BIOTRANSFORMATION OF ANILINE, PARA-CHLROANILINE AND

PENTACHLORONITROBENZENE AND ENVIRONMENTAL IMPLICATIONS

By

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ABSTRACT OF THE DISSERTATION

Biotransformation of Aniline, *Para*-chloroaniline and Pentachloronitrobenzene and

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This dissertation describes research on biotransformation of aniline and para-

chloroaniline (PCA) under anoxic and anaerobic conditions in sediments and

groundwater aquifer solids recovered from a chemical industrial site. Four locations were

examined: lightly and highly contaminated freshwater canal sediments, and lightly and

highly contaminated aquifer sediments. Aquifer and canal microcosms showed loss of

aniline under nitrate-, Fe (III)- and sulfate-amended conditons, and under methanogenic

conditions. Expected intermediates of aniline biotransformation were not detected. Only

5 to 10 percent of ^{14}C was recovered as CO_2 from ^{14}C-labelled aniline during a

mineralization test. Thus, definitive pathways of biodegradation were not determined for

aniline.

PCA loss was observed in nitrate-amended microcosms. Reductive dechlorination of

PCA was observed in canal microcosms under methanogenic conditions and was

concurrent with stoichiometric aniline production. Dechlorination was slow, and was

only observed after increasing the PCA concentration to ~1500 µM and adding the

electron donor/hydrogen source, lactate. PCA loss was not observed under methanogenic

conditions in aquifer sediments. Combined with the observation of aniline loss in

methanogenic microcosms, these results support the possibility for complete dechlorination of monochloroaniline to aniline and subsequent degradation of aniline in canal sediments.

The bacterial phylotypes present in nitrate-aniline-amended microcosms clustered with known aniline degraders (*Delftia*) and nitrate-reducers (*Acidovorax and Variovorax*). The dominant phylotype in the most active aniline-amended transfers had high similarity to *Magnetospirillum*, a genus that is capable of nitrate reduction and degradation of aromatic pollutants. The phylotypes identified in PCA-dechlorinating microcosms included *Dehalobacter*, a dechlorinator that has been linked to dechlorination of chlorinated benzenes at the same site.

This work highlights the potential for anaerobic transformation of aniline and PCA at this industrial site and is one of only a few studies that have identified bacterial community members in sediments and enrichments that carry out these transformations. More work is needed to conclusively identify the biological pathways responsible for the loss of aniline.

Finally, a mixed culture containing *Dehalococcoides mccartyi* strain 195 was tested for ability to transform pentachloronitrobenzene to chlorinated anilines. The culture did not dechlorinate PCA. PCNB was abiotically transformed to pentachloroaniline, which was reductively dechlorinated to dichloroaniline.

ACKNOWLEDGEMENTS

DEDICATION

This dissertation is dedicated to my mother, Mrs. Yuxin Huang and to my late

grandmother, Mrs. Yuli Yan.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

Chapter 1 Introduction

1.1 Rationale

Aniline and *para*-chloroaniline (PCA) have a long history as industrial chemicals for production of dyes, pesticides and pharmaceutical compounds, and have been problematic pollutants of soils and groundwater throughout the history of chemical manufacturing (Travis 1997).

Pentachloroaniline (PeCA) and pentachloronitrobenzene (PCNB) are also widely distributed nitrogen-containing aromatic pollutants [for a recent review see (Okutman Tas and Pavlostathis 2013)]. Their widespread distribution is in part caused by the use of PCNB as a fungicide for crops and because in anoxic environments a major pathway of transformation is its reduction to PeCA (Gile and Gillett 1979). Aromatic amines such as the chlorinated anilines can also be formed by the cleavage of azo dyes or by the biodegradation of other nitroaromatic compounds under anoxic conditions and thus may have additional sources in the environment (Bartha and Pramer 1967; Tan et al. 1999; Spain, J.C. 1995).

Aniline is considered a potential carcinogen and is genotoxic (U.S. EPA (United States Environmental Protection Agency) 1992; U.S. EPA 1991; U.S. EPA 1988; U.S. EPA

1985), while PCA is a known animal carcinogen and is also genotoxic (Pinheiro et al. 2004). The removal of aniline and PCA from contaminated aquifers, sediments and wastewater is problematic and has been attempted by both biological and abiological means (Laha and Luthy 1990; Vandevivere et al. 1998).

Bacterial biotransformation of aniline and monochloroanilines occurs under both aerobic and anoxic conditions. Aniline can be rapidly metabolized via the catechol pathway under aerobic conditions by a variety of bacterial species (Bachofer et al. 1975; Zeyer and Kearney 1982; Latorre et al. 1984; Spain 1995; Liu et al. 2002).

Degradation of aniline also occurs under anoxic conditions. *Desulfobacterium anilini* Ani 1 originally isolated from marine sediment from the North Sea (Schnell, Bak, and Pfennig 1989) utilizes aniline under sulfate-reducing conditions. Anaerobic degradation of aniline proceeds through 4-aminobenzoate which is then converted via CoA-thioesterification and reductive deamination of the 4-aminobenzoyl-CoA to benzoyl-CoA (Schnell and Schink 1991). Strain HY99, closely related to *Delftia acidovorans* (Loidle et al. 1990) an aerobic aniline degrader, was reported to degrade aniline under both aerobic and nitrate-reducing conditions and 4-aminobenzoate was detected in the nitrate-reducing cultures (Kahng, Kukor, and Oh 2000). Only these two reports exist of specific identified aniline degraders under anoxic conditions. However, there are several reports of biologically-linked anaerobic aniline degradation in sediments and sludges by unidentified microbes. Aniline loss was observed under nitrate-reducing conditions in sediment from Newtown Creek, a estuarine channel located in New York, NY USA, and in anaerobic digester sludge from the Berkeley Heights WWTP in Berkeley Heights, NJ

USA, however, no aniline loss was observed under methanogenic conditions after 217

days in the sediments (De, Owen A. O'Connor, and Kosson 1994). De et al. detected 4-

hydroxybenzoate in active aniline-degrading microcosms, but not in killed controls or in

non-active microcosms. Addition of bicarbonate to the medium appeared to lead to more

nitrogen production (from nitrate reduction) compared to controls. Addition of

bicarbonate was thought to allow carboxylation of aniline to produce *p*-aminobenzoate.

Aniline loss in Hudson River sediments was observed under Fe(III)-reducing conditions

within 65 days but the activity was not sustained by refeeding, and thus it could not be

confirmed how the initial aniline loss occurred (Kazumi et al. 1995a). In the

aforementioned environmental studies, microbial communities associated with the

various activities were not characterized. Thus, knowledge of anaerobic aniline

degradation pathways and the responsible microorganisms is quite limited.

Aerobic degradation of PCA in systems co-amended with aniline resulted in formation of

toxic 4-chlorocatechol (Radianingtyas, Robinson, and Bull 2003) while some species

transformed 3-chloroaniline through 3-chlorocatechol (Boon et al. 2003; Boon et al.

2001). Possible reductive deamination of dihaloanilines under nitrate-reducing conditions

resulted in formation of dihalobenzenes by *Rhodococcus* sp. strain 2 (Travkin et al.

2002). Several studies have reported negative results for reductive dechlorination of PCA

or other monochloroanilines in reduced methanogenic enrichments or microcosms which

dechlorinated higher chlorinated anilines or other halogenated aromatics such as

chlorophenols or chlorobenzoates (Kuhn and Suflita 1989; Haggblom and Young 1995;

Sharak Genthner 1999; Okutman Tas and Pavlostathis 2005). Thus, to date, little is

known about the microorganisms involved in the transformation of PCA (or other chloroanilines) in anoxic sediments.

Since the mid-1990's, several anaerobic bacterial genera were reported to be able to reductively dehalogenate halogenated compounds and obtain energy for growth from the dehalogenation reaction (Holliger, Wohlfarth, and Diekert 1999). *Dehalococcoides mccartyi* (Löffler et al. 2013) was initially mainly identified in chloroethene and chlorobenzene dechlorinating enrichments, however members of this genera have varied abilities to dechlorinate a wide array of chlorinated organic compounds. *Dehalococcoides mccartyi* strain 195 dehalorespires tetra- tri and di-chloroethenes and co-metabolizes vinyl chloride to ethene (Maymo-Gatell et al. 1997). This organism also dechlorinates selected congeners of the polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs) and polychlorinated biphenyls (PCBs) (Fennell et al. 2004; Liu and Fennell 2008) and grows through dehalorespiration of chlorophenols (Adrian et al. 2007). Krumins et al. (2009) investigated the use of the crop fungicide PCNB as a co-amended substrate to enhance the dechlorination of weathered PCBs by native bacteria or by bioaugmented strain 195 in Anacostia River, Washington DC sediments. Under both these tested conditions, the rate and extent of PCB dechlorination was enhanced. It is believed that by adding a halogenated co-substrate, the increasing production of the dehalogenase enzymes may maximize catalytic efficiency or stability of PCB dechlorination (See review by Bedard 2008). Interestingly, whereas the native population of the Pinellas subgroup of *Dehalococcoides* spp. increased in Anacostia River sediments treated with PCNB (which was confirmed to be rapidly reduced to PeCA, then dechlorinated to dichloroanilines in the sediments), the bioaugmented strain 195

population decreased over 300 days, even though PCB dechlorination was enhanced (Park et al. 2011). The dechlorination or dehalorespiration of PeCA (the main product of PCNB transformation in anoxic systems) or other chlorinated anilines by *Dehalococcoides mccartyi* strain 195 has not been directly investigated.

1.2 Site under Study

The contaminated site under study is located along the eastern shore of the Delaware River along State Highway 130 (Shell Road) in Deepwater, New Jersey. East of the site are light industrial, residential and recreational areas. North of the site are residential areas, with the Delaware River to the west (Figure 1.1a). Samples were obtained from four locations (Table 1.1) at this large chemical manufacturing site: lightly contaminated groundwater aquifer sediments; highly contaminated groundwater aquifer sediments; lightly contaminated freshwater canal sediments; and highly contaminated freshwater canal sediments (Figure 1.1b). The freshwater canal provides the process water supply for the manufacturing site. The canal runs adjacent to the southern boundary of the site and discharges into the Delaware River. The canal sediments were contaminated under the influence of the adjacent contaminated groundwater aquifer. At the more highly contaminated locations, benzene, monochlorobenzene, dichlorobenzene, polyaromatic hydrocarbons, dyes and other contaminants are present, in addition to aniline and PCA.

1.2.1 "Lightly" contaminated groundwater aquifer location

Four cores of 3 ft by 1.5 in. in diameter were provided from the lightly contaminated groundwater aquifer area. The sample location is labled as GDS-11 and is shown in Figure 1.1c. Cores of sediment were obtained from 0 to 10 ft below ground surface in December 2006. Groundwater was obtained from the groundwater aquifer near this location.

1.2.2 "Highly" contaminated groundwater aquifer location

Five cores of 3 ft by 1.5 in. in diameter were provided from the highly contaminated groundwater aquifer location, which is beneath a former dyes manufacturing area. The sample location is labled as G06-14 and shown in Figure 1.1d**.** Cores of sediment were obtained from 0 to 10 ft below ground surface in December 2006. The yellow to red color gradient on the map (Figure 1.1d) shows the groundwater isoconcentration of aniline. Aniline concentrations in the groundwater range from 6 to 61,000 µg/L. The highly contaminated location is located close to the highest concentration point of aniline.

1.2.3 "Lightly" contaminated freshwater canal location

Three cores of 5 ft by 3.2 in. in diameter were provided from the lightly contaminated freshwater canal area (up stream) in January 2007. The sample location is shown in Figure 1.1d and 1.1e. Additional sediment grab samples were obtained from the bottom of the water column at the upstream lightly contaminated location in April 2011 on a rainy day. At both sampling dates, canal water was also obtained from the upstream lightly contaminated location.

1.2.4 "Highly" contaminated freshwater canal location

Three cores of 5 ft by 3.2 in. in diameter were provided from the highly contaminated area (downstream) in January 2007. The sample location is also shown in Figure 1.1d and 1.1e.

1.3 Goals and Objectives

The overall goal of this study was to investigate biodegradation of aniline and PCA under anoxic conditions in contaminated groundwater and freshwater sediment systems from a chemical manufacturing site. A secondary goal was to investigate the dechlorination of PCA and PeCA by a mixed culture containing *Dehalococcoides mccartyi* strain 195. This research was intended to strengthen our understanding of the fate of aniline and PCA under anoxic conditions and to identify bacterial community members in active aniline- and PCA-degrading systems. This research was focused on solving environmental problems for chemical manufacturing sites where combinations of chemical precursors including aniline and PCA are present as contaminants. The specific objectives of the study were:

(1) To determine if aniline and PCA can be degraded under aerobic, nitrate-amended, Fe(III)-amended, sulfate-amended or methanogenic conditions in sediment collected

from different locations within a large industrial site and to identify specific bacterial phylotypes involved.

(2) To enrich and identify specific bacterial strains involved in aniline and PCA degradation under nitrate-amended and methanogenic conditions.

(3) To determine if a mixed culture containing *Dehalococcoides mccartyi* strain 195 could dechlorinate PCA.

(4) To investigate the transformation of PCNB and dechlorination of PeCA by a mixed culture containing *Dehalococcoides mccartyi* strain 195 and to delineate dechlorination pathways for PeCA by this culture.

1.4 Dissertation Overview

This dissertation is composed of six chapters. In Chapter 1, an introduction to the study and its rationale is provided. Chapter 2 contains a literature review showing how the study relates to previous research and scholarly thought. Chapters 3 through 5 include the detailed design of the research approach and the results. Chapter 6 discusses the significance of the research findings and environmental implications. The transformation of PCNB and dechlorination of PeCA by a mixed culture containing *Dehalococcoides*

mccartyi strain 195 is discussed in Appendix I. Tables and the most informative figures are shown at the end of each respective chapter. Appendix II is a collection of figures showing negative or inconclusive results.

Table 1. 1. Summary of sediment samples from study site.

Location	Sediment sample	Locations ID	Date
Lightly contaminated groundwater aquifer	Four cores of 3 ft × 1.5 in.	GDS-11 (0-10')	Dec. 2006[a]
Highly contaminated groundwater aquifer	Five cores of 3 ft × 1.5 in.	G06-14 (0-10')	Dec. 2006
Lightly contaminated freshwater canal	Three cores of 5 ft × 3.2 in.	SCD-120	Jan. 2007 [b]
	Grab samples	SCD-120	Apr. 2011[c]
Lightly contaminated freshwater canal	Three cores of 5 × 3.2 in.	SCD-7, SCD-8, SCD-25	Jan. 2007

