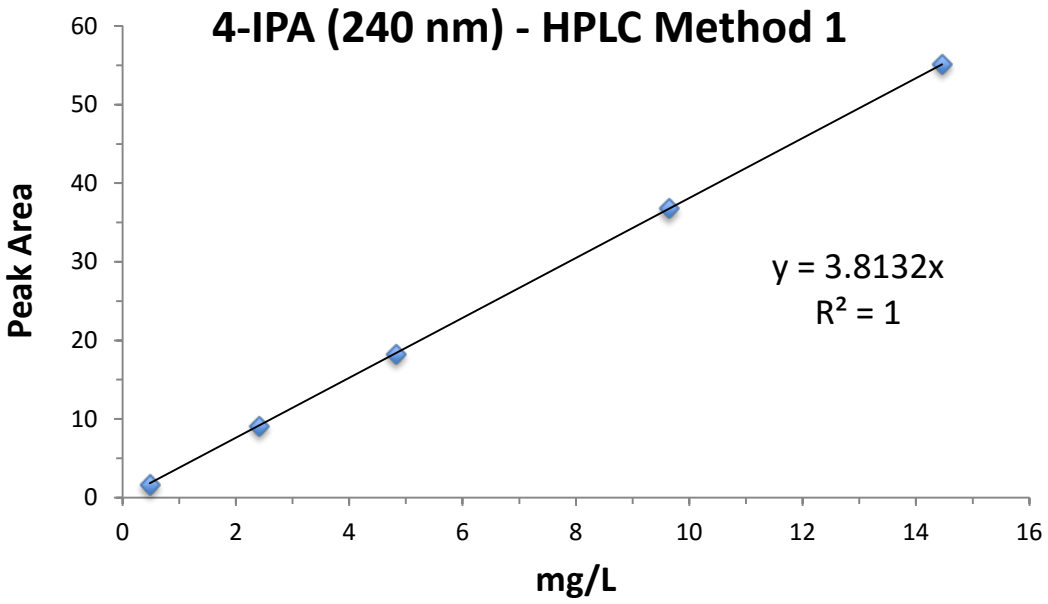

Figure C.11 Calibration curve for 4-IPA for Method 1 in the HPLC Dionex 3000 Ultimate Series.

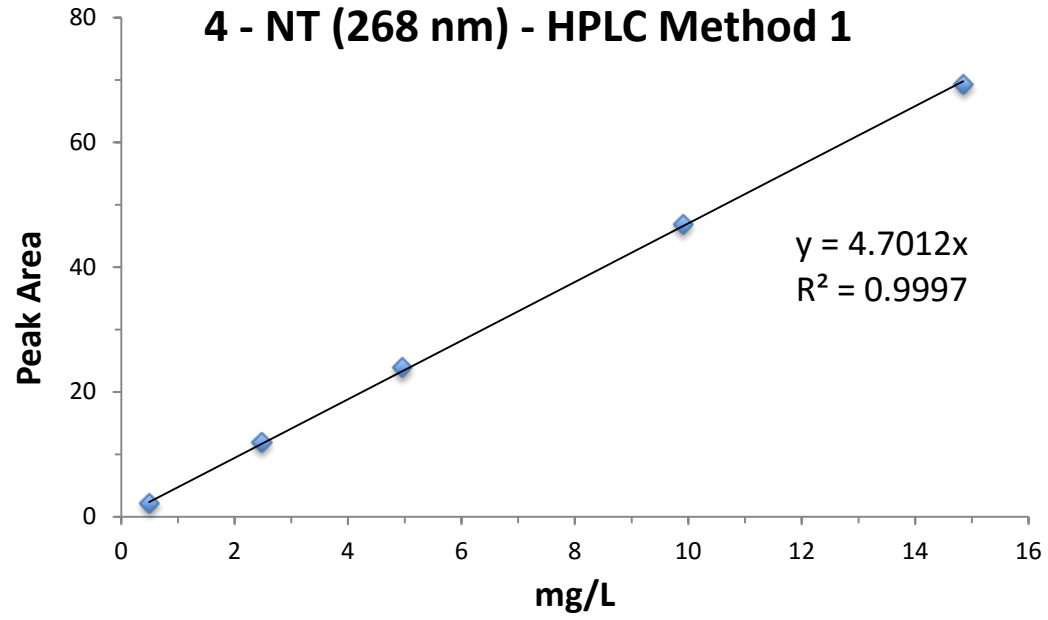

Figure C. 12 Calibration curve for 4-NT for Method 1 in the HPLC Dionex 3000 Ultimate Series.