[a, b, c] *During this sampling event/location groundwater or canal water was also recovered.*
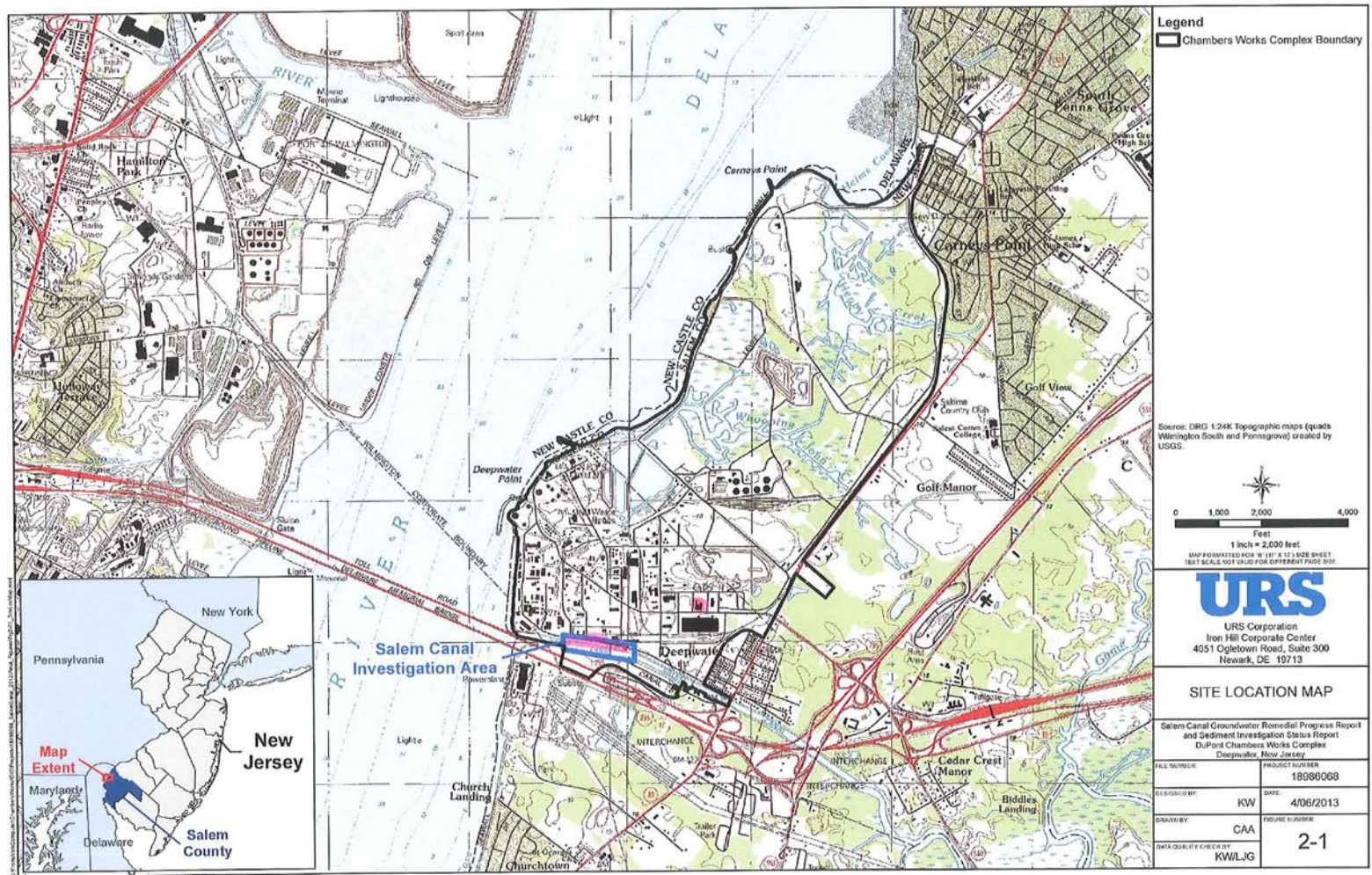
Figure 1.1a. Study site overview.
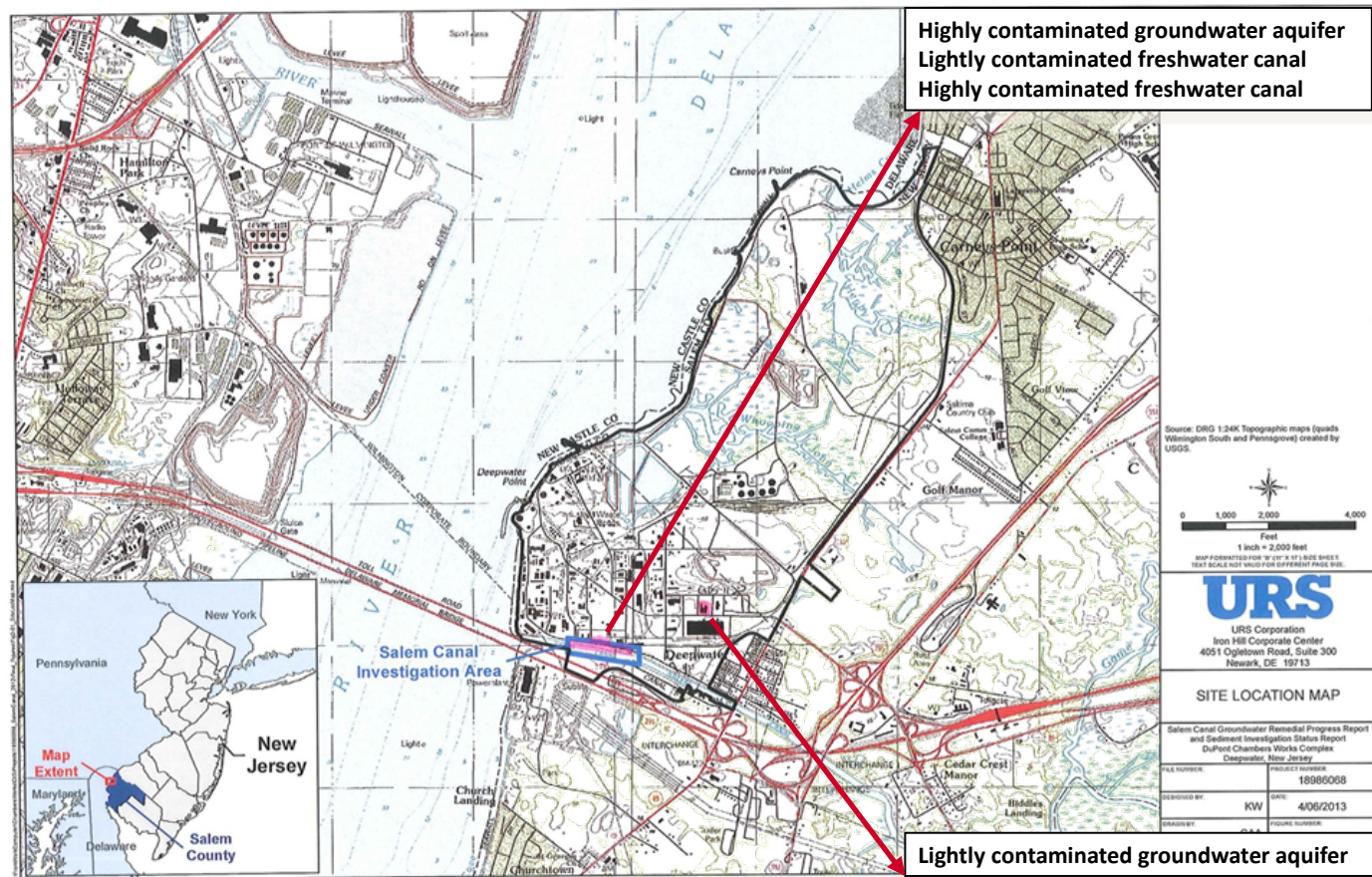
Figure 1.1b. Four sampling locations: lightly contaminated groundwater aquifer sediments; highly contaminated groundwater aquifer sediments; lightly contaminated freshwater canal sediments; and highly contaminated freshwater canal sediments.
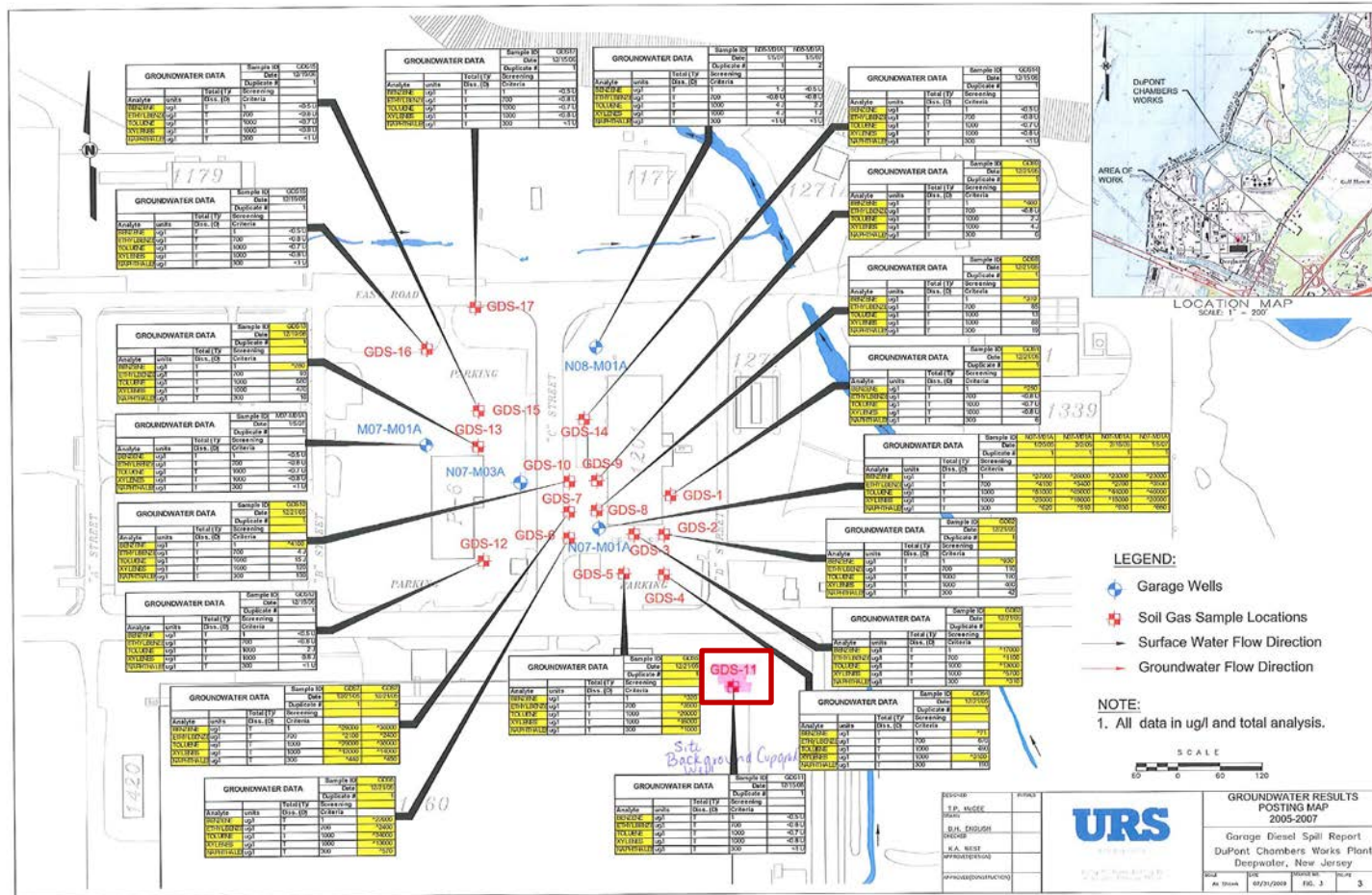
Figure 1.1c. Lightly contaminated groundwater aquifer sample ID: GDS-11. Cores were obtained from 0 to 10 ft below ground
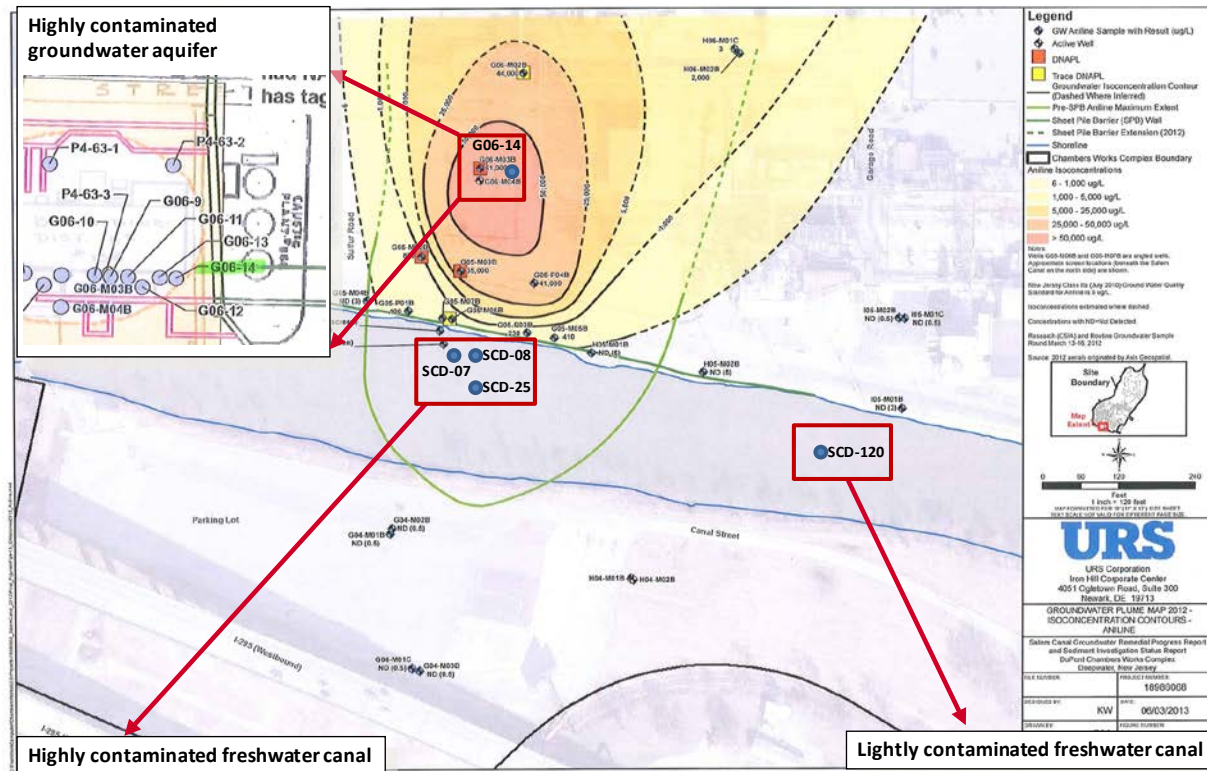
surface in Dec. 2006.

Figure 1.1d. Highly contaminated groundwater aquifer sample ID: G06-14. Cores were obtained from 0 to 10 ft below ground surface in Dec. 2006. Lightly contaminated freshwater canal sample ID: SCD-120. Sediment samples were obtained in Jan. 2007 and Apr. 2011. Highly contaminated freshwater canal sample ID: SCD-7, 8 and 25. Sediment samples were obtained in Jan. 2007. Aniline isoconcentrations range from 6 to 61,000 µg/ L.
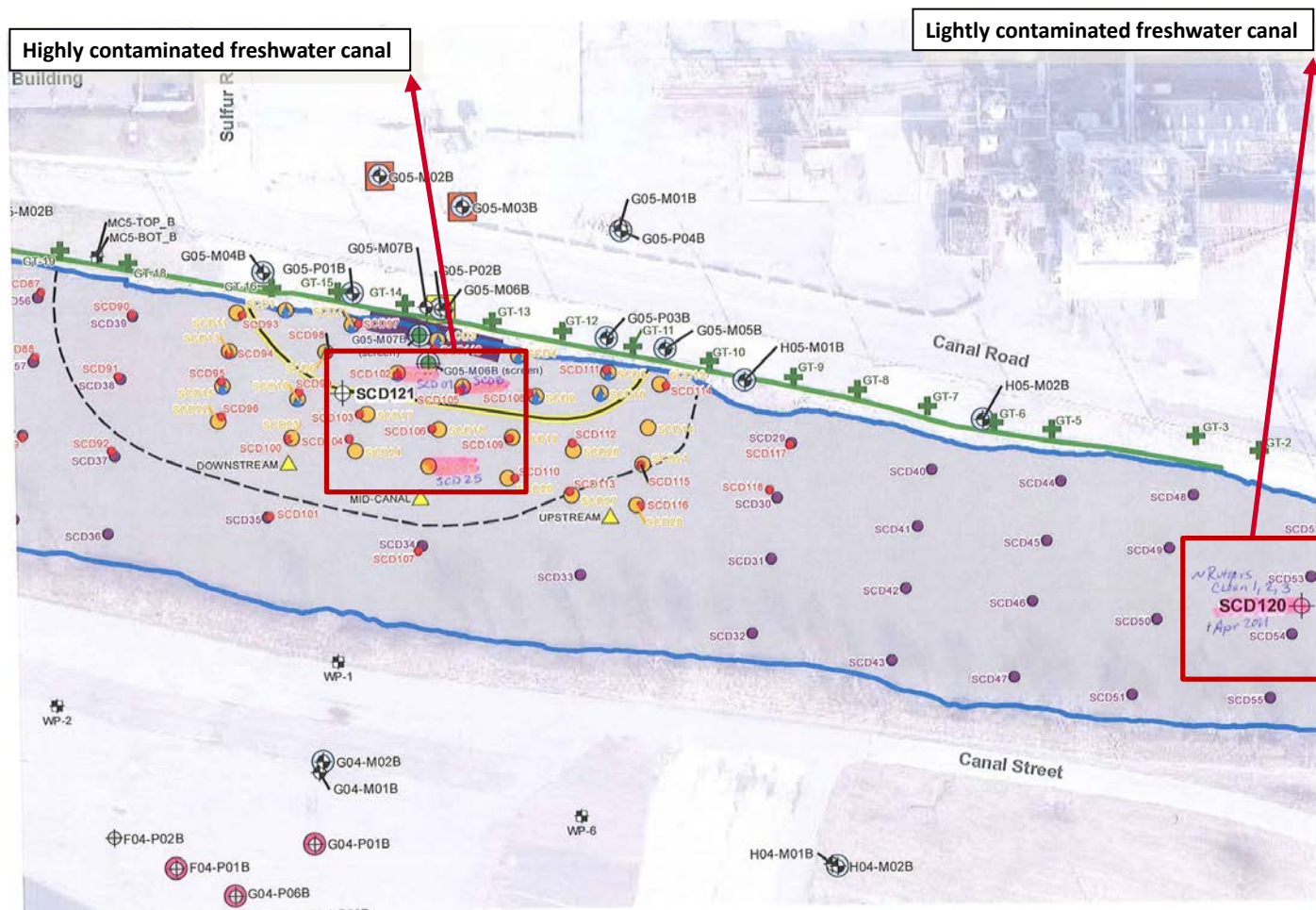
Figure 1.1e. Lightly contaminated freshwater canal sample ID: SCD-120. Sediment samples were obtained in Jan. 2007 and Apr. 2011. Highly contaminated freshwater canal sample ID: SCD-7, 8 and 25. Sediment samples were obtained in Jan. 2007.

Chapter 2 Literature Review

It has been reported that in anoxic environments, nitrogen-containing chlorinated aromatic compounds undergo reductive transformations via a variety of abiotic processes (Glaus et al. 1992; Schwarzenbach et al. 1990). At present, there are only a few reports available on the specific mechanisms of enzymatic attack on such compounds (See Schackmann and Müller 1991; Fetzner and Lingens 1994; Zhen et al. 2006). This literature review addresses what is known about biological transformation of aniline and chlorinated anilines, with a focus on anaerobic biotransformation.

2.1 Physical-Chemical Properties of Aniline and PCA

Aniline and 4-chloroaniline (*p*-chloroaniline, PCA) are aromatic amines (Figure 2.1), which are precursors in the manufacture of synthetic dyes, pesticides, pharmaceutical compounds and other products (Travis 1997; OKutman Tas and Pavlostathis 2013). They may be released accidentally to the environment or when dyes or pesticides are degraded. For example, aniline and its derivatives may be released accidentally to the environment incomplete degradation of linuron and propanid (Sheludchenko et al. 2005). Aniline is considered a potential carcinogen and it is genotoxic (U.S. EPA 1992; U.S. EPA 1991;

U.S. EPA 1988; U.S. EPA 1985), while PCA is a known animal carcinogen and is also genotoxic (Pinheiro et al. 2004).

Tables 2.1 and 2.2 show the physical and chemical properties of PCA and aniline. The moderate water solubility and vapor pressure for each compound suggests low volatility from aqueous solutions (Thomas 1990). Evaporation from soil was reported to be in the range of 0.11–3.65% of applied PCA, depending on soil type and sorption capacity (Kilzer et al. 1979). Thus volatilization is likely not a major pathway of loss from the environment.

2.2 Aniline Biodegradation

Aerobic degradation of aniline is well characterized. The major pathway of aniline biodegradation under aerobic conditions was found to involve oxidative deamination to catechol, which was further metabolized through an *ortho* or *meta* cleavage pathway and the citric acid cycle to CO_2 (Liu et al. 2002; Sheludchenko et al. 2005; Shin and Spain 2009; Xiao et al. 2009).

Bacteria including *Delftia* sp. AN3 (Liu et al. 2002), *Delftia* sp. XYJ6 (Xiao et al. 2009), *Delftia tsuruhatensis* 14S (Sheludchenko et al. 2005), *Delftia tsuruhatensis* AD9 (Liang

et al. 2005), *Pseudomonas* sp. (Helm and Reber 1979; Konopka et al. 1989; Hinteregger et al. 1992), *Rhodococcus* sp. (Aoki et al. 1983; Nwinyi et al.2008), *Acinetobacter* sp. (Kim et al. 1997; Kim et al. 2002), *Frateuria* sp. (Murakami et al. 1999), and *Moraxella* sp. (Zeyer et al. 1985) have been shown to be able to degrade aniline under aerobic conditions (Table 2.3).

Delftia sp. AN3 was reported to be able to use aniline as a sole source of carbon and nitrogen, and as an energy source, and metabolized aniline via the catechol pathway (*ortho*-cleavage) (Figure 2.2). This organism could survive on concentrations of aniline up to 53.8 mM (5000 mg/l) (Liu et al. 2002).

Delftia sp. XYJ6 was reported to be able to use aniline as a sole source of carbon and nitrogen, and as an energy source, and also metabolize aniline via the catechol pathway (*meta*-cleavage) (Figure 2.3).

Delftia tsuruhatensis 14S was also reported to be able to completely degrade aniline by the catechol *ortho*-cleavage pathway. The strain grew on catechol and *p*-hydroxybenzoic acid and was capable of consuming aniline up to 3200 mg/L (approximately 34.4 mM) (Sheludchenko et al. 2005). The gene cluster responsible for the complete metabolism of aniline to TCA-cycle intermediates in *Delftia tsuruhatensis* AD9 was cloned into *Escherichia coli* JM109. The genes were predicted to encode a multi-component aniline dioxygenase, a LysR-type regulator and *meta*-cleavage pathway enzymes for catechol degradation (Liang et al. 2005).