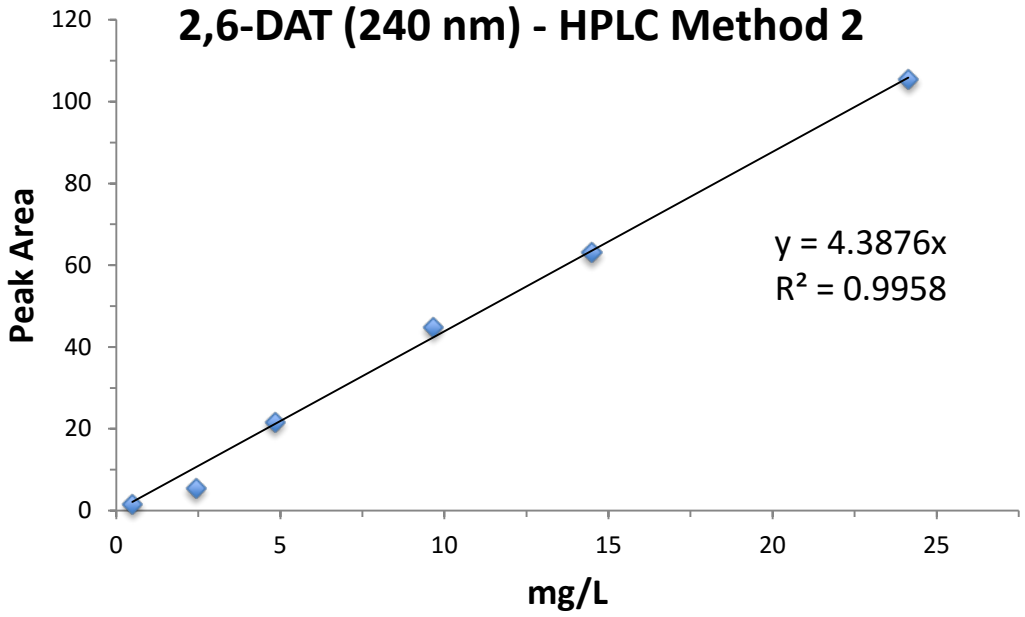

Figure C.13 Calibration curve for 2,6-DAT for Method 2 in the HPLC Dionex 3000 Ultimate Series.

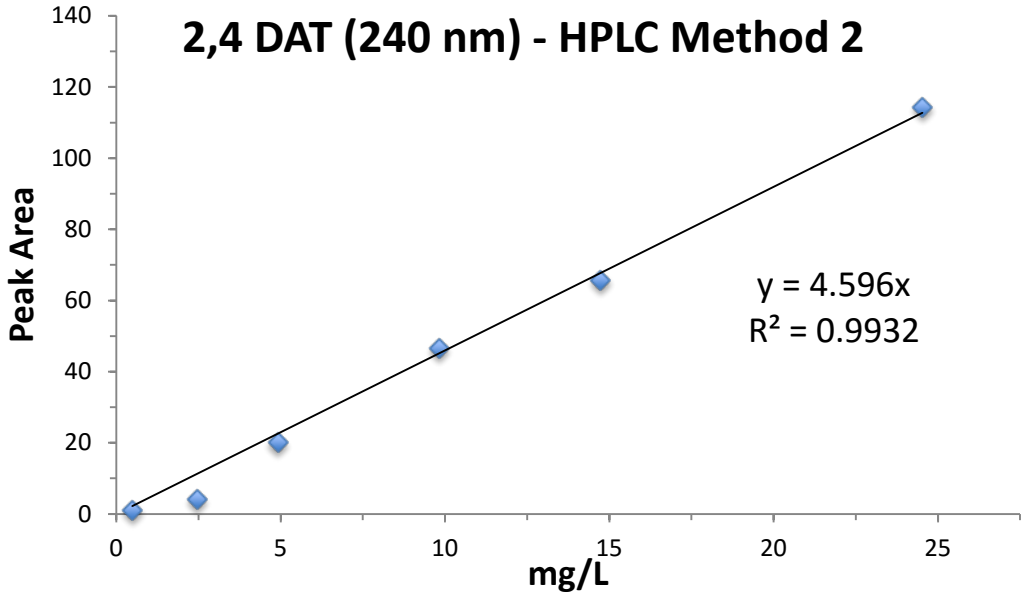

Figure C.14 Calibration curve for 2,4-DAT for Method 2 in the HPLC Dionex 3000 Ultimate Series.

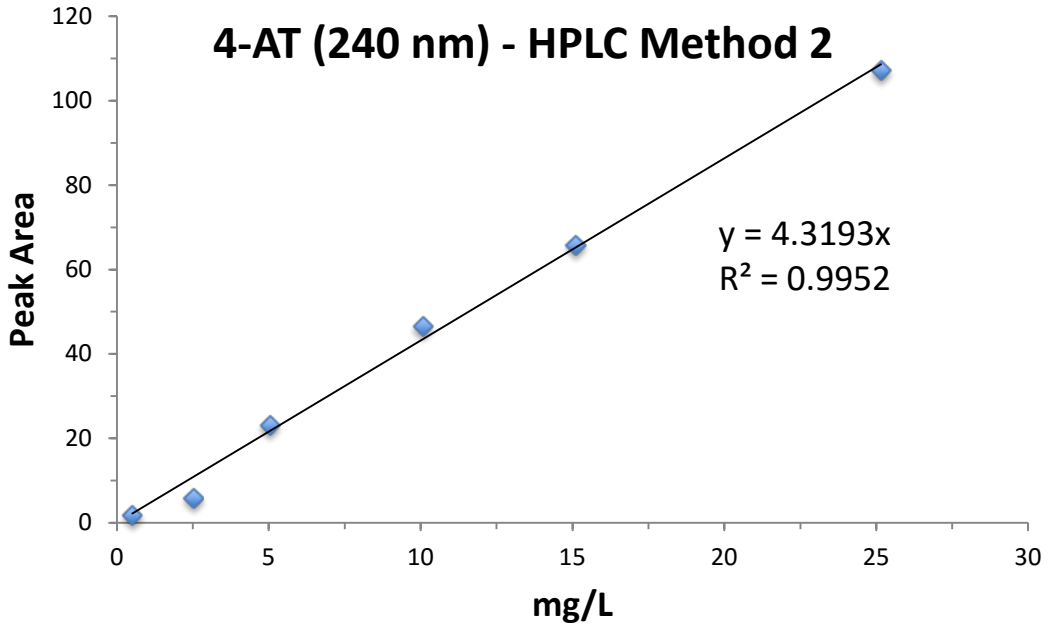

Figure C.15 Calibration curve for 4-AT for Method 2 in the HPLC Dionex 3000 Ultimate Series.

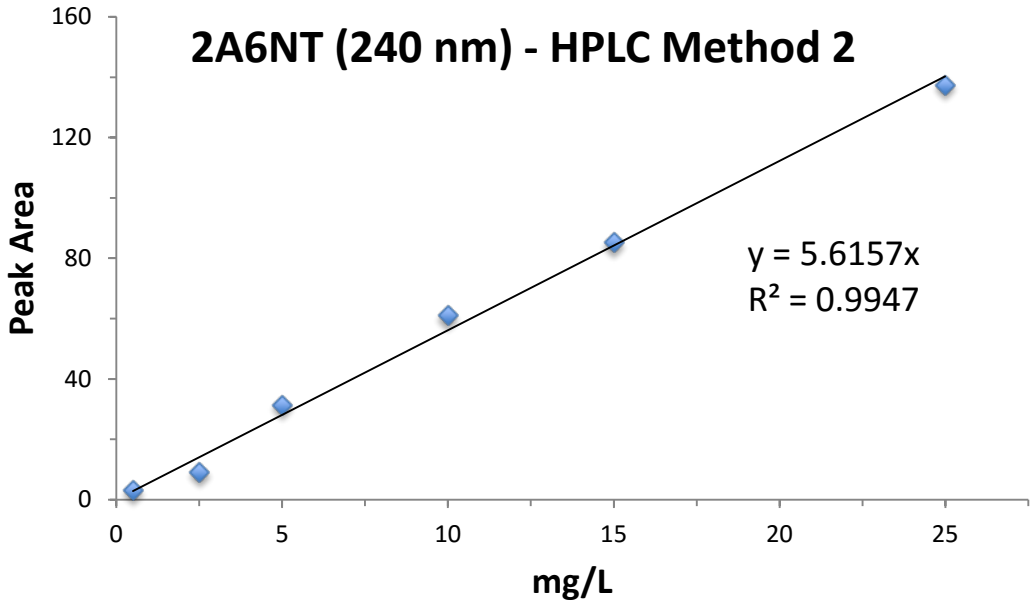

Figure C.16 Calibration curve for 2A6NT for Method 2 in the HPLC Dionex 3000 Ultimate Series.

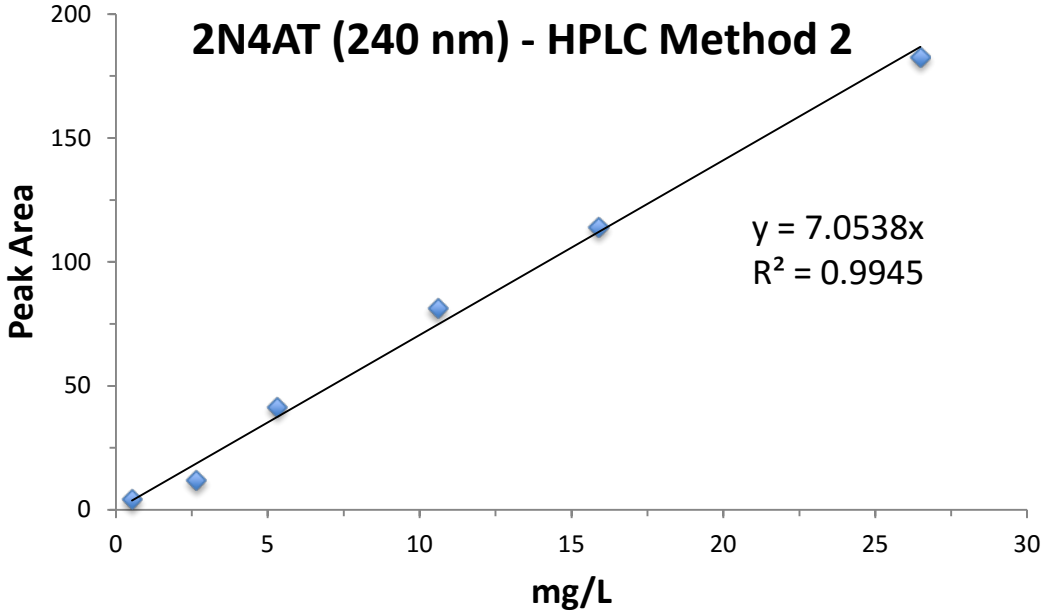

Figure C.17 Calibration curve for 2N4AT for Method 2 in the HPLC Dionex 3000 Ultimate Series.