Burkholderia sp. strain JS667 (Table 2.3) was reported to be able to convert diphenylamine to aniline and catechol, which were further biodegraded by the well-established aerobic degradation pathways (Shin and Spain 2009).

Aniline can be carboxylated and activated through the benzoyl-CoA pathway under anaerobic conditions (Heider and Fuchs 1997). Only two isolates—*Desulfobacterium anilini* (Schnell et al. 1989; Schnell and Schink 1991) and strain HY99 (Kahng et al. 2000)—were directly shown to be able to fully degrade aniline under anoxic conditions (Table 2.4). *Desulfobacterium anilini* Ani 1 (= DSM 4660^T) degrades aniline under sulfate-reducing conditions via 4-aminobenzoate. Another sulfate-reducing *Desulfobacterium anilini* strain AK1 degrades phenol but not aniline (Ahn et al., 2009). Strain HY99 was identified as most similar to *Delftia acidovorans* (originally described by Loidle et al. 1990), which can degrade aniline only under aerobic conditions, but strain HY99 has been shown to be able to degrade aniline under both aerobic and denitrifying conditions. Under denitrifying conditions, the concentration of nitrite increased (through denitrification of nitrate) and aniline was apparently metabolized via the *p*-aminobenzoate pathway (Kahng et al. 2000). Figure 2.4 shows the anaerobic pathway through which aniline is transformed to benzoyl-CoA.

Three photoheterotrophic purple non-sulfur bacterial strains (Table 2.4) were reported to produce indole derivatives when grown in mineral anaerobic medium with aniline as carbon or nitrogen source. The intermediate products were detected as 14 chromatographically distinct peaks observed by HPLC with UV detection at 275-280 nm (Mujahid et al. 2010). Finally, aniline loss was also observed under sulfate-reducing,

nitrate-reducing and Fe(III)-reducing conditions in a variety of anoxic systems (De,

Owen A. O'Connor, and Kosson 1994; Kazumi et al. 1995a) however, no specific

responsible bacteria were identified during these studies.

2.3 Biotransformation of Chloroanilines

Chloroanilines have been used for many years in the production of paints, pesticides,

plastics, pharmaceuticals and other materials (Dejonghe et al. 2002). They are known to

be toxic and carcinogenic to living organisms and inhibit the addition of a phosphate

group to a protein or other organic molecule and thus prevent microbial growth

(Mysyakina and Funtikova 2000). Chloroanilines may be converted to toxic

chlorocatechols during aerobic degradation (Radianingtyas et al., 2003), however,

monochloroanilines can be rapidly metabolized via chlorocatechol as an intermediate

under aerobic conditions (Bachofer et al., 1975; Zeyer and Kearney 1982; Latorre et al.,

1984; Spain et al., 2000; Liu et al., 2002). Several strains were isolated and were able to

degrade monochloroaniline by the *ortho*-cleavage pathway to the corresponding

chlorocatechol (Radianingtyas et al., 2003) (Table 2.5). Aniline was identified as

metabolite during degradation of dichloroanilines and PCA (suggesting a dechlorination

step) under aerobic conditions by *Acinetobacter baylyi* strain GFJ2 which was isolated

from soil in areas where herbicides had been applied (Hongsawat and Vangnai 2011).

Under nitrate-reducing conditions *Rhodococcus* sp. strain 2 (Table 2.6) transformed

dichloroanilines into dichlorobenzenes via a reductive deamination pathway (Travkin et

al. 2002). Reducing agents, nitrogen headspace and the redox indicator rezasurin were

used to control and monitor the redox potential. However, the author failed to provide

evidence to indicate that the deamination of 3,4-dihaloanilines was coupled to nitrate

reduction.

Despite several studies investigating the dechlorination of polychlorinated anilines, to

date there are no identified bacterial strains that dechlorinate monochloroaniline. No loss

of monochloroanilines was observed under anoxic conditions where dechlorination of

higher chlorinated anilines was observed (Kuhn and Suflita 1989; Struijs and Rogers

1989; Haggblom and Young 1995; Genthner 1999; Okutman Tas and Pavlostathis 2005;

Okutman Tas et al. 2006). Kazumi et al. (1995a) also reported that there was no loss of 2-

, 3-, or 4-chloroanilines under Fe(III)-reducing conditions in Hudson River sediments.

Microorganisms have evolved over geological time to exploit natural organic compounds

as sources of carbon and energy for growth. In contrast, xenobiotic compounds released

into the environment only recently could be resistant to microbial degradation.

Monochloroanilines such as 4-chloroaniline may be regarded as more resistant to

microbial attack under reducing conditions than dichloroanilines due to the electron-

withdrawing properties of the amino group and the single chlorine molecule.

2.4 Biotransformation of Chloronitrobenzenes

Chloronitrobenzenes (CNBs) are environmental pollutants known to be toxic and mutagenic (Linch 1974; Weisburger et al. 1978; Shimizu et al., 1983). They are resistant to microbial degradation because of the electron-withdrawing properties of the nitro and chlorine groups. Schackmann and Müller reported that under aerobic conditions, *Pseudomonas* sp. strain CBS3 could reduce 4-chloronitrobenzene to 4-chloroaniline (4-CA), *N*-acetyl-4-chloroaniline, and 4-chloronitrosobenzene at low rates without any further degradation (Schackmann and Müller 1991). There is only limited information available on chloronitrobenzenes and transformation pathways in anoxic environments (Murthy and Kaufman 1978; Heijman et al. 1993; Susarla et al., 1996). Table 2.7 lists the detected intermediates of reduction of the nitro group of chloronitrobenzenes under different redox conditions from several references.

Heijman *et al*. (1993) reported a rapid transformation of 4-chloronitrobenzene (4-CNB) to 4-chloroaniline (4-CA) with a half-life of a few minutes and when a selective FeOOH medium was employed. Susarla *et al*. (1996) reported the results of laboratory studies of the transformations of 3-chloronitrobenzene (3-CNB), 3,4-dichloronitrobenzene (3,4-DCNB), 2,3,4-trichloronitrobenzene (2,3,4-TCNB) and pentachloronitrobenzene (PCNB) under sulfate-reducing conditions in estuarine sediment. The disappearance of PCNB and the appearance of its transformation products 2,3,4,5-tetrachloroaniline (2,3,4,5-TeCA) (*ortho* Cl removal), 3,4,5-trichloroaniline (3,4,5-TCA) (*ortho* Cl removal), 3,5-

dichloroaniline (3,5-DCA) (*para* Cl removal) was reported. 2,3-DCA and 3,4-DCA were further transformed to 3-CA, 2-CA and 4-CA. So far, there are only limited reports on single bacterial cultures capable of mineralizing 4-chloronitrobenzene (Corbett and Corbett 1981; Katsivela et al. 1999) and 2-chloronitrobenzene (Liu et al. 2005). Corbett and Corbett (1981) reported that the yeast *Rhodosporidium* sp. transformed 4-chloronitrobenzene (also called 1-chloro-4-nitrobenzene, or 4-CNB) to the metabolite 4-chloro-2-hydroxyacetanilide by a Bamberger rearrangement (Corbett and Corbett 1981).

The bacterial strain LW1utilizes 4-chloronitrobenzene (4CNB) as a sole source of carbon, nitrogen, and energy (Katsivela et al. 1999). Under anoxic conditions LW1 transformed 4CNB into 2-amino-5-chlorophenol indicating that there was partial reduction of the nitro group to the hydroxylamino substituent, followed by Bamberger rearrangement. Liu et al. (2005) also reported the mineralization of 2-CNB by *Pseudomonas stutzeri*.

In another degradation experiment, 2-, 3- or 4-CNB in aerobic batch culture were reduced to the corresponding monochloroanilines by *Pseudomonas acidovorans* strain CA50 only in the presence of an additional carbon and nitrogen source (Lenke and Knackmuss 1996; Kuhlmann and Hegemann 1997) .

Tas and Pavlostathis (2005) described the PCNB biotransformation/dechlorination pathway in a mixed methanogenic enrichment culture. The authors stated that PCNB was transformed to PeCA in a sodium azide inhibited methanogenic culture, where dechlorination of PeCA was not observed. PCNB was also abiotically transformed to PeCA in the killed control at the same rate observed in the azide-amended culture and the active culture (Okutman Tas and Pavlostathis 2005). It has been reported that in anoxic

environments, the reduction of the nitro group is one of the first steps (Macalady et al., 1986) during the transformation or mineralization of nitroaromatic compounds.

Theoretically possible compounds that could be formed by abiological or biotransformation of PCNB to PeCA and subsequent reductive dechlorination of PeCA are shown in Figure 2.5.

There is great interest in identifying the specific dechlorinating organisms responsible for transformation of toxic halogenated compounds so that their activities can be harnessed for environmental cleanup. Identification of organisms responsible for dechlorination of chlorinated anilines thus has great intrinsic value. There are other reasons for this interest, however. A previous study investigated bioaugmentation with a mixed culture containing *Dehalococcoides mccartyi* strain 195 and biostimulation with a halogenated co-substrate, pentachloronitrobenzene (PCNB) to enhance dechlorination of weathered polychlorinated biphenyls (PCBs) in Anacostia River, Washington, DC, sediments (Krumins et al. 2009; Park et al. 2011; Liu et al., 2013). PCNB was investigated as an alternate halogenated co-substrate because of its use as a food crop fungicide and potential acceptability as a sediment amendment or growth substrate for stimulating PCB dehlaogenators. *Dehalococcoides mccartyi* strain 195[T], formerly designated '*Dehalococcoides ethenogenes*' strain 195, was isolated on chloroethenes (Maymo-Gatell et al. 1997) but is also known for its ability to reductively dehalogenate persistent environmental pollutants such as PCBs and PCDD/Fs (Fennell et al. 2004; Liu and Fennell 2008). In the PCB microcosm experiment, *Dehalococcoides* spp. were monitored using polymerase chain reaction coupled to denaturing gradient gel electrophoresis. The

results showed an increase in the population of the native *Dehalococcoides* spp., but not of *Dehalococcoides mccartyi* strain 195 during incubation of sediments over hundreds of days. Strain 195 was slowly lost from the sediments (Krumins et al. 2009; Park et al. 2011). It is believed that by adding a halogenated co-substrate, the increasing production of the degradative enzymes (in this case dehalogenases) may maximize catalytic efficiency or stability of the microbial system (Vangnai and Petchkroh 2007). It was assumed that PCNB co-substrate supplementation may have improved growth rates and biodegradation efficiency of different *Dehalococcoides* sub-populations that differ by dechlorination activity (Krumins et al. 2009). Although PCNB was no longer detected in the Anacostia River sediments after 135 days, the pathway of its transformation was not delineated (Krumins et al. 2009). Since both native *Dehalococcoides* and the bioaugmented strain 195 were stimulated by PCNB to increase PCB dechlorination, it may be that some *Dehalococcoides* have the ability to dehalorespire the reduction products of PCNB. To our knowledge, there has been no report of attempts to test the dechlorination of PCNB or chloroanilines by *Dehalococcoides* strain 195 or other *Dehalococcoides mccartyi* spp. Since *Dehalococcoides* spp. have been utilized in field scale bioremediation of chlorinated solvents (Hendrickson et al. 2002), discovery of other potential pollutants that could be removed from the environment by bioaugmentation with *Dehalococcoides* (e.g., chloroanilines) should be investigated.

2.5 Abiotic and Biotic Mechanisms Leading to Humus Binding of Aniline Compounds

Organic compounds become incorporated or bound into the naturally occurring humic structures of soil (Bollag 1992). The phenomena controlling the incorporation of aniline and chloroaniline into soils via oxidative coupling reactions are mediated both by abiotic catalysts such as birnessite (δ-MnO_2) and by enzymes including laccases and peroxidases (Park et al., 1999; Kong et al. 2013). These reactions generally result in the compounds becoming covalently bound to soil humic material. Further, substituted aniline (e.g., chlorinated aniline) has been shown to oligomerize via similar processes and these oligimerized compounds may subsequently bind to humus materials in the soil (Simmons et al., 1987). The presence of soluble humic substances, which undergo the same reactions, and serve as coupling partners, enhances the binding process (Park et al., 1999). Weber et al. (1996) described covalent binding of aniline with dissolved organic matter to be governed by second order kinetics. While such binding has been proposed as a remediation approach (Bollag 1992) it is possible under some environmental conditions (e.g., low pH) that the pollutants could be re-released (Kong et al., 2013).

In studies of the biodegradation of aniline and chlorinated aniline in microcosms, binding to the soil (or sediment) could thus occur naturally. Under aerobic conditions, fungal produced oxidoreductases could mediate the oxidation of aniline or chloroaniline that leads to the ability to form covalent bonds (Bollag, 1992).

Table 2. 1. Physical and chemical properties of aniline.

Property		Value	Source
FW	Formula weight	93.13	(Dean 1985)
ρ	Density, g/mL, liquid	1.0217	(Dean 1985)
T_m	Melting point, °C	−5.98	(Dean 1985)
		184.40	(Dean 1985)
T_b	Boiling point at 101.325 kPa, °C	184.1	(Lide 2002)
pK_a	Dissociation constant	4.87	(Lide 2002)
Log K_{oc}	Octanol-Water partition coefficients	0.90	(Lide 2002)
V_p	Vapor pressure	183.5 (100 kPa)	(Lide 2002)
k_H	Henry's law constants, kPa m^3/mol	14	(Lide 2002)
S_w	Solubility in water at 25°C	3.38%	(Lide 2002)

Table 2. 2. Physical and chemical properties of PCA.

Property		Value	Source
FW	Formula weight	127.57	(Dean 1985)
ρ	Density	1.169 at 16°C relative to water at 4°C	(Dean 1985)
T_m	Melting point, °C	72.5	(Dean 1985)
T_b	Boiling point, °C	232	(Dean 1985)
pK_a	Dissociation constant	3.96 (25 °C) 3.99 (+1)	(Lide 2002) (Dean 1985)
K_{ow}	n-octanol/water partition coefficient	1.83 2.05	(Boehncke et al. 2003)
V_p	Vapor pressure, Pa	0.5 (10°C) 1.4 to 2.1 (20°C)	(Boehncke et al. 2003)
k_H	Henry's law constant, Pa m^3/mol	0.1[a]	(Boehncke et al. 2003)
S_w	Solubility in water at 20°C, g/L	3.9	(Kilzer et al. 1979)

[a] *the Henry's law constant was calculated from the water solubility and vapor pressure of PCA*

Table 2. 3. Aniline-degrading bacteria identified under aerobic conditions.

Bacterial species	[a]Substrate Information	Identified intermediate	Reference
Delftia sp.AN3	Grows on aniline up to 53.8 mM	Catechol *meta*-cleavage	(Liu et al. 2002)
Delftia sp.XYJ6	Aniline	Catechol *ortho*-cleavage	(Xiao et al. 2009)
*Delftia tsuruhatensis*14S	Consumed aniline up to ~ 34.4 mM	Catechol *ortho*-cleavage	(Sheludchenko et al. 2005)
Delftia tsuruhatensis AD9	Aniline	Catechol *meta*-cleavage	(Liang et al. 2005)
Pseudomonas multivorans strain An 1	>16 mM aniline was toxic	NA	(Helm and Reber 1979)
Pseudomonas sp. strain Kl	Metabolized aniline as low as 50 nM	Catechol *meta*-cleavage	(Konopka et al. 1989)
Pseudomonas acidovorans CA28	Grows on aniline and 3-CA	*ortho*-cleavage	(Hinteregger et al. 1992)
Rhodococcus erythropolis AN 13	Grows on 0.65 to 2.6 mg/mL aniline	Catechol	(Aoki et al. 1983)
Rhodococcus	>10 mM aniline was toxic	NA	(Nwinyi et al. 2008)
Acinetobacter lwoffii K24	Aniline	Catechol *ortho*-cleavage	(Kim et al. 1997)
Frateuria sp. ANA-18	Aniline	Catechol *ortho*-cleavage	(Murakami et al. 1999)
*Moraxella*sp. strain G	Aniline, PCA, 2-CA, 3-CA, 4-fluoroaniline and 4-bromoaniline	*ortho*-cleavage	(Zeyer et al. 1985)
Burkholderia sp. strain JS667	Diphenylamine	Aniline, catechol	(Shin and Spain 2009)
Strain HY99	Aniline	Catechol *meta*-cleavage	(Kahng et al. 2000)
Acinetobacter baylyi strain GFJ2	3,4-DCA, PCA	PCA, aniline, 4-chlorocatechol, *ortho*-cleavage pathway	(Hongsawat and Vangnai 2011)

[a]*3-CA (3-chloroaniline); PCA (para-chloroaniline); 2-CA (2-chloroaniline); 3,4-DCA (3,4-dichloroaniline)*

Table 2. 4. Aniline-degrading bacteria identified under anoxic conditions.

Bacterial species	Substrates	Identified intermediate	Condition	Reference
Desulfobacterium anilini Ani 1	Aniline	4-aminobenzoate	Sulfate-reducing	(Schnell et al.,1989; Schnell and Schink 1991)
strain HY99	Aniline	4-aminobenzoate	Nitrate-reducing	(Kahng et al., 2000).
Purple non-sulfur bacteria *Rhodospirillum rubrum* ATCC 11170	Aniline	Tryptophan, indole-3-ethanol	Anaerobic, 30 °C; light 2,400 lux	(Mujahid et al., 2010).
Purple non-sulfur bacteria *Rhodobacter sphaeroides* DSM 158	Aniline	Tryptophan, indole-3-aldehyde, anthranilic acid	Anaerobic, 30 °C; light 2,400 lux	(Mujahid et al., 2010).
Purple non-sulfur bacteria *Rubrivivax benzoatiliticus* JA2	Aniline	Tryptophan, indole-3-acetic acid, indole-3-aldehyde	Anaerobic, 30 °C; light 2,400 lux	(Mujahid et al., 2010).

Table 2. 5. Chloroaniline-degrading bacteria identified under aerobic conditions.

Bacterial species	[a]Substrates	Identified intermediate	Reference
Delftia tsuruhatensis H1	2-CA, 3-CA & PCA	4-Chlorocatechol, *ortho*-cleavage pathway	(Zhang et al. 2010)
Moraxella sp. strain G	Aniline, PCA, 2-CA, 3-CA, 4-fluoroaniline & 4-bromoaniline	*ortho*-cleavage pathway	(Zeyer et al., 1985)
Pseudomonas sp. strain JL2	PCA	4-Chlorocatechol, *ortho*-cleavage pathway	(Latorre et al., 1984)
Delftia (Previously *Pseudomonas*) *acidovorans* CA28	aniline & 3-CA	*ortho*-cleavage	(Hinteregger et al. 1992)
Comamonas testosteroni I2	3-CA	Yellow intermediate	(Boon et al. 2003)
Acinetobacter baumannii CA2	PCA from 0.2 to 1.2 mM	modified *ortho*-cleavage pathway	(Vangnai and Petchkroh 2007)
Pseudomonas putida CA16	PCA from 0.2 to 1.2 mM	modified *ortho*-cleavage pathway	(Vangnai and Petchkroh 2007)
Klebsiella sp. CA17	PCA from 0.2 to 1.2 mM	modified *ortho*-cleavage pathway	(Vangnai and Petchkroh 2007)
Pseudomonas sp. *Acidovorax* sp. *Delftia* sp. *Achromobacter* sp. *Comamonas* sp.	3,4-DCA; 3-CA	NA	(Dejonghe et al. 2002)
Pseudomonas putida	Co-metabolic transformation of 3,4-DCA, aniline	4,5-dichlorocatechol, 3,4-dichloromuconate, 3-chlorobutenolide, 3-chloromaleyl acetate, and 3-chloro-4-ketoadipate	(You and Bartha 1982)
Pseudomonas sp. KB35B	3,4-DCA	chlorocatechol-2,3-dioxygenase activity	(Kim et al. 2007)
Acinetobacter baylyi strain GFJ2	dichloroanilines PCA	chlorocatechol aniline	(Hongsawat and Vangnai 2011)

[a]*3-CA (3-chloroaniline); PCA (para-chloroaniline); 2-CA (2-chloroaniline); 3,4-DCA (3,4-dichloroaniline)*

Table 2. 6. Chloroaniline-degrading bacteria identified under anoxic conditions.

Bacterial species	Substrates	Identified intermediate	Reference
Rhodococcus sp. strain 2	Co-metabolic transformation of 3,4-DCA	3,4-dichloroacetanilide, 3,4-dichloro-N-(3,4-dichlorophenyl)benzamide and 1,2-dichlorobenzene	(Travkin et al. 2002)

Table 2. 7. Intermediate metabolites for chloronitrobenzene biodegradation under

different redox conditions.

Parent compound	Identified intermediate	Condition	Reference
4-CNB[a]	4-CA, N-acetyl-4-chloroaniline, and 4-chloronitrosobenzene	Aerobic by *Pseudomonas* sp. strain CBS3	(Schackmann and Müller 1991)
1-Chloro-2,4-dinitrobenzene	4-chloro-l,3-diaminobenzene	Aerobic by *Pseudomonas* sp. strain CBS3	(Schackmann and Müller 1991)
4-CNB	PCA	Iron-reducing	(Heijman et al. 1993).
3-CNB	3-CA, aniline	Sulfate-reducing	(Susarla et al., 1996)
3,4-DCNB	3,4-DCA, 3-CA	Sulfate-reducing	(Susarla et al., 1996)
2,3,4-TCNB	2,3,4-TCA, 2,3-DCA, 3,4-DCA, 2-CA, 3-CA, PCA	Sulfate-reducing	(Susarla et al., 1996)
PCNB	PeCA, 2,3,4,5-TeCA, 3,4,5-TCA, 3,5-DCA, 3-CA, 2-CA, PCA	Sulfate-reducing	(Susarla et al., 1996)
PCNB	PeCA, 2,3,4,5- and 2,3,5,6-TeCA, 2,4,5- and 2,3,5-TCA, 2,4-, 2,5-, and 3,5-DCA, 3-CA and PCA	Methanogenic	(Okutman Tas and Pavlostathis 2005)
4-CNB	4-chloro-2-hydroxyacetanilide	Nitro reduction by the yeast *Rhodosporidium* sp.	(Corbett and Corbett 1981)

[a] 4-CNB, 4-chloronitrobenzene; 4-CA, 4-chloroaniline; 3-CNB, 3-chloronitrobenzene; 3-CA, 3-chloroaniline; 3,4-DCNB, 3,4-dichloronitrobenzene; 3,4-DCA, 3,4-dichloroaniline; 2,3,4-TCNB, 2,3,4-trichloronitrobenzene; 2,3,4-TCA, 2,3,4-trichloroaniline; 2,3-DCA, 2,3-dichloroaniline; 3,4-DCA, 3,4-dichloroaniline; 2-CA, 2-chloroaniline; PCNB, pentachloronitrobenzene; PeCA, pentachloroaniline; 2,3,4,5-TeCA, 2,3,4,5-tetrachloroaniline; 2,3,5,6-TeCA, 2,3,5,6-tetrachloroaniline.
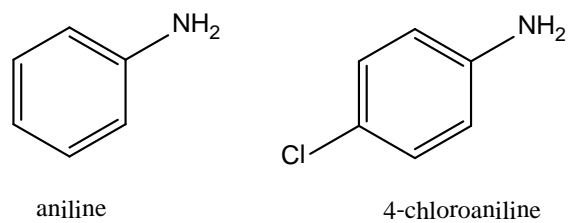
Figure 2. 1. Molecular structures of aniline and 4-chloroaniline (4-CA, PCA).
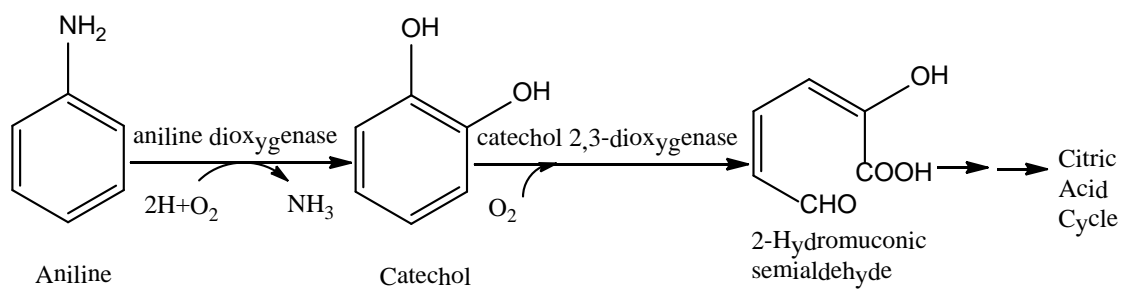
Figure 2. 2. Proposed *meta*-cleavage pathway of aniline degradation by *Delftia* sp. AN3

(modified from Liu et al. 2002).
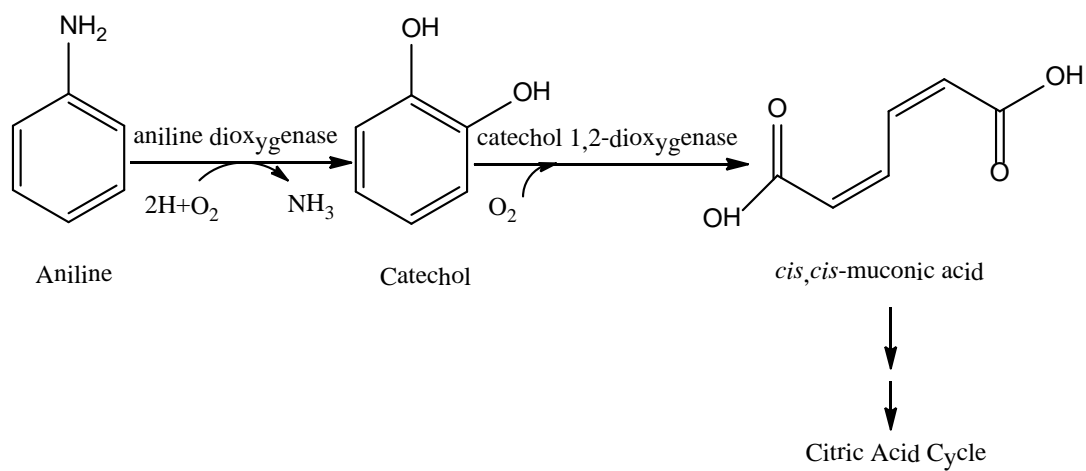
Figure 2. 3. Proposed *ortho*-cleavage pathway of aniline degradation by *Delftia* sp. XYJ6

(modified from Xiao et al. 2009).
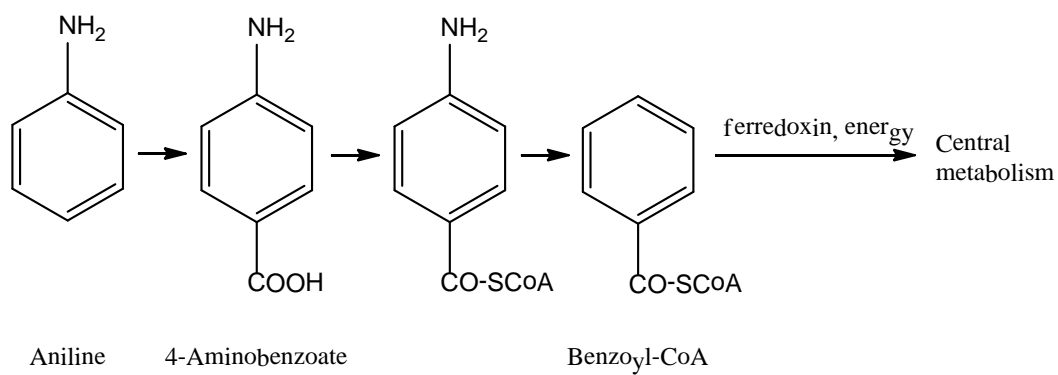
Figure 2. 4. Anaerobic pathway transforming aniline to benzoyl-CoA (modified from (Harwood et al. 1998; Carmona et al. 2009).
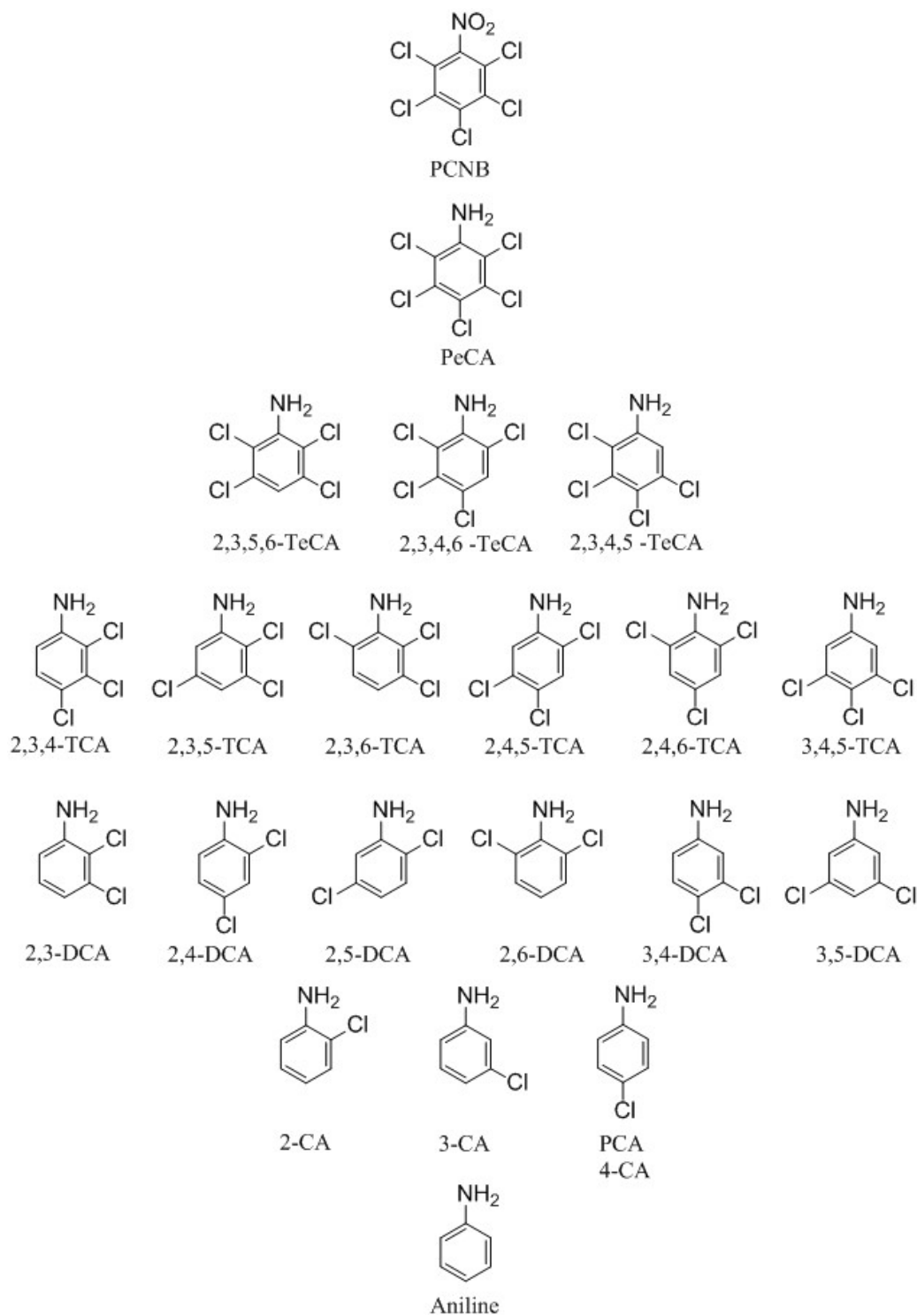
Figure 2. 5. All possible compounds that could theoretically be formed by

biotransformation of PCNB and reductive dechlorination of PeCA.

Chapter 3   Aerobic and Anaerobic Degradation of Aniline and *Para*-chloroaniline in Contaminated Sediments from an Industrial Site

3.1 Abstract

Aniline and 4-chloroaniline (para-chloroaniline, PCA) are prevalent environmental contaminants associated with chemical production facilities. Aerobic biotransformation of these compounds has been reported extensively, but their fate under anoxic conditions is less well studied. Microcosms amended with aniline or PCA were established for groundwater aquifer and canal sediments under nitrate-amended, Fe (III)-amended, sulfate-amended and methanogenic conditions (i.e. no other terminal electron acceptor added other than bicarbonate) using 20% sediment slurry in site water. Two locations were studied for each of the sites—highly contaminated and lightly contaminated.

The more contaminated sediments contained co-contaminants such as azo dyes, PAHs, benzene and chlorinated benzenes. Microcosms were established under nitrate-amended, Fe (III)–amended, sulfate-amended and methanogenic conditions and were repeatedly re-

amended with up to 1 mM aniline or PCA. Bacterial community analysis was performed using polymerase chain reaction analysis coupled to denaturing gradient gel electrophoresis (PCR-DGGE). Groundwater aquifer and freshwater canal sediment microcosms showed loss of aniline under nitrate-, Fe (III)- and sulfate–amended conditions and methanogenic conditions. PCA loss was observed under nitrate-amended conditions. PCA loss was not observed under methanogenic conditions in microcosms prepared using lightly contaminated or highly contaminated groundwater aquifer sediments, or highly contaminated freshwater canal sediment. PCR-DGGE of aquatic sediment under nitrate- and sulfate-amended conditions yielded distinct population profiles that appeared to share at least one community member. Active systems were re-amended several times, and enriched to identify the responsible microorganisms. The terminal daughter products of dechlorination of highly chlorinated anilines have previously been reported to be di- or monochloroanilines. Results from this study support the possibility for complete dechlorination of the monochloroaniline, PCA, to aniline and subsequent degradation of aniline under anoxic conditions. It was also suggested that aniline and PCA in sediment and aquifers were amenable to biological treatment.

3.2 Introduction

Bacterial metabolism is driven by chemical oxidation-reduction (redox) reactions. The cells obtain energy by oxidizing reduced compounds (e.g., organic molecules or H_2) and transferring electrons to terminal electron acceptors (e.g., oxygen, nitrate, sulfate, Fe(III), organic molecules or organohalides) (Lovley and Goodwin 1988; McCarty 1965; McCarty 1964; McCarty 1971; Thauer et al. 1977). Electron acceptors with higher potentials (eg. oxygen) yield more energy than those with lower ones. The ease of reduction of aromatic compounds in particular depends on the nature of the substituents on the ring and on the redox potential of the environment. Electron-withdrawing groups activate the molecule for the reduction, whereas electron-donating groups make the ring unresistant to electrophilic attack (Spain 1995).

Thus, in the environment, bacteria that use a specific electron acceptor that yields a greater amount of energy may be more competitive than one that used a less energetically valuable electron acceptor. Furthermore, when oxygen is present, it is toxic to many anaerobes or may inhibit anoxic microbial processes. Further, biotransformation of compounds under anoxic conditions may require long incubation times, to allow slow-growing anaerobes to increase in population. These are some of the reasons why there are more reports about aerobic biotransformations than anaerobic ones. However, anaerobic biodegradation processes have several advantages over aerobic ones. For example, anaerobic mineralization of carbonaceous matter produces methane, which is a biofuel.

Another useful application for anaerobic microbes is for biodegradation of chemical contaminants in polluted environments. Often, environmental pollutants occur in aquifers, aquatic sediments and flooded soil areas that are anoxic (Khomenkov et al. 2008). Under these conditions, the biodegradation of pollutants must be carried out by microorganisms that use an electron acceptor other than oxygen. These include nitrate (for nitrate reducers), sulfate (for sulfate reducers), ferric iron (Fe(III), for iron reducers), carbon dioxide (for methanogens) and many others (e.g. chlorate, Mn, Cr, U, organohalides, etc.) (Khomenkov et al. 2008).

This study examined the biodegradation of aniline and *para*-chloroaniline (PCA) in sediments from an industrial site where both compounds are present as pollutants. Aniline and PCA have a long history as industrial chemicals for production of dyes, pesticides and pharmaceutical compounds, and have been problematic pollutants of soils and groundwater throughout the history of chemical manufacturing (Travis 1997).

Aniline is considered a potential carcinogen and is genotoxic (U.S. EPA 1992; U.S. EPA 1991; U.S. EPA 1988; U.S. EPA 1985), while PCA is a known animal carcinogen and is also genotoxic (Pinheiro et al. 2004). The removal of aniline and PCA from contaminated aquifers, sediments and wastewater is problematic and has been attempted by both biological and abiological means (Laha and Luthy 1990; Vandevivere et al. 1998). At the industrial site under study, biodegradation was examined in aquatic sediments and groundwater, both of which are anoxic.

Bacterial biotransformation of aniline and monochloroanilines occurs under both aerobic and anoxic conditions. Aniline can be rapidly metabolized via the catechol pathway

under aerobic conditions by a variety of bacterial species (Bachofer et al. 1975; Zeyer and Kearney 1982; Latorre et al. 1984; Spain 1995; Liu et al. 2002) (see Table 2.3).

Degradation of aniline also occurs under anoxic conditions (see Table 2.4), however, much less is known about bacteria that mediate this process. *Desulfobacterium anilini* Ani 1 originally isolated from marine sediment from the North Sea (Schnell et al. 1989) utilizes aniline under sulfate-reducing conditions. Degradation of aniline proceeds through 4-aminobenzoate which is then converted via CoA-thioesterification and reductive deamination of the 4-aminobenzoyl-CoA to benzoyl-CoA (Schnell and Schink 1991). Strain HY99, isolated from orchard-soil samples in Cheju, Korea and closely related to *Delftia acidovorans,* an aerobic aniline degrader (originally described by Loidle et al. 1990), was reported to degrade aniline under both aerobic and nitrate-reducing conditions and 4-aminobenzoate was detected in the nitrate-reducing cultures (Kahng et al. 2000). Only these studies have reported specific aniline degraders under anoxic conditions. There are, however, several reports of aniline degradation in sediments and sludges by unidentified microbes. The other studies do not show intermediates or growth as evidence to back up their results. Aniline loss was observed under nitrate-reducing conditions in sediment from Newtown Creek, a estuarine channel located in New York, NY USA, and in anaerobic digester sludge from the Berkeley Heights WWTP in Berkeley Heights, NJ USA. However, no aniline loss was observed under methanogenic conditions after 217 days in the Newtown Creek sediments (De et al. 1994). Aniline loss in Hudson River sediments was observed under Fe(III)-reducing conditions within 65 days but the activity was not sustained by refeeding (Kazumi et al. 1995a). Thus, information regarding anoxic and anaerobic aniline degraders is very limited.