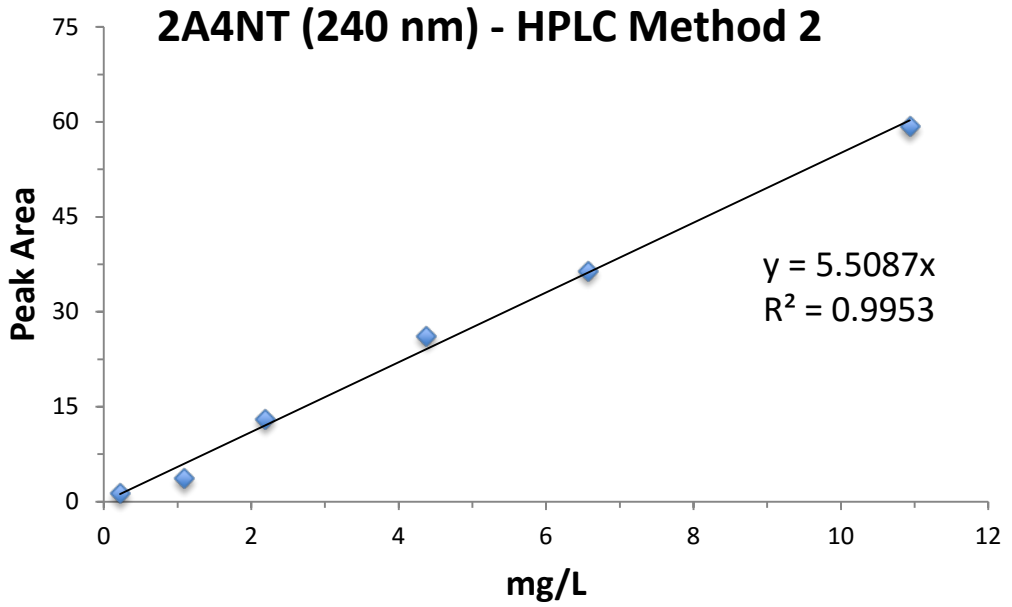

Figure C.18 Calibration curve for 2A4NT for Method 2 in the HPLC Dionex 3000 Ultimate Series.

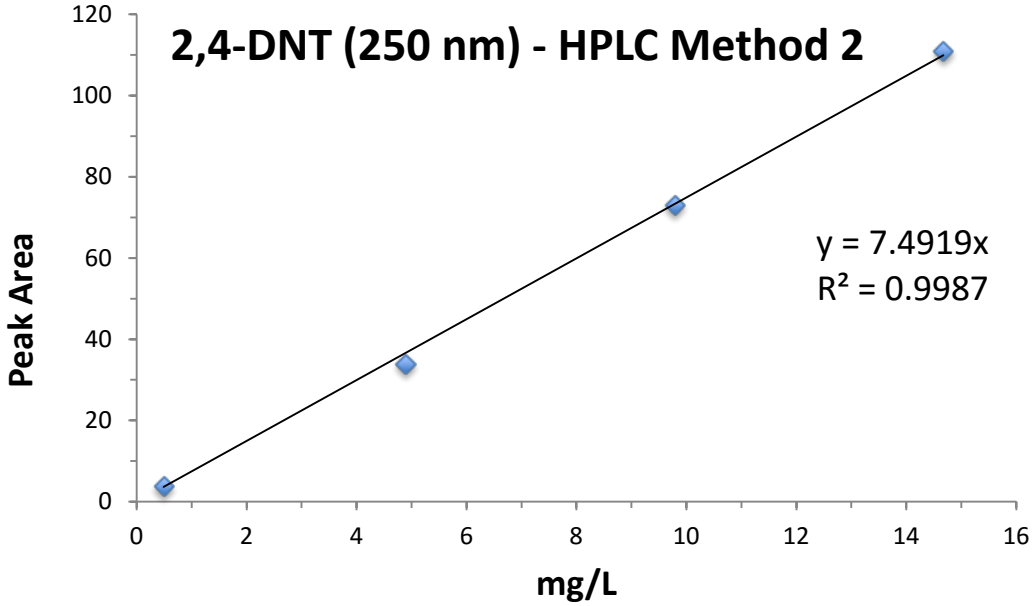

Figure C.19 Calibration curve for 2,4-DNT for Method 2 in the HPLC Dionex 3000 Ultimate Series.