PCA can also be transformed under aerobic and anoxic conditions. Aerobic degradation of PCA in systems co-amended with aniline resulted in formation of 4-chlorocatechol (Radianingtyas, Robinson, and Bull 2003) while some species transformed 3-chloroaniline through 3-chlorocatechol (Boon et al. 2003; Boon N. et al. 2001) (see Table 2.5). Possible reductive deamination of dihaloanilines under nitrate-reducing conditions resulted in formation of dihalobenzenes by *Rhodococcus* sp. strain 2 (Travkin et al. 2002) (see Table 2.6). Several studies have reported negative results for reductive dechlorination of PCA or other monochloroanilines in reduced methanogenic enrichments or microcosms which dechlorinated higher chlorinated anilines or other halogenated aromatics such as chlorophenols or chlorobenzoates (Kuhn and Suflita 1989; Haggblom and Young 1995; Sharak Genthner 1999; Okutman Tas and Pavlostathis 2005). Aniline *was* identified as metabolite during dechlorination of dichloroanilines and PCA under aerobic conditions by *Acinetobacter baylyi* strain GFJ2 which was isolated from soil in areas where herbicides had been applied (Hongsawat and Vangnai 2011). Additionally, aniline was identified as a metabolite during dechlorination of dichloroanilines, ortho-chloroaniline and PCA under anoxic conditions in estuarine sediment (Susarla et al. 1997). To date, however, little is known about the bacteria involved in the transformation of poly- or monochloroanilines in anoxic sediments.

In this study the degradation of aniline and PCA was examined in microcosms established to produce aerobic (aniline only), nitrate-reducing, Fe(III)-reducing, sulfate-reducing and methanogenic conditions in contaminated aquatic sediments and groundwater aquifer sediments from a chemical industrial site. The hypothesis was that by adding an excess supply of alternative electron acceptors, each of the reducing redox

potential conditions would provide a specific selective condition for certain native

microbes, which may degrade aniline and PCA. Aniline and PCA could theoretically act

as both electron donor and carbon source (and even nitrogen source) while PCA could act

as an electron acceptor during dechlorination during this process.

3.3 Materials and Methods

3.3.1 Chemicals and reagents

Aniline (99.5+%, A.C.S. Reagent), 4-chloroaniline (sublimed, 99+ %) and resazurin

(certified, dye content 91%) were purchased from Sigma-Aldrich (St. Louis, MO). Ferric

chloride hexahydrate (lumps/ certified A.C.S.), potassium nitrate (certified A.C.S.),

sodium sulfate (certified A.C.S.), sodium hydroxide (pellets/ certified A.C.S.) and

acetonitrile (certified A.C.S.) were obtained from Fisher Scientific (Pittsburgh, PA).

3.3.2 Sediment microcosm set up

The site under study and the materials obtained at each location are described in detail in

Chapter 1. The sediment core sleeves were cut, capped and labeled in the field prior to

shipment to Rutgers University on ice. Groundwater from the uncontaminated area of the

groundwater aquifer was provided in sterile 1 L amber jars. Canal water was provided in

a 10 L polyethylene carboy. All materials were stored at 4°C until use.

The sediment cores (see Table 1.1, Section 1.2) were composited under anoxic conditions

in disposable glove bags (Cole Parmer Instrument Company, Veron Hills, IL) purged

with nitrogen. A separate glovebag was used for each location. Stainless steel spoons and

mixing container, glass jars and lids, spatulas, pipettes and all other materials used to

composite the cores were sterilized by autoclaving prior to placement in the glovebag.

The blade of the hacksaw used to open cores was sterilized with 70% ethanol before

cutting the cores. Cores from each lightly and heavily contaminated location were

removed from the acetate sleeves and mixed thoroughly in a sterile 20-L stainless-steel

container. For the groundwater aquifer cores, the upper approximately two ft unsaturated

zone was not included in the composited material. After compositing, three 15 mL

samples were removed per location and placed in 15 mL polypropylene tubes at -80°C

for later microbial molecular analysis. Each set of composited material was packed into

clean sterile glass jars and stored at 4°C until use.

Jars of composited sediment, site water, and autoclaved serum bottles, gray butyl

Teflon™-lined septa, spatulas, and other material were placed inside the disposable glove

bag in a chemical fume hood and were purged with sterile nitrogen overnight.

Microcosms were prepared under different redox conditions for each site as shown in

Table 3.1. No alternate electron acceptor, Na_2SO_4 (20 mM), freshly precipitated

amorphous iron as ferric oxyhydroxide (200 mM), or KNO_3 (30 mM) was added and

intended to promote methanogenic, sulfate-, Fe(III)-, or nitrate-reducing conditions,

respectively according to previously published methods (Monserrate and Haggblom 1997). The Fe(III)-amended, nitrate-amended, sulfate-amended and methanogenic microcosms were set up in volumes of 100 mL in sterile 160 mL serum bottles. A 100 mL volume of sediment slurry (20% composited aquifer sediment and 80% groundwater for the two locations of the groundwater aquifer; or 20% composited canal sediment and 80% canal water for the two locations for the freshwater canal) was prepared for the methanogenic, nitrate-amended and sulfate–amended microcosms by gravimetrically adding sediment and volumetrically adding site water. Groundwater was added directly and canal water was purged with sterile, anaerobic $70\%N_2/30\%CO_2$ prior to use. The bottles were capped with Teflon™-coated butyl rubber stoppers and crimped with aluminum seals under a N_2/CO_2 headspace. Stock solutions of nitrate or sulfate were added as shown in Table 3.2.

The synthesis of amorphous FeOOH was performed by dissolving 64.88 g of $FeCl_3$ in 1200 mL of DI water and adjusting the pH to 7 with 5 N NaOH. Flocculated FeOOH was allowed to settle overnight, then it was washed with 10 volumes of DI water prior to use in microcosms (Lovley and Phillips 1986) to remove Na^+. The iron floc was centrifuged at 1000 rpm for 10 min to remove more water. Next the iron floc was mixed with 1 L of sediment slurry (20% sediment and 80% of site water) and the volume was adjusted to 1200 mL using sterile anoxic DI water. The Fe (III)-amended microcosms were prepared by adding 100 mL of the mixture to each serum bottle.

Aerobic microcosms (for the aquifer locations only) of 100 mL (20% composited aquifer sediment and 80% groundwater) were established in sterile 250 mL Erlenmeyer flasks

and were plugged with sterile foam stoppers (27 to 34 mm) to prevent contamination and allow air exchange.

100 μM of aniline or PCA and 0.5 mg/L resazurin (as a redox indicator) were added to each serum bottle or flask based upon the final enrichment volume of 100 mL. Killed controls were prepared by autoclaving prepared microcosms at 121°C for 30 min on each of three consecutive days. All microcosms were incubated at 28°C in the dark and only the aerobic microcosms were shaken on a platform shaker at 120 rpm.

3.3.3 Enrichment set up

Enrichments were prepared under strictly anoxic conditions in 160 mL serum bottles (containing 100 mL of medium). Four different sets of medium were set up to enrich for aniline and PCA degradation under nitrate-amended, Fe(III)-amended, sulfate-amended and methanogenic conditions. All media contained the following (per liter): KCl, 1.3 g; KH_2PO_4, 0.2 g; NaCl, 1.17 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $MgCl_2 \cdot 6H_2O$, 0.18 g; $NaHCO_3$, 2.5 g; resazurin stock solution (1 g/L), 1 mL; vitamin solution, 5 mL; trace salts solution I, 1 mL; and trace salts solution II, 0.1 mL. Vitamin solution contained the following (per 500 mL): d-biotin, 0.01 g; folic acid, 0.01 g; pyridoxine hydrochloride, 0.05 g; thiamine hydrochloride, 0.025 g; riboflavin, 0.025 g; nicotinic acid, 0.025 g; DL-calcium pantothenate, 0.025 g; vitamin B12, 0.005 g, *p*-aminobenzoic acid, 0.025 g; lipoic acid (thiotic acid), 0.025 g; 1,4-naphthaquinone, 0.02 g; nicotinamide, 0.05 g; and hemin, 0.005 g. Trace salts solution I contained the following (per liter): $MnCl_2 \cdot 6H_2O$, 5 g; H_3BO_3, 0.5 g; $ZnCl_2$, 0.5 g; $CoCl_2 \cdot 6H_2O$, 0.5 g; $NiCl_2 \cdot 6H_2O$, 0.46 g; $CuCl_2 \cdot 2H_2O$, 0.3

g; NaMoO$_4$· 2H$_2$O, 0.1 g; and FeCl$_2$·4H$_2$O, 1.49 g. Trace salts solution II contained per liter 0.03 g of NaSeO$_3$ and 0.08 g of Na$_2$WO$_4$.

The nitrate-amended medium for enrichments was set up as shown in Table 3.3. The first and second sets of nitrate-amended medium contained NH$_4$Cl, 0.5 g/L; the second and third sets of nitrate-amended medium contained 0.9 mM S^{2-} as reducing agents; in addition to KNO$_3$, 3.3 g/L. The sulfate-amended medium contained Na$_2$SO$_4$ 2.84 g/L and the Fe (III)-amended media was prepared as described in Section 3.3.1, except the basal medium was mixed with the appropriate amount of freshly prepared and rinsed FeOOH slurry. For methanogenic enrchments, no additional electron acceptor (other than HCO$_3^-$) was added. To prepare media, briefly, KCl, KH$_2$PO$_4$, NaCl, NH$_4$Cl, CaCl$_2$· 2H$_2$O, MgCl$_2$· 6H$_2$O, and appropriate electron acceptor were first added to a pressure-resistant 1L bottle along with a few mLs less than 1 liter of DI water. Media was then purged with nitrogen through a fine diffusing stone for 30 to 45 min then was purged with 70%N$_2$/30%CO$_2$ and NaHCO$_3$ was added. After 15 min, the bottle was sealed with a 6.5 stopper and a temperature resistant cap with a drilled hole. A purged 60 mL syringe with a 1.5 in. needle was used to remove 180 mL of gas from the media bottle. The bottle was placed in a tub with about 3 inches of water, autoclaved for 30 min and the bottle was cooled on the bench at room temperature. A sterile, purged syringe with 1.5 inch needle was used to add anoxic, sterile 70%N$_2$/30%CO$_2$ until bottle was re-pressurized. Finally, resazurin stock solution and trace salts solutions I and II were added via sterile, anoxically flushed syringe. Two mL of the final media was removed with an anoxic, sterile syringe and the pH was measured with pH meter to confirm the medium had a pH near neutrality.

The medium was transferred from the reservior via sterile tubing, while under under pressure from a stream of sterile, anoxic $70\%N_2/30\%CO_2$, to autoclaved serum bottles which were capped with Teflon™-coated butyl rubber stoppers and crimped with aluminum seals.

The serum bottles were inoculated with 10% (vol/vol) of sediment-slurry from active microcosms or from a previous enrichment culture using a sterile, anoxic syringe and incubated at 27°C without agitation. Na_2S and/or cysteine stock solutions were added to the medium as reductants before adding the inoculum.

3.3.4 Analytical methods

All microcosms were sampled for aniline or PCA, nitrate, and sulfate, initially weekly and thereafter, periodically. For aniline and PCA analysis, one mL of well-mixed slurry was withdrawn from each serum bottle using a sterile 1 mL plastic syringe equipped with an 18 gauge needle that had been pre-flushed with sterile nitrogen. Samples were placed in 1.5 mL Eppendorf tubes and either frozen or extracted immediately. Extractions were performed using slight modification of previously described methods (Struijs and Rogers 1989) by adding 1 mL of acetonitrile, mixing, and centrifuging at 10,000 rpm for 3 min. The supernatant was removed using a plastic syringe, filtered through a 0.45 μm nylon filter, and placed in a glass sample vial sealed with a Teflon™-lined butyl rubber septum. Analysis was by an Agilent 1100 high performance liquid chromatography (HPLC) system (Agilent Technologis, Inc., Santa Clara, CA) equipped with a diode array detector operating at 244 nm for detection of aniline and PCA. Diphenylamine and azobenzene standards were prepared in acetonitrile (potential polymerization products), and these

eluted at 9.6 and 6.1 min, respectively, using this method. Isocratic separations were made on a Luna 5µ C18 (2) 120 column (250*2 mm) (Phenomenex, Torrance, CA). The column was held at 40°C. A water: acetonitrile (ACN) mixture (45:55 volume:volume) was supplied at a flow rate of 0.33 mL min^{-1} as the mobile phase. Aniline eluted at 2.8 min and was identified by comparison of the retention time to a known standard. Aniline concentrations in samples were quantified using a five point calibration. Microcosms were established initially amended with 500 µM aniline or PCA and thus, the calibration concentration of the standards initially ranged from 50 µM to 800 µM. After the aniline or PCA concentration was increased to approximately 1500 µM, the calibration concentration of the standards ranged from 100 µM to 2000 µM. The detection limit was estimated to be approximately 0.15 µM.

Potential metabolites of aniline transformation were selected for analysis so that pathways could be investigated. Extracted samples from certain time points collected from active microcosms or enrichments were analyzed using other methods to determine if the potential metabolites could be detected. For 4-aminobenzoate, benzoic acid and phenol (potential transformation products), a solvent system of acetonitrile (ACN): 0.1% acetic acid buffer (25:75) was used as the mobile phase and supplied at a flow rate of 0.35 mL min^{-1}. Analysis was by the Agilent 1100 high performance liquid chromatography (HPLC) system (Agilent Technologies, Inc., Santa Clara, CA) equipped with a diode array detector operating at 220, 230, 254, 280 nm and the wavelength of the fluorescence detector (FLD) was operated at: excitation wavelength (Ex) = 244 and emission wavelength (Em) = 419 nm. Isocratic separations were made on a HyperClone 5µ ODS (C18) 250 × 2.00 mm 5µ column (Phenomenex®, Torrance, CA). Standard

curves were not prepared for these compounds, but pure standards were analyzed to determine retention times.

Lactic, acetic, propionic, butyric and oxalic acids (potential fermentation products) were analyzed on a Beckman Coulter® System Gold™ HPLC (Beckman-Coulter, Inc., Fullerton, CA) equipped with a Bio-Rad® Aminex HPX-87H organic acid analysis column (Bio-Rad Laboratories, Hercules, CA). UV absorption detection was operated at a wavelength of 210 and 280 nm. The column was held at 60°C. Filtered 5.0 mM H_2SO_4 was supplied at a flow rate of 0.6 mL min^{-1} as the eluent. For analyses of these potential metabolites the detection limit was approximately 1 mM.

The concentrations of nitrate and sulfate were monitored by ion chromatography. The samples were analyzed using a Dionex ICS-1000 (Sunnyvale, CA) Ion Chromatography system equipped with an AS9-HC guard in column and an AG9-HC guard out column. The mobile phase was 9 mM Na_2CO_3 with a flowrate of 1 mL min^{-1}. A linear calibration curve for nitrate and sulfate was obtained over the range from 0.5 to 5 mM. A 0.5 mL sample was removed from a microcosm or enrichment using a sterile nitrogen-flushed syringe, diluted 20 times with DI water and filtered through a 0.45 μm nylon filter. The method detection limit was approximately 3 mM (prior to dilution) for nitrate or sulfate.

Headspace samples were analyzed for methane content using a gas chromatography system (Agilent 6890N G1530N network GC system) equipped with flame ionization detector. The injection block was maintained at 250 °C, the detector was maintained at 250 °C and the oven setpoint was 150 ° C. The colmnn (Agilent 113-4332, GS-GasPro, capillary: 30 m x 0.32 mm I.D.) was used with a helium carrier gas flow rate of 1.3 mL

min^{-1}. The hydrogen flow to the detector was 40 mL min^{-1} and air flow was 45 mL min^{-1}.

Under these conditions, methane was eluted as a symmetrical peak at 1.6 min.

3.3.5 Stoichiometry of aniline degradation

To examine whether aniline and PCA degradation were coupled to reduction of the

electron acceptor under each condition, predicted values based on stoichiometric

equations were applied to compare to those measured in microcosms and enrichments.

These stoichiometric equations (Equations 3.1 to 3.10) assume that aniline/PCA was

completely mineralized to CO_2 (Struijs and Rogers 1989) and ignore the production of

cells:

Equations 3.1 to 3.10

Aerobic:

$$C_6H_7N + 28O_2 + 4H_2O \rightarrow 6HCO_3^- + NH_3 + 6H^+ \tag{1}$$

$$C_6H_6NCl + 6.75O_2 + 4.5H_2O \rightarrow 6HCO_3^- + NH_3 + Cl^- + 6H^+ \tag{2}$$

Denitrifying:

$$C_6H_7N + 5.6NO_3^- + 1.2H_2O \rightarrow 6HCO_3^- + NH_3 + 0.4H^+ + 2.8N_2 \tag{3}$$

$$C_6H_6NCl + 5.4NO_3^- + 1.8H_2O \rightarrow 6HCO_3^- + NH_3 + Cl^- + 0.6H^+ + 2.7N_2 \tag{4}$$

Iron-reducing:

$$C_6H_7N + 28Fe^{3+} + 18H_2O \rightarrow 6HCO_3^- + NH_3 + 34H^+ + 28Fe^{3+} \tag{5}$$

$$C_6H_6NCl + 27Fe^{3+} + 18H_2O \rightarrow 6HCO_3^- + NH_3 + Cl^- + 33H^+ + 27Fe^{3+} \tag{6}$$

Sulfidogenic:

$$C_6H_7N + 3.5SO_4^{2-} + 4H_2O \rightarrow 6HCO_3^- + NH_3 + 2.5H^+ + 3.5HS^- \tag{7}$$

$$C_6H_6NCl + 3.375SO_4^{2-} + 4.5H_2O \rightarrow 6HCO_3^- + NH_3 + Cl^- + 2.625H^+ + 3.375HS^- \tag{8}$$

Methanogenic:

$$C_6H_7N + 7.5H_2O \rightarrow 2.5HCO_3^- + 3.5CH_4 + NH_3 + 2.5H^+ \tag{9}$$

$$C_6H_6NCl + 7.875H_2O \rightarrow 2.625HCO_3^- + 3.375CH_4 + NH_3 + Cl^- + 2.625H^+ \tag{10}$$
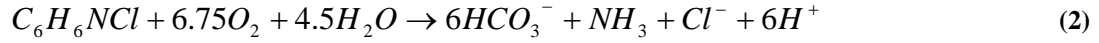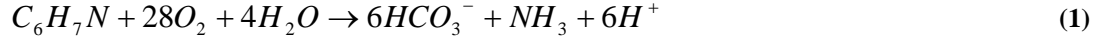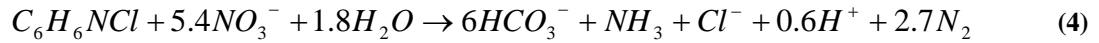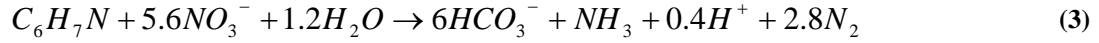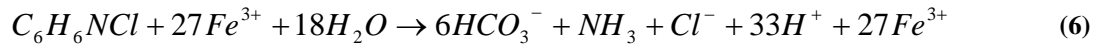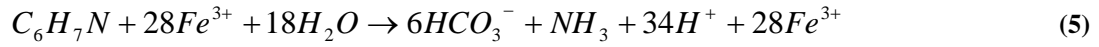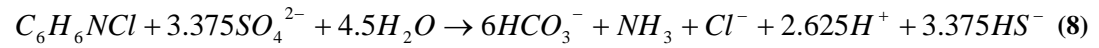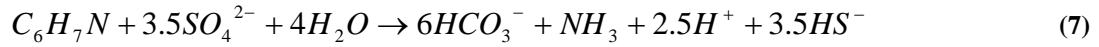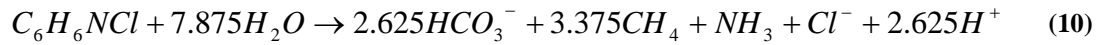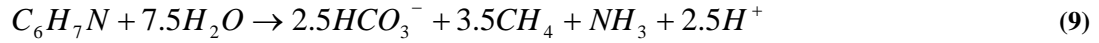
3.3.6 DNA extraction and PCR amplication of 16S rRNA genes

Total genomic DNA extraction was performed with the PowerSoil[TM] DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) from pellets collected following centrifugation of 1 mL slurry samples from sediment microcosms or from 0.25 to 1 g original sediment. Final DNA was eluted in 50 µL of PowerSoil[TM] Solution C6.

The polymerase chain reaction (PCR) products were generated with Bac338f-GC and Univ519r primers (See Table 3.4), resulting in an expected 200 bp DNA fragment of amplified bacterial 16S rRNA gene. Each 50 µL PCR reaction mixture contained the following: 1x PCR buffer (all reagents were obtained from USB Corp., Cleveland, OH, USA. All primers were obtained from Integrated DNA Technology, Coralville, IA, USA.), 2.5 mM of $MgCl_2$, 20 nmol of deoxynucleoside triphosphate, 10 pmol of each primer, 1.5 U of Taq polymerase, and 4 µL of template DNA. 10 pmol $µL^{-1}$ primers were made from 100 pmol $µL^{-1}$ original stock by diluting in PCR quality water. The thermocycling program was as follows: initial denaturation at 94°C for 5 minutes, then 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds (30 cycles), and a final elongation at 72 °C for 7 minutes. Final hold was set at 4 °C for an indefinite time. The expected PCR amplicon sizes were verified by running the PCR products on a 1.5 % agarose gel. A blank (negative control) using DI water as template was run with each set of samples.

3.3.7 DGGE and sequence analysis of DNA bands excised from DGGE gels

Bacterial community analysis was performed using PCR-denaturing gradient gel electrophoresis (PCR-DGGE) of DNA obtained from active aniline or PCA amended microcosms. The hypothesis was that microcosms set up under different redox conditions would support bacterial communities that indicate the specific redox system. For example, nitrate-reducing bacteria would be the dominant species in the nitrate-amended microcosms and sulfate-reducing bacteria would be the dominant species in the sulfate-amended microcosms. No archaeal-specific community analysis was performed.

The PCR products were subjected to denaturing gradient gel electrophoresis (DGGE) according to the protocol of Muyzer *et al*. (1993) and adapted by using an 8% polyacrylamide gel with a 35 to 55% urea-formamide gradient, and electrophorizing for 290 min at 150 V and 60°C using a DCode mutation detection system (Bio-Rad Laboratories, Hercules, CA, USA). The PCR primers used are shown in Table 3.4. DNA was visualized after 0.1% ethidium bromide staining by UV transillumination on a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories). Each prominent band detected in the DGGE gel was excised using a sterile razor blade and placed in 20 μL of sterilized DI water overnight at 4°C. The DNA eluted from the gel slice to the DI water was then used as template DNA and re-amplified using universal primer 338f and 519r (Table 3.4). The resulting amplicons were sized on a 1.5 % agarose gel and purified with an UltraClean PCR Clean-up kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Purified PCR products were then sequenced by Genewiz (Genewiz Inc., South Plainfield, NJ).

3.3.8 Construction of phylogenetic trees

The 16S rRNA gene fragments were analyzed and compared to sequences in the

GenBank database by BLAST® (BlastN, National Center for Biotechnology Information

Database; http://www.ncbi.nlm.nih.gov). Phylogenetic trees were constructed from

unique sequences and closely related sequences of isolates or environmental phylotypes

selected from the GenBank database by Mega (www.megasoftware.net). Bootstrap values

were determined from 100 iterations of the maximum likelihood calculation.

3.4 Results

3.4.1 Background concentrations of aniline and PCA in microcosms

After set up of microcosms (Day 0) and before spiking with aniline/PCA stock solutions

(Day 1), samples were taken from three random microcosms from each location and

analyzed for background aniline and PCA concentrations. The concentrations of aniline

in the lightly contaminated groundwater aquifer material ranged from below the detection

limit (0.15 µM) to 1.1 µM.   The concentrations of aniline in the highly contaminated

groundwater aquifer material were 1.8 to 10.3 µM. The concentrations of PCA in both

groundwater aquifer sites ranged from 0.2 µM to 0.3 µM. The concentrations of aniline

and PCA in the freshwater canal materials were near the detection limit of the HPLC
method (0.15 µM).

3.4.2 Biological microcosm results

The results for the most active microcosm sets for aniline or PCA are shown in Figures
3.1 through Figure 3.25. Results for less active microcosms are shown in Figure B.1
through B.50. Note that the results for the nitrate-amended-aniline and methanogenic-
aniline microcosms prepared using lightly contaminated sediments from the freshwater
canal are discussed in Chapter 4 and are shown in Figures 4.1 through 4.6. The
methanogenic-PCA results from microcosms of the lightly contaminated sediments from
the freshwater canal are discussed in Chapter 5 and the results are presented in Figures
5.1 and 5.2.

Microcosms established under anoxic conditions were initially amended with
approximately 100 µM of aniline or PCA and then later increased to 1500 µM aniline or
PCA. In the figures, aniline or PCA loss is shown. Further analysis as described in
Section 3.3.3 was performed to detect potential metabolites of aniline and PCA
biodegradation. No peaks corresponding to benzoic acid, phenol, 4-aminobenzoate,
diphenylamine or azobenzene were observedand all monitored organic acids were less
than the detection limit of 1 mM.

A summary of observed activity under each redox condition from day 0 to day 340 and
from day 340 to day 1200 is shown in Table 3.5 and 3.6, respectively.

3.4.2.1 Lightly contaminated groundwater aquifer location

The results for the groundwater aquifer microcosms prepared using lightly contaminated material are shown in Figures 3.1 through 3.13 and Figures B.1 through B.7. The summaries of methane production for the groundwater aquifer microcosms are shown in Table 3.7 and Table 3.8.

3.4.2.1.1 Aniline amended microcosms

Groundwater aquifer microcosms from the lightly contaminated location showed loss of aniline under aerobic conditions with no lag (Figure 3.1). Microcosms were initially amended with 100 µM and by day 8, only 1.4 µM of aniline was detected in aerobic live microcosms. A second spike of 156 µM of aniline was added in aerobic live treatment on day 31. By day 37, only 1.6 µM of aniline was detected. Loss of aniline was also noted in the aerobic killed controls. By day 8, 54% loss of aniline was detected; however, this loss slowed and it took 90 days for the remaining aniline to decrease from 47.7 µM to 10 µM in killed aerobic control microcosms. On day 111, aniline was below the detection limit in all triplicate aerobic killed controls suggesting substantial abiotic losses. The live and killed aerobic microcosms were discontinued after 120 days.

Loss of aniline without a lag was observed in all triplicate microcosms prepared using nitrate-amended conditions (Figure 3.2). Loss of aniline in the killed anaerobic controls was about 26% at the first sampling interval between days 1 and 8. The nitrate-amended microcosms were re-amended with aniline on days 31, 144, 223 and 342 with subsequent loss of the added aniline. The aniline concentration in the microcosms after the aniline

concentration was increased (days 450 to 1350) is shown in Figure 3.3. Nitrate

concentration in these microcosms is shown in Figure 3.4. According to the stoichiometry

shown in Eq. 3.3, consumption of nitrate concomitant with the spike of aniline on day

144 would have consumed approximately 0.866 mM nitrate. Nitrate concentration

showed approximately 0.976 mM loss between days 144 and 151, similar to what was

expected. Consumption of nitrate concomitant with spike of aniline on day 223 should

have consumed approximately 0.846 mM nitrate on average, however the average nitrate

concentration decreased by 7.69 mM between day 222 and 304, perhaps indicating

degradation of other contaminants or natural organic matter linked to denitrification.

Nitrate was depleted in triplicate #1 and loss of aniline ceased likely as a result of the

depletion of the electron acceptor. This observation indicated that nitrate must be present

for aniline loss to proceed, an indication that microbial activity was responsible for

aniline degradation. Re-amendment of nitrate to that microcosm is indicated by the

arrow in Figure 3.4. Triplicate microcosms #1 and #2 were amended with approximately

1500 µM aniline for the sixth and seventh re-amendments (Figure 3.3). The slurry in

triplicate #3 was used for enrichment transfers. Enrichment cultures were started using

10% inoculum from triplicate #3 at day 469 (See Section 3.4.3 and Figure B.51). Loss of

aniline from triplicates #1 and #2 was show and compared to anaerobic killed controls

occurred at similar rates between day 495 and 1308.

Loss of aniline in two of the triplicates (#1 and #2) was noted under Fe(III)-amended

conditions after a lag of 8 and 14 days (Figure 3.5). The Fe(III)-amended microcosms

were re-amended with aniline on days 93 and 174 with subsequent loss of the added

aniline. 40% loss of aniline was noted in the Fe(III)-amended killed controls within the

first 340 days of incubation but the loss in live microcosms was far greater and exhibited a different pattern. Microcosms were amended with approximately 1500 µM aniline for the fourth re-amendment event. 46% loss of aniline in one of the triplicates #1 was noted under Fe(III)-amended conditions by day 429 (Figure 3.6). From day 429 to 778, the concentration of aniline in this active microcosm decreased from 802 to 33 µM. There was no sampling time point between day 429 and 778, so the time progression of the removal of the aniline is not known for that time period. The active microcosm was spiked for the fifth time on day 941. Loss of aniline was noted from 1003 µM to 25 µM only 28 days after the re-spike. Enrichment cultures were started using 10% inoculum from this microcosm on day 885 (See Section 3.4.3 and Figure B.61). Little loss of aniline was noted in the Fe(III) amended killed controls between day 343 to day 1284 suggesting that the rapid loss of aniline in triplicate #1 was biologically mediated.

After a lag of approximately 37 days, aniline loss was noted in two of the sulfate-amended triplicates and after a lag of 135 days, loss in the third triplicate was observed (Figure 3.7). This loss was rapid after it began and was not similar in rate to the slower loss observed in the killed controls. The sulfate-amended microcosms were re-spiked on days 223 with subsequent loss of the added aniline in two of the triplicates (#1 and #3). Microcosms were amended with approximately 1500 µM aniline for the third and fourth re-amendments (Figure 3.8). No loss of aniline in triplicates was noted compared to anaerobic killed controls between day 495 and 1308 at the higher aniline concentration. Sulfate concentration in these microcosms is shown in Figure 3.9. Sulfate was present in excess throughout the incubation period. According to the stoichiometry shown in Eq. 3.7, consumption of sulfate concomitant with the first spike of aniline degradation would

have consumed 0.24 mM sulfate in sulfate-amended aniline 1 microcosm from day 54 to 111. Analysis of the sulfate data showed 1.28 mM sulfate loss between days 54 and 111, more than expected based on theoretical stoichiometry. The time points for re-amendment of sulfate to the sulfate-amended microcosms is shown in Figure 3.9.

The results from cultures set up under conditions meant to enhance methanogenesis are shown in Figures 3.10 and B.1. [Note that triplicate #3 was inadvertantly not amended with aniline during the initial incubation period.] Loss of aniline occurred in triplicate #2 after a 54 day lag, while no loss was noted in the remaining triplicate bottle. Further, no loss of aniline in triplicates was noted when compared to anaerobic killed controls between day 495 and 1308, after the aniline concentration had been increased to 1500 µM.

Methane was monitored in microcosm headspaces. Table 3.7 shows that there was only a trace of methane in microcosms under nitrate-, sulfate- or Fe(III)-amended conditions by day 366 compared to the higher concentrations in the headspaces of those established under methanogenic conditions.

3.4.2.1.2 PCA amended microcosms.

In aerobic microcosms prepared with material from the lightly contaminated groundwater aquifer location, substantial, rapid loss of PCA was observed in both live and killed controls (Figure B.2). The live and killed aerobic microcosms were discontinued after 120 days.

In nitrate-amended microcosms, PCA loss occurred without any lag (Figure 3.11). PCA loss was also observed when PCA was re-amended on days 31, 111, 144 and 234, although in nitrate replicate #3, the loss was incomplete after third spike of PCA. According to the stoichiometry shown in Eq. 3-4, consumption of nitrate concomitant with PCA degradation would have consumed approximately 4.45 mM nitrate in triplicate #1, which received a total of 800 µM of PCA dosage from day 0 to day 495 (assuming that the losses from sampling can be ignored). Although there is variability in the nitrate concentrations measured, there was an overall loss of nitrate. There was a decrease of approximatedly 11 mM nitrate between days 54 and 510 in triplicate #1 (Figure 3.13). This indicates that nitrate loss could have been coupled with biodegradation processes related to other compounds besides PCA. Microcosms were re-amended with approximately 1500 µM PCA on day 495 (Figure 3.12). 95% loss of PCA in triplicate #1 was noted by day 923. In triplicate #2, the concentration of PCA decreased from 3570 to 2320 µM between day 495 and day 923. There was no sampling time point between day 504 and 923 and thus the time progression of the loss is not known. The active microcosms were re-spiked with PCA two more times. Loss of PCA was from 2270 µM to 600 µM in triplicate #3 between days 495 to 1308. Note that a gradual 30% loss of PCA was also observed in the killed controls between days 495 and 1308. Nitrate was present in excess during entire the incuabtion period (nitrate concentration after day 800 not shown in Figure 3.12).

In Fe(III)-amended , sulfate-amended and methanogenic microcosms prepared with material from the lightly contaminated groundwater aquifer location, no loss of PCA was observed in comparison to killed controls (Figures B.3 through, B.6). Sulfate

concentration in the sulfate-amended microcosms is shown in Figure B.7. Sulfate was present in excess throughout the incubation with only a slow decrease over this long incubation period, suggesting no strong sulfate-reducing activity in these microcosms.

Note that methane production was minimal in all microcosms, except those established to produce methanogenic conditions (Table 3.8).

3.4.2.2 Highly contaminated groundwater aquifer location.

The results for the groundwater aquifer microcosms prepared using the highly contaminated material are shown in Figures 3.14 through 3.16 and Figures B.8 through B.26.

3.4.2.2.1 Aniline amended microcosms.

The day 0 aniline concentration in groundwater aquifer microcosms from the highly contaminated location, set up under aerobic conditions, were calculated based on the volume of injected aniline stock solution and was estimated to by 100 μM. By day 1, the average aniline concentration in killed aerobic controls dropped to 19 μM (Figure B.8) and aniline was completely depleted in live triplicate #3. By day 8, an average of 1.7 μM of aniline was detected in aerobic live microcosms. A second amendment of ~160 μM of aniline was added to live and killed controls on day 31. By day 37, loss of aniline was observed with no lag in loss in the live treatments (Figure B.8). Loss of aniline was also

noted in the aerobic killed controls, but this loss was far less rapid than that observed in the live treatments. The aerobic microcosms were discontinued after 120 days.

Loss of aniline was not observed in triplicate microcosms prepared using nitrate-amended conditions (Figures B.9 and B.10). Loss of aniline in the killed anaerobic controls was also minimal. Nitrate concentration in these microcosms is shown in Figure B.11. Nitrate was present in excess throughout the incubation, and no depletion of nitrate was observed suggesting little biological activity under these conditions. Further, low concentrations (1 to 4.6 µM) methane were detected in microcosms prepared under all redox conditions, with no enhancement of methanogenesis noted in those microcosms prepared under methanogenic conditions (Table 3.7).

Loss of aniline in all live triplicate microcosms was noted under Fe(III)-amended conditions after 100 days (Figure B.12). The Fe(III)-amended microcosms were re-amended on day 174. Although loss of aniline was observed, it was not complete and ~100 µM aniline persisted. Only 52% loss of aniline was observed in the Fe(III)-amended killed controls in the first 340 days of incubation. Microcosms were amended with approximately 1500 µM aniline for the third re-amendment on day 343. In contrast with Fe(III)-amended groundwater aquifer microcosms from the lightly contaminated location (Figure 3.6), no loss of aniline was noted in either the Fe(III)-amended killed or live microcosms between day 343 to day 1284 in groundwater aquifer microcosms from the highly contaminated location after the aniline concentration was increased to 1500 µM (Figure B.13). Slow loss of aniline was observed throughout the remainder of the incubation and live microcosms and killed controls behaved similarly. As previously

noted, Table 3.7 shows that there was insubstantial accumulated methane under Fe(III)-amended conditions by day 366.

Loss of aniline occurred in triplicate #3 in the sulfate-amended microcosms after a 300 day lag, while no loss was noted in the remaining triplicate bottles (Figures 3.14). Microcosms were amended with approximately 1500 μM aniline for the second amendment (Figure B.14). 85% loss of aniline in triplicate #3 was noted by 335 days after the re-amendment. There was no sampling time point between day 504 and 815 and thus the rapidity of the loss is not known. The active microcosm was re-amended for a third time on day 964, but no loss of aniline was observed by day 1308. Sulfate concentrations in these microcosms is shown in Figure B.15. Sulfate was present in excess throughout the entire incubation period. According to the stoichiometry shown in Eq. 3-7, consumption of sulfate concomitant with the first spike of aniline degradation would have consumed 6.2 mM sulfate in triplicate #3 between day 495 to 830. The variability in the sulfate concentration measured in the microcosms does not allow conclusive computational evidence for the concomitant loss of sulfate concentration between days 112 and 852. The examination of sulfate concentration in triplicate #3 indicates that aniline loss was not apparently coupled to sulfate-reduction, at least following the second spike of 1500 μM aniline. Table 3.5 shows that there was no substantial accumulated methane production under sulfate-amended conditions from this site by day 376.