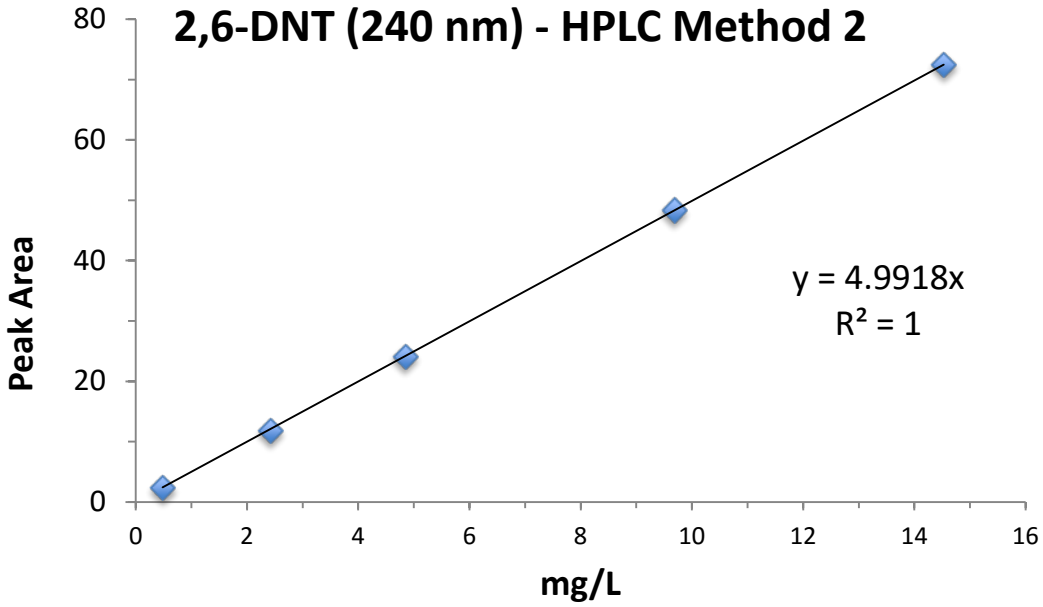

Figure C.20 Calibration curve for 2,6-DNT for Method 2 in the HPLC Dionex 3000 Ultimate Series.

Table C.2 Filter test for 4-IPA, 2,4-DNT, 2,6-DNT and 4-NT.

	4-IPA		DNTs (2,4 and 2,6-DNT)		4-NT	
	Peak Area	Concentration (mg/L)	Peak Area	Concentration (mg/L)	Peak Area	Concentration (mg/L)
Unfiltered - Sample 1	69.99	16.05	171.20	-	77.74	15.05
Unfiltered - Sample 2	68.90	15.81	173.69	-	77.55	15.02
Unfiltered - Sample 3	69.29	15.89	177.36	-	78.68	15.23
Filtered - Sample 1	68.75	15.77	178.88	-	77.43	14.99
Filtered - Sample 2	61.79	14.17	159.39	-	70.59	13.67
Filtered - Sample 3	66.48	15.25	175.16	-	76.73	14.86
***t*test**		0.15		0.66		0.24

Table C.3 Filter test for 2,6-DAT, 2,4-DAT, 4-AT, 2A6NT, 2N4AT, 2A4NT.

	Concentration (mg/L)					
	2,6 DAT	2,4 DAT	4 AT	2A6NT	2N4AT	2A4NT
Unfiltered - Sample 1	8.94	8.17	8.79	8.89	9.44	3.90
Unfiltered - Sample 2	9.11	8.49	8.81	8.90	9.44	3.81
Unfiltered - Sample 3	8.35	7.31	8.76	8.87	9.46	3.88
Filtered - Sample 1	8.33	7.33	8.78	8.88	9.43	3.90
Filtered - Sample 2	8.41	7.41	8.80	8.86	9.41	3.88
Filtered - Sample 3	8.44	7.44	8.78	8.85	9.39	3.89
***t*test**	0.15	0.17	0.80	0.08	0.05	0.39

Appendix D: COD Colorimetric Method and Calibration Curve

1. Preparation of Standards

a) Dry ~0.5 g of KHP in an oven (120°C) for several hours, then cool in a desiccator; KHP = potassium hydrogen phthalate ($KOCOC_6H_4$-2-COOH, MW = 204.22; Crystal AR (ACS), Primary Standard); 1.1752 mg COD/mg KHP.

b) Prepare a stock solution of 500 mg/L COD by dissolving 0.4250 g of KHP in 1 L of DDI water. Record the actual weight of the KHP added.

c) Prepare standards by making the following dilutions

Table D.1 Preparation of standards for COD analysis

Standard (mg/L COD)	Dilution
150	15.0 mL diluted to 50.0 mL
125	25.0 mL diluted to 100.0 mL
100	20.0 mL diluted to 100.0 mL
50	10.0 mL diluted to 100.0 mL
10	2.0 mL diluted to 100.0 mL

2. Analysis of the Standards and Samples

a) Preheat the COD heat block in a lab hood to 150°C.

b) For each sample to be analyzed obtain and number a COD digestion reagent vial to allow later sample identification.

c) Wearing gloves, lab coat and eye protection, digest the samples and standards in the following manner:

- Remove the cap of a COD digestion reagent vial. Hold the vial at a 45° angle pointing away from you. Slowly add 2.5 mL of sample into the vial, allowing the sample to run down the side. The sample should form a layer on top of the reagents. Note: Spilled reagent will affect test accuracy and is hazardous to skin and other materials.
- Replace the vial cap tightly.
- Using a heat resistant glove, hold the vial by the cap and over a sink. Invert gently several times to mix the contents. The vial will get very hot. Rinse the outside of the COD vial with DDI water and wipe the vial clean with a lab wipe. It is important to remove any material from the outside of the vial before it is heated.
- Place the vials in the preheated (150°C) COD heat block. Heat the vials for 2 hours. Check the heat block temperature periodically. If the heat block temperature drops below 150°C, extend the reaction time to compensate for the reduced reactor temperature.
- Very carefully remove the vial from the heating block and place it in a rack to cool.
- Invert each vial several times while still warm.
- Wait until the vials have cooled to room temperature and any precipitate has settled.