The results from bottles set up under conditions meant to enhance methanogenesis are shown in Figure 3.15 and 3.16. Rapid loss of aniline occurred in triplicate #1 after a 150-

day lag. The other two triplicates also showed loss beginning after day 150. Microcosms were amended with approximately 1500 µM aniline for the second re-amendment (Figure 3.16). Loss of aniline in triplicate #3 was noted 652 days following the second re-amendment, while no loss was noted in the remaining triplicate microcosms.

Examination of methane accumulation in these microcosms indicates that there were 4.3, 5.5, 1.6 µM methane in the headspace of triplicates #1, 2 and 3, respectively on day 376. According to the stoichiometry in Equation 3.9, an average of 100 µM aniline could be completed mineralized and produce an average of 350 µM methane in each triplicate microcosm (ignoring the production of cells). This indicates that aniline might not be completely mineralized to methane or that part of the carbon source was consumed in cell production.

3.4.2.2.2 PCA amended microcosms.

In aerobic microcosms prepared with material from the lightly contaminated groundwater aquifer location, loss of PCA occurred in both killed aerobic controls and live micocosms at similar rates. The live and killed aerobic microcosms were discontinued after 120 days (Figure B.16).

No loss of PCA was observed relative to the controls under any anoxic condition in microcosms from the highly contaminated groundwater aquifer location (Figures B.17 to B.26). Nitrate and sulfate concentrations in these microcosms are shown in Figure B.19 and Figure B.24. Nitrate and sulfate were present in excess throughout the incubation period and thus could have supported the corresponding activities if present. Sulfate

depletion was noted in triplicates #1 and #3 at the end of the incubation (between day 850 and 1150). Table 3.8 shows that there was insubstantial accumulated methane under nitrate-, Fe(III)-, sulfate-amended or methanogenic conditions for this location. Thus, the highly contaminated groundwater aquifer location exhibited very little biological activity overall.

3.4.2.3 Lightly contaminated freshwater canal location.

The results for the freshwater canal microcosms prepared using lightly contaminated material are shown in Figures 3.17 through 3.24, Figures 4.1 through 4.5 and B.27 through B.31. The summaries of methane production for freshwater canal microcosms are shown in Table 3.9 and Table 3.10. No aerobic treatments were established for canal sediments.

3.4.2.3.1 Aniline amended microcosms.

The aniline–nitrate-amended-microcosms prepared using lightly contaminated freshwater canal materials were the most active microcosms observed in this study with repeated loss of aniline upon re-amendment. Extensive efforts to enrich for aniline degraders were performed using these microcosms. The full results for these microcosms and corresponding enrichments are described in detail in Chapter 4 (Figures 4.1, 4.2 and 4.5). and are not discussed here.

Loss of aniline without lag was noted under Fe(III)-amended conditions in all triplicate microcosms (Figure 3.17). By day 8, an average loss of 70% was noted in Fe(III)-amended live microcosms and 47% loss of aniline was also noted in the Fe(III)-amended

killed controls. By day 14, less than 10 µM of aniline was detected in live microcosms.

Loss of aniline in two of the triplicates was noted under Fe(III)-amended conditions after

a second re-amendment. Table 3.9 summarizes the methane production under Fe(III)-

amended conditions as measured on day 217. There was 32.6 µM methane detected in

the head space of Fe(III)-amended triplicate #1 and 15.2 and 3.0 µM methane detected in

triplicates #2 and 3, respectively. The average methane production in Fe(III)-amended

killed controls was 9.2 µM with 0.5 µM standard deviation. The production of methane

in the Fe(III)-amended microcosms (and as evidenced in some other microcosm sets

established with nitrate, Fe(III) or sulfate) could be due to the depletion of the electron

acceptor (note that loss of Fe(III) was not monitored during this study) or to development

of Fe(III)-depleted microenvironments during static incubation. Methane production was

still much lower than that observed under methanogenic conditions. Further, methane

was also detected in autoclaved controls for this location. To ensure that the controls

were inactive, the Fe(III)-amended killed controls were re-autoclaved at 121°C for 30

min on each of three consecutive days after day 379. Microcosms were amended with

approximately 1500 µM aniline for the third re-amendment on day 343. Loss of aniline in

triplicate #1 was noted 86 days after this addition (Figure 3.18). The active microcosm

was spiked for the fourth and fifth times on days 551 and 941 with accompanying loss of

aniline. There was no sampling time point between days 551 and 778, and thus it is not

known how rapidly this loss may have occurred. 18.5% loss of aniline was also noted in

the Fe(III)-amended killed controls between day 343 to day 1109, similar to the loss in

live replicates #2 and #3.

After a lag of approximately 20 days, aniline loss was observed in all triplicate microcosms prepared using sulfate-amended conditions (Figure 3.19). The sulfate-amended microcosms were re-amended on days 100 and 174 with subsequent loss of the added aniline in all triplicates. Sulfate concentration in these microcosms is shown in Figure 3.21. Sulfate was maintained in excess throughout the entire incubation period, by respiking sulfate after day 400, however, notable loss of sulfate did occur, especially between days 100 and 400. Microcosms were amended with approximately 1500 µM aniline for the fourth to sixth re-amendment events (Figure 3.20). Loss of aniline in triplicates was noted after each re-amendment between days 343 and 1109. [There was no sampling point between day 350 and 775.] Loss of aniline in the corresponding killed anaerobic controls was minimal. According to the stoichiometry shown in Eq. 3-7, consumption of sulfate concomitant with the degradation of the initially added aniline would have consumed 0.83 mM sulfate from day 1 to 328. Analysis of sulfate data showed an average of 8.65 mM sulfate loss between days 1 and 328 (Figure 3.21), more than 10-fold the amount required for aniline degradation. This indicates that sulfate reduction was active and likely coupled to degradation of other pollutants or natural organic matter in the sediments, in addition to aniline.

The methanogenic-aniline microcosm results prepared using lightly contaminated freshwater canal materials are described in detail in Chapter 4 (Figure 4.3 and Figure 4.4). These microcosms were also among the most active observed during this study, with repeated loss of aniline upon re-amendment.

3.4.2.3.2 PCA amended microcosms.

Nearly complete loss of PCAwas observed in nitrate-amended microcosms from day 0 to day 90 (Figure B.27). A slower 45% loss of PCA was also noted in the killed controls between day 1 and 90. The microcosms were re-amended on day 174 with subsequent average 86% loss of the added PCA in live triplicates by day 350. Loss of PCA was minimal in the killed controls between day 67 and day 345. Microcosms were amended with approximately 1500 µM PCA for the third re-amendment event (Figure B.28). No loss of PCA was observed relative to the controls from day 345 to day 1281 at this higher PCA concentration. Nitrate concentration in these microcosms is shown in Figure B.29. Nitrate was depleted in the live replicates by day 350.There was also 6.8 µM accumulated methane production in triplicate #1 on day 357, but less than 1 µM accumulated methane production in the other triplicates (Table 3.10). Note that there was also accumulated methane in anaerobic killed controls on day 357 (2.8±3.5 µM). Nitrate was re-amended periodically throughout the incubation period and its subsequent depletion suggests nitrate reduction was quite active and may have been coupled to degradation of other organic matter or contaminants besides PCA to account for this extra stoichiometric loss.

PCA in Fe(III)-amended microcosms prepared with material from the lightly contaminated freshwater canal location are shown in Figures B.30 and 3.22. By day 14, 58% loss of PCA was noted in both Fe(III)-amended live microcosms and Fe(III)-amended killed controls. No loss of PCA was observed with respect to killed controls from day 14 to day 345 (Figure B.30). Microcosms were amended with approximately

1500 μM PCA for the second re-amendment event (Figure 3.22). Interestingly, loss of 1200 μM PCA in triplicate #1 was noted with concomitant 800 μM aniline accumulation on day 932 (the aniline concentration is not shown in Figure 3.22). There was 130 μM ±200 μM of methane production in these microcosms, indicating depletion of Fe (III) in these microcosms (Table 3.10). There was 365 μM, 5 μM and 20 μM methane accumulation in triplicates #1, 2 and 3, respectively. The accumulation of aniline in replicate #1 could indicate the onset of strongly reducing methanogenic conditions, which was more amenable to reductive dechlorination. From day 427 to day 804, the concentration of PCA in this active microcosm decreased from 1350 to 260 μM. [There was no sampling time point between day 427and 804.] The active microcosm was re-spiked for the third time on day 939. The concentration of PCA from this active microcosm decreased again from 1000 μM to 410 μM 177 days after re-spiking. Because the apparent depletion of Fe(III) as the electron acceptor and the dechlorination of PCA to aniline, the results from bottles set up under Fe(III)-amended conditions were perhaps more likely to reflect the onset of methanogenesis and the results mirror those of the original methanogenic microcosms, which also actively dechlorinated PCA to aniline (see a full description in Chapter 5).

By day 67, 64% loss of PCA was noted in both sulfate-amended live microcosms and anaerobic killed controls. No loss of PCA was observed with respect to killed controls from day 67 to day 345 (Figure B.31). There was 2100 μM methane in the head space of sulfate-amended triplicate #1 on day 217 (Table 3.10). Sulfate concentration in this bottle decreased from 3.0 mM to 1.76 mM from day 325 to day 427 indicating that sulfate was strongly depleted (Figure 3.24), allowing onset of methane production. There were 83.4

and 6.3 µM methane detected in the headspaces of sulfate-amended triplicates #2 and #3 on day 217, respectively. Sulfate concentration decreased from 11.6 mM to 9.0 mM and from 18.5 mM to 14.4 mM in triplicate #2 and #3 from day 325 to day 427, respectively, but was never completely depleted. Sulfate was re-spiked on day 427. Microcosms were re-amended with approximately 1500 µM PCA for the second re-amendment (Figure 3.23). Loss of PCA in triplicate #2 was noted after a 500 day lag, but no aniline was detected. No loss of PCA was noted in the other two triplicates. Thus, despite depletion of sulfate and onset of methanogenesis in replicate #1, dechlorination of PCA to aniline was not observed as it was for methanogenic and Fe(III)-amended microcosms.

The methanogenic-PCA microcosm results prepared using the lightly contaminated freshwater canal materials are described in detail in Chapter 5 (Figure 5.1 and 5.2). After increasing the PCA concentration and adding lactic acid as an electron donor and hydrogen source, these microcosms became active. Repeated amendments of PCA were dechlorinated to aniline slowly over long time periods.

3.4.2.4 Highly contaminated freshwater canal location.

The results for the freshwater canal microcosms prepared using sediments from the highly contaminated location are shown in Figure 3.25 and Figures B.32 through B.50.

3.4.2.4.1 Aniline amended microcosms.

Loss of aniline in microcosms prepared using nitrate-amended, sulfate-amended and methanogenic conditions were not significantly different from anaerobic controls

(Figures B.32, B.33, B.36, B.37, B.39, B.40). Loss of aniline in killed anaerobic controls (though not in killed Fe(III)-amended controls) was observed for this location. The killed anaerobic control and Fe(III)-amended killed controls were re-autoclaved at 121°C for 30 min on each of three consecutive days after day 379, but losses continued to be observed subsequent to this attempt to kill biological activity. This observed activity despite vigorus efforts to kill the microorganisms may indicate that substantial abiotic mechanisms were responsible for aniline loss in the killed controls.

Despite the possibility of abiotic mechanisms, in examing the results in detail, some indicators of biologically-mediated activity were noted. The nitrate-amended and sulfate-amended microcosms and killed anaerobic controls were re-spiked with aniline on day 174 with complete loss of the added aniline observed in one nitrate-amended and one sulfate-amended triplicate by day 328. There was no sampling time point between day 198 and 328. A 70% loss of aniline was observed in triplicate anaerobic killed controls from day 174 to 343. The triplicate nitrate-amended and sulfate-amended microcosms were re-amended for the fourth time on day 343 and 775 to achieve approximately 1500 µM aniline (Figure B.33 and Figure B.37). Complete loss of aniline was noted in nitrate-amended triplicate #2 and in sulfate-amended triplicate #3 after the fourth re-amendment. The active microcosms were re-amended for the fifth time on day 941 and loss of aniline was noted again after this addition in these active microcosms. No loss of aniline was noted in the other two of the triplicates under nitrate-amended conditions or under sulfate-amended conditions after day 343. 76% loss of aniline was noted in the killed controls between day 343 and day 1109, however the loss was slow compared to loss in the active replicates. [There was no sampling time point between day 429 and 775.]

Nitrate and sulfate were maintained in excess throughout the incubation period (Figure B.34 and Figure B.38). In triplicate sulfate-amended microcosms, sulfate was re-amended on day 969. Sulfate loss was evident in replicate #3 (Figure B.38), concomitant with loss of aniline (Figure B.37).

A 26% loss of aniline was observed in both the Fe(III)-amended microcosms and Fe(III)-amended killed controls in the first 22 days incubation (Figure B.35). However, no loss of aniline occurred in the live microcosms relative to the killed anaerobic controls from day 1 to 343. Table 3.9 shows there was no accumulated methane under Fe(III)-amended conditions as measured on day 217. Microcosms were amended with approximately 1500 µM aniline for the second amendment event. Loss of aniline in triplicate #3 was noted after this second amendment (Figure 3.25). The active microcosm was amended for the third time on day 941 with loss of aniline observed after 28 days. Only 10% loss of aniline was noted in the Fe(III)-amended killed controls between day 343 to day 1109. [There was no sampling time point between day 429 and 778.]

The cultures set up under conditions meant to enhance methanogenesis showed similar results as the nitrate-amended and sulfate-amended microcosms in the first year of incubation (Figure B.39), i.e. with similar losses of aniline in live and killed microcosms. Again, after long lag periods, some indication of biological activity was observed in some replicates. Microcosms were amended with approximately 1500 µM aniline on day 343 (Figure B.40). Complete loss of aniline was noted in methanogenic triplicate #3 by day 775. [There was no sampling time point between day 429 and 775.] Triplicate #3 was re-amended for the fifth time on day 941 and loss of aniline was noted again after re-

amendment in this active microcosm. Loss of aniline was also observed in methanogenic triplicate #2 beginning after 800 days. No loss of aniline was noted in methanogenic triplicate #1 from day 343 to 1109. Thus, at this location activity was not homogeneous and replicates behaved differently. Killed anaerobic controls exhibited substantial, slow, apparently abiotic, losses. Losses in active live microcosms (when observed) occurred at rapid rates relative to the slow losses in the killed controls.

3.4.2.4.2 PCA amended microcosms.

Loss of PCA under nitrate-amended conditions occurred and PCA was re-amended on day 174 (Figure B.41). Loss of PCA occurred in triplicates after this second amendment. However, 81% loss of PCA in the killed anaerobic controls was also observed. Table 3.10 shows there was no substantial methane production in killed anaerobic controls as measured on day 357. Nitrate-amended microcosms and killed anaerobic controls were re-amended with approximately 1500 µM PCA for the third re-amendment (Figure B.42). 63% loss of PCA was noted in the nitrate-amended microcosms between day 345 to day 1281. A 49% loss of PCA was also observed in the killed anaerobic controls between day 345 to day 1281. Nitrate loss was observed in triplicates #1 and #2 over the first 200 days of incubation (Figure B.43). However, no further loss in any replicate was observed thereafter.

No loss of PCA was observed relative to the controls in microcosms amended with Fe(III) from the highly contaminated freshwater canal location (Figures B.44 and B.45). Loss of PCA with respect to the killed anaerobic controls was difficult to discern in triplicate microcosms prepared under nitrate-amended, sulfate-amended and

methanogenic conditions (Figures B.41, B.42, B.46, B.47, B.49, B.50). However, aniline was detected in triplicates #1 and #2 of the methanogenic microcosms and this detection corresponded to the onset of more rapid loss of PCA (Figure B.50). There was 285 and 507 µM aniline detected in triplicate #1 on days 890 and 1281, respectively, and there was 404 µM, 542 µM and 187 µM aniline detected in triplicate #2 on days 890, 1116 and 1281, respectively. No aniline was detected in killed controls. The detection of aniline indicates that reductive dechlorination of PCA likely occurred in these microcosms, similar to that observed in methanogenic microcosms of the lightly contaminated sediments (see Chapter 5).

Sulfate concentration in the microcosms is shown in Figure B.48. Sulfate concentration decreased over the first 400 days in all triplicates and was re-amended on day 427.

3.4.3 Enrichments from aniline and PCA amended microcosms

Enrichments were developed from active microcosms by transferring into fresh medium as described in Section 3.3.2. The complete results from the most successful enrichments are described in detail in Chapters 4 and 5. In brief, the most successful enrichments were those established from the nitrate and aniline amended lightly contaminated freshwater canal location microcosms (Figure 4.7 and 4.8) and from active methanogenic-PCA amended microcosms from the lightly contaminated freshwater canal sediment location (Figure 5.5 and 5.6). Other enrichments were attempted and the results from those where little or no activity was observed are shown in Figures 5.4 and 5.7 and Figures B.51 through B.63.

3.4.4 Bacterial community analysis of microcosms

Bacterial community structures were studied in microcosms amended with aniline or PCA under different redox conditions. The DGGE images showing DNA fragments resulting from PCR amplification of bacterial 16S rRNA genes from aniline-amended microcosms under different redox conditions is shown in Figure 3.26 and for PCA-amended microcosms in Figure 3.28. Multiple visible bands suggest a broad diversity within the domain bacteria. The native lightly contaminated freshwater canal sample, which was analyzed to provide an initial background community is shown and discussed in Chapter 4, Figure 4.9. In brief, no dominant DGGE bands were observed in the original sediment (See Section 4.4.4), only numerous light bands which could not be successfully excised or sequenced. This result is suggestive of a highly diverse original sediment bacterial community in the lightly contaminated sediment.

Microcosm sequences (<190 bp) were compared by BLAST® with sequences existing in the GenBank® database (Tables 3.11 and 3.13). The phylogenetic tree produced to compare site phylotypes to previous isolates and environmental sequences is shown in Figures 3.27 and 3.29.

3.4.4.1 Bacterial community analysis of aniline-nitrate-amended microcosms.

Phylogenetic analysis of the 16S rRNA gene sequences obtained with primers 338fGC and 519r from aniline-amended microcosms suggested a broad diversity within phyla Beta and Gamma-proteobacteria, Chloroflexi, Nitrospirae and Firmicutes. DGGE band 18 was excised from an aniline-nitrate-amended highly contaminated freshwater canal

microcosm day 939 sample (Figure 3.26). The sequence of band 18 had 99% similarity

(Table 3.12) with *Variovorax ginsengisoli* sp., a denitrifying bacterium isolated from soil

of a ginseng field (Im et al. 2010). DGGE band 24 was excised from an aniline-nitrate-

amended lightly contaminated freshwater canal microcosm day 939 sample (Figure 3.26).

The sequence of band 24 had 100% similarity (Table 3.12) with uncultured *Xylophilus*

sp., a phylotype recovered from a pilot-scale bioremediation process for hydrocarbon-

contaminated soil (Militon et al. 2010).