- Determine the COD for the standards with a Genesis 20 spectrophotometer (Thermo Scientific).
    - Make sure the correct “holder” is installed. Two are available; one is for cuvettes, the other is for COD vials. Make sure the latter is in place. If it isn’t, pull the cuvette holder straight up and replace it with the COD vial holder, which should be on the bench top next to the instrument.
    - Turn on the spectrophotometer using the switch at the back of the instrument and allow it to warm up for 15 min.
    - Adjust the spectrophotometer wavelength control to 440 nm using the key button “nm” on the front panel.
    - Insert the 150 mg/L COD standard into the sample compartment. Adjust the zero by pressing the “0 ABS” key on the front panel.
    - Read the absorbance of each standard and sample twice, rotating the tube 90° between readings. Average your readings for each tube.
    - Prepare a calibration curve by plotting the absorbance of the standards versus their known concentrations.
- Compare sample absorbance to the calibration curve to determine COD concentration.

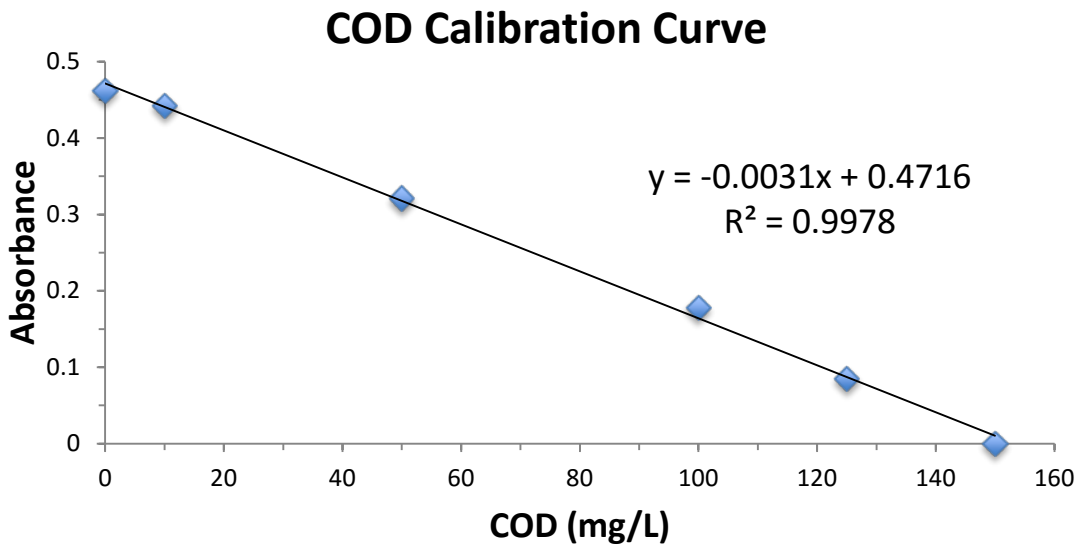

Figure D.1 Calibration curve for CB for Method 1 in the HP 6890 Series II GC.

Table D.2 COD measurements in groundwater samples and chemical oxidation stoichiometric treatment.

Sample ID	ABS (0º)	ABS (90º)	Average ABS	COD (mg/L)
GW - Trip 1	0.422	0.418	0.420	15.9
GW - Trip 2	0.423	0.425	0.424	14.6
GW - Trip 3	0.433	0.433	0.433	11.7
ChemOx 1X - Trip 1	0.021	0.023	0.022	145.0
ChemOx 1X - Trip 2	0.003	0.005	0.004	150.8
ChemOx 1X - Trip 3	0.009	0.009	0.009	149.2
ChemOx 0.5X - Trip 1	0.239	0.240	0.240	74.9
ChemOx 0.5X - Trip 2	0.209	0.210	0.210	84.5
ChemOx 0.5X - Trip 3	0.229	0.226	0.228	78.7

Appendix E: SYBR Green qPCR Protocol

(General Bacteria)

1. Materials

Applied Biosystems® StepOnePlus™ real-time PCR system, positive pressure hood

ABI MicroAmp® fast optical 96-well reaction plate and optical adhesive film

ice block, pipette + tips, centrifuge for the plate, microcentrifuge tubes, vortex and microcentrifuge.

2. Sterilization

Before you start, autoclave the pipette tips and the 2mL microcentrifuge tubes (also the DNA free water and IDTE or TE buffer, if used). Clean the positive pressure hood with 70% ethanol solution, place the plate, film, and the small microcentrifuge tubes (~500uL) inside and turn on the UV light for 15min to 1 hour.

3. Primers

For bacteria qPCR, we use the following primer pair:

PRBA338F 5'-AC TCC TAC GGG AGG CAG CAG -3'

PRUN518R 5'-ATT ACC GCG GCT GCT GG -3'

4. Standards

The standard was obtained from IDT and diluted with IDTE buffer to a final concentration of 10^{10} gene copies/µL. Each time qPCR is performed; make a series of dilution from this stock solution.

5. qPCR reaction composition

To prepare the qPCR reaction mix (25 µL) combine master mix, primers and water in a microcentrifuge vial and tap it. Dispense the mixture into the wells in the reaction plate, followed by the addition of the DNA templates

Table E.1 Components of a 25 µL qPCR reaction mix.

Component	Stock Soln. (µM)	µL stock soln. /25 µL rxn.	Final Conc.	Final amount /25 µL rxn.
Master Mix[a]	2x	12.5	1x	-
Forward Primer[b]	6 (20x)	1.25	300 nM	7.5 pmole
Reverse Primer[b]	6 (20x)	1.25	300 nM	7.5 pmole
DNA template	-	2.5	-	100pg-1µg
Water[c]	-	7.5	-	-

[a] Power SYBR Green Master Mix from Life Technologies.
[b] Primers can be ordered from IDT as customized double strand oligo.
[c] RNase-free, molecular biology grade water.

6. Plate preparation

Place the 96-well plate in an ice block (everything should be in the ice-block when not in use). Dispense the premixed qPCR mix in each reaction well. Add your DNA template and use the pipette tips to remove trapped bubbles (as much as possible). Seal the plate with an optical adhesive film. The plate can be centrifuged briefly in the salad spin to collect the content at the bottom of the plate and eliminate any trapped air bubbles.

Each plate should contain negative controls (water instead of DNA template), samples and standards. Triplicate samples are recommended.

7. Load the plate and run program

Load the plate in StepOnePlus™. Choose “Standard mode thermal cycling conditions” and select “SYBR Green” and "Standard" ramp speed in the "Method & Materials”.

Set up the plate arrangement.

Set up the temperature program (3 stages are included):

Stage I: 10 min at 95°C (to activate the AmpliTaq Gold® DNA Polymerase)

Stage II: 40~45 cycles of:

30s at 95°C

20s at 62°C

30s at 72°C

Stage III: Melting curve from 50°C to 99 C at 1°C increment.

REFERENCES

Chen J. 2012. Development of Enrichment Cultures for Anaerobic Reductive Dechlorination of Tetrachloroethene Under Low pH Conditions. M.S. Thesis, Clemson University, Clemson, SC.

Droste, E., Marley, M., Parikh, J., Lee, A., Dinardo, P., Woody, B., Hoag, G., & Chheda, P. 2002. Observed enhanced reductive dechlorination after in situ chemical oxidation pilot test. Remediation of Chlorinated and Recalcitrant Compounds. Proceedings of the Third International Conference on Remediation of Chlorinated and Recalcitrant Compounds (Monterey, CA).

Elango, V., Cashwell, J. M., Bellotti, M. J., Marotte, R., & Freedman, D. L. 2010. Bioremediation of hexachlorocyclohexane isomers, chlorinated benzenes, and chlorinated ethenes in soil and fractured dolomite. *Bioremediation Journal*, *14*(1), 10–27.

Elango, V., Kurtz, H. D. & Freedman, D. L. 2011. Aerobic cometabolism of trichloroethene and *cis*-dichloroethene with benzene and chlorinated benzenes as growth substrates. *Chemosphere*, *84*(2), 247-253.

Fung, J. M., Weisenstein, B. P., Mack, E. E., Vidumsky, J. E., Ei, T., & Zinder, S. H. 2009. Reductive dehalogenation of dichlorobenzenes and monochlorobenzene to benzene in microcosms. *Environmental Science and Technology*, *43*(7), 2302–2307.

Gray, D., Lantzy, R. 2014. Evaluation of combined in-situ remedial technologies alternatives for VOC/SVOC constituents: bench scale studies. Remediation of Chlorinated and Recalcitrant Compounds - The ninth interational conference (Monterey, CA).

Haigler, B. E., Pettigrew, C. A., & Spain, J. C. 1992. Biodegradation of mixtures of substituted benzenes by *Pseudomonas* sp. strain JS150. *Applied and Environmental Microbiology*, *58*(7), 2237–2244.

Hareland, W. a., Crawford, R. L., Chapman, P. J. & Dagley, S. 1975. Metabolic function and properties of 4-hydroxyphenylacetic acid 1-hydroxylase from *Pseudomonas acidovorans. J.* Bacteriol. 121, 272–285.

Heidrich, S., Weiß, H., & Kaschl, A. 2004. Attenuation reactions in a multiple contaminated aquifer in Bitterfeld (Germany). *Environmental Pollution*, *129*(2), 277–288.

Hongsawat, P., & Vangnai, A. S. 2011. Biodegradation pathways of chloroanilines by *Acinetobacter baylyi* strain GFJ2. *Journal of Hazardous Materials*, *186*(2-3), 1300–1307.

Huang, K. C., Zhao, Z., Hoag, G. E., Dahmani, A. & Block, P. 2005. Degradation of volatile organic compounds with thermally activated persulfate oxidation. *Chemosphere* **61,** 551–560.

Hudcova, T., Halecky, M., Kozliak, E., Stiborova, M., & Paca, J. 2011. Aerobic degradation of 2,4-dinitrotoluene by individual bacterial strains and defined mixed population in submerged cultures. *Journal of Hazardous Materials*, *192*(2), 605–613.

Jacob, H. 1970. Chapter IV Redox potential. Methods in Microbiology 2:91-123.

Kurt, Z., & Spain, J. C. 2013. Biodegradation of chlorobenzene, 1,2-dichlorobenzene, and 1,4-dichlorobenzene in the vadose zone. *Environmental Science and Technology*, *47*(13), 6846–6854.

Leahy, J. G., Tracy, K. D., & Eley, M. H. 2003. Degradation of mixtures of aromatic and chloroaliphatic hydrocarbons by aromatic hydrocarbon-degrading bacteria. *FEMS Microbiology Ecology*, *43*(2), 271–276.

Lendenmann, U., Spain, J. C., & Smets, B. F. 1998. Simultaneous biodegradation of 2,4-dinitrotoluene and 2,6 dinitrotoluene in an aerobic fluidized-bed biofilm reactor. *Environmental Science & Technology*, *32*(1), 82–87.

Leungsakul, T., Keenan, B. G., Yin, H., Smets, B. F., & Wood, T. K. 2005. Saturation mutagenesis of 2,4-DNT dioxygenase of *Burkholderia* sp. strain DNT for enhanced dinitrotoluene degradation. *Biotechnology and Bioengineering*, *92*(4), 416–426.

Mujahid, M., Sasikala, C. & Ramana, V. C. 2014. Aniline is an inducer, and not a precursor, for indole derivatives in *Rubrivivax benzoatilyticus* JA2. *PLOS One* **9,** 1–10.

Nelson, J. L., Fung, J. M., Cadillo-Quiroz, H., Cheng, X., & Zinder, S. H. 2011. A role for *Dehalobacter spp*. in the reductive dehalogenation of dichlorobenzenes and monochlorobenzene. *Environmental Science and Technology*, *45*(16), 6806–6813.

Nishino, S. F., Paoli, G. C., & Spain, J. C. 2000. Aerobic Degradation of dinitrotoluenes and pathway for bacterial degradation of 2,6-dinitrotoluene. *Applied and Environmental Microbiology*, *66*(5), 2139–2147.

Nitisakulkan, T., Oku, S., Kudo, D., Nakashimada, Y., Tajima, T., Vangnai, A. S., & Kato, J. 2014. Degradation of chloroanilines by toluene dioxygenase from *Pseudomonas putida* T57. *Journal of Bioscience and Bioengineering*, *117*(3), 292–297.

Peroxychem. Activating Klozur® SP with a 25% Sodium Hydroxide Solution. (n.d.): n. pag. Web. 15 Jan. 2016. <http://www.peroxychem.com/media/191078/peroxychem-klozur-sp-activation-guide-alkaline-25.pdf>.

Peroxychem. Peroxygen Talk - Klozur ® Persulfate: The Impact of Sulfate Residual. 2007. pag. Web. 15 Jan. 2016. < http://www.peroxychem.com/media/179424/peroxychem-peroxygen-talk-2007-2-klozur-persulfate-the-impact-of-sulfate-residual.pdf >.

Peroxychem. Peroxygen Talk - The Effect of Klozur® Activated Persulfate ISCO on Microbial Populations. 2009. pag. Web. 15 Jan. 2016. <http://www.peroxychem.com/media/179431/peroxychem-peroxygen-talk-2009-1-the-effect-of-klozur-activated-persulfate-isco-on-microbial-popul.pdf>

Sander, R. Compilation of Henry's Law Constants for Inorganic and Organic Species of Potential Importance in Environmental Chemistry. 1999. n. pag. Max-Planck Institute of Chemistry. Web. 3 May 2015. <http://www.henrys-law.org/henry-3.0.pdf>.

Scheunert, I., & Reuter, S. 2000. Formation and release of residues of the ^{14}C-labelled herbicide isoproturon and its metabolites bound in model polymers and in soil. *Environmental Pollution, 108*(1), 61–68. 7491(99)00202-X.

Shin, K. H., Lim, Y., Ahn, J. H., Khil, J., Cha, C. J., & Hur, H. G. 2005. Anaerobic biotransformation of dinitrotoluene isomers by *Lactococcus lactis* subsp. *lactis* strain 27 isolated from earthworm intestine. *Chemosphere*, *61*(1), 30–39.

Spiess, T., Desiere, F., Fischer, P., Spain, J., Knackmuss, H. & Lenke, H. 1998. A new 4-nitrotoluene degradation pathway in a *Mycobacterium* strain. *Appl. Environ. Microbiol.* **64,** 446–452.

The Interstate Technology & Regulatory Council. 2005. Technical and regulatory guidance for In-Situ Chemical Oxidation for contaminated soil and groundwater. 171 (2005). at <G:\bibliotheek\digitale documenten\D2 ISCO 2.pdf>

Yang, H., Halasz, A., Zhao, J. S., Monteil-Rivera, F. & Hawari, J. 2008. Experimental evidence for in situ natural attenuation of 2,4- and 2,6-dinitrotoluene in marine sediment. *Chemosphere* **70,** 791–799.

Zhang, J., Hong, Li, Q., Cao, L., Sun, J. Q., Yan, X. & Li, S. P. 2012. Characterization of isoproturon biodegradation pathway in *Sphingobium* sp. YBL2. International Biodeterioration and Biodegradation, 70, 8–13.