16S rRNA gene sequence analysis showed that the sequences of DGGE band 18 and 24

were closely related to aniline-degrading bacterium HY99 and *Acidovorax* AJ012071

(Figure 3.27). Aniline-degrading bacterium HY99 is a denitrifying Comamonadaceae and

was identified as most similar to *Delftia acidovorans*. *Delftia acidovorans* can degrade

aniline only under aerobic conditions (originally described by Loidle et al. 1990), but

strain HY99 has been shown to be able to degrade aniline under both aerobic and nitrate-

reducing conditions (Kahng, Kukor, and Oh 2000). *Acidovorax* AJ012071 is notable

because it is the closest relative matched to a phylotype identified by Bio-Trap® samplers

used during in situ groundwater sampling at the site and analyzed using DGGE by

Microbial Insights (IM), Inc. (Knoxville, TN, USA). [Drs. Erin Mack and Eleanor

Jennings, personal communitation.] These two DGGE bands cluster within the family

Comamonadaceae in the Betaproteobacteria comprising the genera of *Acidovorax,*

Variovorax and *Xylophilus*. Bacteria of the genus *Variovorax* are common community

members isolated from soil. For example, *Variovorax ginsengisoli* sp. is a denitrifying

bacterium isolated from soil of a ginseng field (Im et al. 2010). *Acidovorax* was generally

thought to be comprised of aerobes until relatively recently. For example, *Variovorax* is

also closely related to *Acidovorax caeni*, which is a known denitrifying species (Heylen et al., 2008).

DGGE band 17 was excised from an aniline-nitrate-amended highly contaminated freshwater canal microcosm sample. It has 100% similarity with *Ammoniphilus oxalivorans* an aerobic ammonium-dependent obligately oxalotrophic bacterium (Zaitsev et al. 1998). It was reported that *Ammoniphilus oxalivorans* requires a high concentration of ammonium ions and that it can use oxalate as the sole organic source of carbon and energy. No oxalic acid was detected in the aniline amended highly contaminated freshwater canal microcosms or the concentrations were lower than the detection limit (approximately 0.5 mM). Further, these microcosms were reduced (as indicated by the absence of a pink tint indicative of oxidizing redox potential in the resazurin-amended microcosms). Thus, it is not known why this organism would be detected after long-term anoxic incubation. It is possible that the phylotype detected has different physiological characteristics than previously reported.

DGGE band 25, which had 100% similarity with a sulfate reducer *Desulfotomaculum* sp. 175, was observed in two of the triplicate samples from aniline amended lightly contaminated freshwater canal nitrate-amended microcosms at day 969. Figure 4.6 (Chapter 4) shows the depletion of nitrate in these two triplicate samples from aniline-nitrate-amended microcosms. These two nitrate-amended microcosms were re-amended with nitrate on day 969. However, it is possible that the depletion of nitrate allowed the growth of these sulfate-reducers. Some *Desulfotomaculum* may also grow fermentatively when electron acceptors such as sulfate are not present (Aüllo et al. 2013).

3.4.4.2 Bacterial community analysis of aniline-sulfate-amended microcosms.

DGGE band 20, which had 99% similarity with uncultured *Thermodesulfovibrio* sp., was observed in two of the triplicate samples from aniline-sulfate-amended lightly contaminated freshwater canal microcosm day 969. Uncultured *Thermodesulfovibrio* sp. was a sulfate-reducing bacterial species sequenced from a biomat in a rock outcrop (Table 3.12). Figure 3.20 shows aniline loss in all triplicate sulfate-amended sediment microcosms from the lightly contaminated freshwater canal location, from day 775 to 1109. Sulfate concentrations in these microcosms were also present in excess (Figure 3.21). There was one DGGE (19) sequence in one of the triplicate samples from this treatment, which had 99% similarity with *Dehalococcoides mccartyi* 195, a strict anaerobe reductively dechlorinating PCE to ethene (See Section 2.5 or Appendix I). It is known from site data obtained through *in situ* sampling that there are *Dehalococcoides* detected at the site [Drs. Erin Mack and Eleanor Jennings, personal communitation]. It is possible that *Dehalococcoides* had other substrates in these particular sediments from co-contaminants or from naturally occurring organohalides.

One sample from the aniline-sulfate-amended highly contaminated freshwater canal microcosms was analyzed by PCR-DGGE. The sequence of band 14 cut from this treatment had 100% similarity (Table 3.12) with *Thiomonas* sp. Ynys3, which was reported to be able to oxidize either iron or arsenic (Chen et al. 2004). Chen *et al*. also isolated a sulphur-oxidizing *Thiomonas* sp., which could use CO_2 as a sole carbon source and which rapidly oxidized H_2S (Chen et al. 2004).

3.4.4.3 Bacterial community analysis of aniline-methanogenic microcosms.

The DGGE bands for the aniline-amended methanogenic highly contaminated freshwater canal sediment microcosms day 1011 were very light (Lanes I, J, K, Figure 3.26) and no bands could be successfully excised.

3.4.4.4 Bacterial community analysis of PCA-methanogenic microcosms.

Band 6 was the dominant DGGE band from the highly contaminated freshwater canal, PCA-amended methanogenic microcosms on day 932 (Figure 3.28). The sequence of band 6 had 99% similarity (Table 3.14) with a methanogenic enrichment culture clone recovered from Ebro River Delta sediment (van der Zaan et al. 2009). Reductive dechlorination of 1,2-dichloroethane was observed in these microcosms containing sediment sourced from the European rivers Ebro. DGGE bands 7, 8, 12 were the dominant DGGE bands from the PCA-amended lightly contaminated freshwater canal methanogenic microcosms on day 1046 (Figure 3.28). These three bands had high similarity with *Clostridium* sp. (99% similarity to *Clostridium* sp. C5S17 and 100% similarity to *Clostridium crotonatovorans*) (Table 3.14). A phylogenetic tree in Figure 3.29 shows that the *Clostridium* phylotypes detected are intermixed between different genera. Bacteria of *Clostridium* genus are obligate anaerobes and some of them produce highly active cellulolytic complexes (Collins et al. 1994). For example, *Clostridium bifermentans* was isolated from the sludge that contained large quantities of polysaccharides and proteins. It was observed that *C. bifermentans* produces hydrogen, which can be further used by methanogens to produce methane under anaerobic conditions (Wang et al. 2003). Hydrogen also could also then be available to stimulate

dehalogenating bacteria, in systems where halogenated substrates (e.g., PCA) are available.

DGGE band 10 had 100% similarity with uncultured *Pelospora* sp. isolated from a fatty acid-oxidizing consortium (Chauhan and Ogram 2006). Bacteria of the *Pelospora* genus belong to the family Syntrophomonadaceae. It was reported that *Pelospora glutarica* strain WoGl3T, a strictly anaerobic bacterium, required glutarate, methylsuccinate and succinate to grow (Matthies et al. 2000). Thus, the methanogenic mcrocosms amended with lactate and PCA contained fermenting and organic-acid oxidizing bacteria. This indicated that a fatty acid-oxidizing consortium was presented along with dechlorinators under methanogenic conditions.

DGGE band 9 was also excised from PCA-amended lightly contaminated freshwater canal methanogenic microcosms on day 1046. 16S rRNA gene sequence analysis showed that the band 9 phylotype is closely related to an uncultured *Dehalobacter* sp., which was isolated from a 1,2-dichloroethane contaminated aquifer (Marzorati et al. 2010). Based on the dechlorination of PCA and the direct detection of the dechlorinator, *Dehalobactor*, using 16S rRNA gene universal primers, *Dehalobacter*-specific PCR was also performed for these systems. This approach also detected *Dehalobacter* sp. from the same microcosm samples taken on the same day (day 1046) (These results are discussed in depth in Chapter 5, Section 5.4.4). Thus different primer sets (universal bacterial and *Dehalobacter*-specific) confirmed detection of *Dehalobacter* sp. directly from the microcosms, where PCA was dechlorinated to aniline. The direct detection suggests that *Dehalobacter* was present in abundance. In general, a nested PCR approach has been

used to detect dehalogenating bacteria directly from sediments (Fennell et al. 2001) or from microcosms (Krumins et al. 2009).

3.4.4.5 Bacterial community analysis of PCA-nitrate-amended microcosms.

DGGE band 1 was from PCA-nitrate-amended lightly contaminated groundwater aquifer microcosms. This phylotype had 100% similarity with a denitrifying bacterium *Rhodanobacter* sp. 3LS1,which was isolated using lactate as electron donor and carbon source (Green et al. 2010).

3.4.4.6 Bacterial community analysis of PCA-Fe(III)-amended microcosms.

No dominant DGGE bands were observed or excised from the lightly contaminated freshwater canal PCA-Fe(III)-amended microcosms on day 932 (Lanes G, H, I, Figure 3.28).

The presence of distict phylotypes supported the evidence that different bacterial communities were enhanced under different redox conditions. In particular, that a known dechlorinator, *Dehalobacter*, was detected in the methanogenic, PCA-amended canal sediment microcosms where dechlorination of PCA to aniline occurred (see Chapter 5), is a novel finding.

3.5 Discussion

The aim of this study was to assess the potential for biodegradation of aniline and PCA by microbial communities from four locations at a chemical manufacturing site: lightly contaminated groundwater aquifer; highly contaminated groundwater aquifer; lightly contaminated freshwater canal sediments; and highly contaminated freshwater canal sediments under different redox conditions (see results summarized in Tables 3.3 and 3.4). Dozens of microcosms were constructed to support this effort and community analysis via PCR-DGGE was performed to identify bacteria that were enriched in the microcosms.

For the lightly contaminated location of the groundwater aquifer, initial aniline loss was observed under aerobic, nitrate-amended, sulfate-amended, Fe(III)-amended and methanogenic conditions, however, when the aniline concentration was increased to 1500 µM (day 340 to 1200), activity ceased (except for one replicate of the Fe(III)-amended microcosms.

In contrast, for the lightly contaminated location of the groundwater aquifer, PCA loss was observed only under nitrate-amended conditions and, no loss of PCA was detected under sulfate-amended, Fe(III)-amended or methanogenic conditions from microcosms prepared from materials from this location, either when 500 µM or 1500 µM PCA was added.

At the lightly contaminated groundwater aquifer location, loss of the electron acceptors nitrate and sulfate was observed (Figures 3.4 and 3.9). Further, methane production occurred in the methanogenic microcosms (Tables 3.7 and 3.8). These observations support the establishment of different redox conditions in accordance with the microcosm set up protocol.

In comparison, for the highly contaminated groundwater aquifer location, aniline loss was observed under Fe(III)-amended conditions, sulfate-amended conditions (one replicate) and methanogenic conditions initially (day 0 to 340) at 500 µM, however, when the aniline concentration was increased to 1500 µM (day 340 to 1200), activity ceased except for one replicate each of the of the sulfate-amended and methanogenic microcosms. Lack of activity for aerobic or nitrate-amended conditions at the highly contaminated location may indicate that the heavily contaminated aquifer is highly reduced. This could be because higher organic pollutant concentrations have historically resulted in depletion of oxygen and other electron acceptors. Thus, anaerobes are dominant at this location. For the highly contaminated groundwater aquifer location no PCA loss was observed (relative to controls) for any redox condition or concentration tested.

At the highly contaminated groundwater location, nitrate depletion did not occur (Figures B.11 and B.19). Sulfate loss occurred after long incubation in PCA-amended microcosms (Figure B.24). Surprisingly, no substantial methane production occurred either. This suggests an alternate hypothesis that biological activity was generally inhibited in this location (see Tables 3.7 and 3.8). The inhibition could be related to high contaminant

concentrations that apparently could constitute many different specific compounds including more soluble ones such as aniline and PCA, but also dye compounds that are more likely to have bound to the aquifer sediment.

In contrast to the aquifer, the freshwater canal sediments (adjacent to the aquifer) exhibited a far greater range and robustness of activities. For the lightly contaminated freshwater canal site, removals of aniline were detected under all tested redox conditions (nitrate-amended, Fe(III)-amended, sulfate-amended and methanogenic conditions; no aerobic assay was performed) at both 500 µM and 1500 µM aniline levels.

When amended with 500 µM PCA, loss was observed only under nitrate-amended conditions, and no loss occurred under Fe(III)-amended, sulfate-amended or methanogenic conditions. As described extensively in Chapter 5, dechlorination of PCA to aniline in methanogenic microcosms from this location was observed when the PCA concentration was increased to 1500 µM and lactate was added as an electron/hydrogen donor. Lack of activity on PCA at the lower concentration suggested that the proper enzyme might only be induced at higher concentration and that these microbes may not act upon the low dosage of PCA. Such "thresholds" for dechlorination have been reported for other halogenated compounds including Aroclor 1254, Aroclor 1248 and hexachlorobenzene (Rhee et al. 1993; Magar et al. 2005; Yuan et al. 1999).

The accumulation of methane in killed anaerobic controls (Tables 3.9 and 3.10) indicate that autoclaving at 121°C for 30 min on each of three consecutive days had not killed all microorganisms, or that re-innoculation of these controls inadvertently occurred. Even so, no dechlorination of PCA to aniline was detected in killed anaerobic controls from the

lightly contaminated freshwater canal site. Loss of PCA was observed under Fe(III)-amended (triplicate #1) and sulfate–amended (triplicate #2) conditions in the lightly contaminated freshwater canal sediments at 1500 µM. It is thought that this might be linked to onset of methanogenic conditions in these microcosms after prolonged incubation (see Table 3.10). Note that aniline was not detected in the active sulfate-amended triplicate #2, but there was 800 µM aniline detected in the active Fe(III)-amended triplicate #1, suggesting the loss of PCA was by a dechlorination pathway. Nitrate and sulfate loss was observed in microcosms from the lightly contaminated canal sediments (Figures 3.21 and 4.6). Further, methane was produced in all microcosms, but the highest methane production was in sulfate-amended and methanogenic systems (Table 3.9 and 3.10).

In microcosms from the highly contaminated freshwater canal location removals of aniline were detected under all tested redox conditions (nitrate-amended, sulfate--amended and methanogenic conditions) at both 500 µM and for a few replicates at the 1500 µM concentration levels. For PCA, no loss was observed at 500 µM under any conditons, however after an increase in PCA to 1500 µM, dechlorination of PCA to aniline in methanogenic microcosms from this site were observed in two of the live triplicates. No methane production or aniline was detected in killed anaerobic controls from this site, although substantial, slow PCA loss did occur.

By comparing with the reference isolates and phylotypes, the nitrate-aniline community members in lightly and highly contaminated freshwater canal microcosms were closely related to *Acidovorax* AJ012071 (the closest GenBank match to a phylotype detected by

BioTrap® sampling in groundwater at the site [E.-E. Mack and E. Jennings, personal communication] and was also closely related to a denitrifying species *Variovorax ginsengisoli*. The sulfate-aniline community members in lightly contaminated freshwater canal sediment were closely related to sulfate-reducing bacteria *Thermodesulfovibrio* sp. *Dehalobacter* sp. were detected in samples taken from the PCA-dechlorinating microcosms. Additionally, *Dehalobacter* spp. was detected by *Dehalobacter* specific-PCR. These results indicated that a relevant bacterial genus also recently identified in chlorobenzene-dechlorinating enrichments from this same site (Nelson et al. 2011) is present in the sediments enriched on PCA. These organisms are therefore important PCA dechlorination candidates from the site and activity of *Dehalobacter* spp. for PCA dechlorination should be investigated further.

This research shows that aniline and PCA biotransformation capacity was present in different sediments at this large chemical industrial site, and that biodegradation potential with associated microbial communities in sediments varies with the imposed geochemical properties of the sediments.

The physical, chemical, and biological characteristics of the sediment may select certain microorganisms to survive and reproduce rapidly. The microcosm microbial communities were enriched by amending with aniline or PCA in both the aquifer and canal sites. Microbial growth may be inhibited by high concentrations of aniline in the lightly contaminated groundwater aquifer site, as evidenced by lack of aniline loss when the aniline concentration was increased. This indicated that areas of the site with a higher concentration of aniline may be toxic to indigenous organisms and that aniline may

accumulate in the environment, while lower concentrations might be biotransformed and the intermediate product might be degraded via central metabolism pathway. In this study, high dosages of aniline and PCA were removed by indigenous microbes present in canal sediments. These compounds may be biotransformed or metabolized by various types of bacteria under different redox conditions. Bacterial community analysis suggested that the addition of different electron acceptors favored different microbial species under certain redox conditions.

Although it could not be concluded which bacteria or degradation pathways were directly responsible for the observed aniline and PCA transformation, this study provided information about the presence of denitrifying bacteria and sulfate-reducing bacteria in nitrate- or sulfate-amended microcosms, respectively. The results also confirmed the consumption of nitrate or sulfate was associated with aniline or PCA loss. It was also observed that other contaminants may have exerted a significant electron acceptor demand in these samples.   Finally, the fact that little biological activity or contaminant loss was observed in microcosms from the highly contaminated groundwater aquifer location suggests that inhibition or toxicity could play a role in the fate of the compounds.

Table 3. 1. Description of microcosms and length of incubation time (days).

Microcosm Treatment	Groundwater Aquifer		Freshwater Canal	
	Aniline	PCA	Aniline	PCA
Aerobic	120	120	na[a]	na[a]
Aerobic Killed Control	120	120	na[a]	na[a]
Nitrate Amended	1300	1300	1200	1300
Sulfate Amended	1300	1300	1300	1300
Fe(III) Amended	1350	1300	1200	1300
Fe(III) Killed Control	1350	1300	1200	1300
Methanogenic	1300	1300	1200	1300
Anaerobic Killed Control	1300	1300	1300	1300

[a]*na: aerobic tests were not performed for freshwater canal sediments*

Table 3. 2. Stock solutions for aerobic and anaerobic aniline and PCA microcosms.

Stock Solution	Solvent	Stock Concentration	Volume added	Microcosm Concentration	Solubility in water
Aniline 1	DI[a] water	10 mM (stored at 4°C)	1 mL	100 µM	3.38% at 25°C (Lide 2002)
PCA 1	DI water	10 mM (stored at 4°C)	1 mL	100 µM	3.9 g/L at 20°C (Kilzer et al. 1979)
Aniline 2	DI water	100 mM (stored at 4°C)	1 mL	1000 µM	3.38% at 25°C (Lide 2002)
PCA 2	DI water	20 mM (stored at 4°C)	5 mL	1000 µM	3.9 g/L at 20°C (Kilzer et al. 1979)
Resazurin	DI water	80 mg/L	1 mL	800 µg/L	NA[b]
KNO_3	DI water	3.3 M (stored at 27.3°C)	1 mL	33 mM	38.3 g/100mL at 25°C (Lide 2002)
Na_2SO_4	DI water	2 M (stored at 27.3°C)	1 mL	20 mM	28.1 g/100 mL at 25°C (Lide 2002)
L-lactate, sodium salt	DI water	0.5 M (stored at 4°C)	1 mL	5 mM	completely soluble (PURAC)

[a]*DI: deionized water*
[b]*NA: not available*

Table 3. 3. Nitrate-amended enrichment first, second and third transfer set up and activity summary. Transfers were inoculated with 10% (vol/vol) of slurry from active microcosms or from active transfers.

Set[a] (bottle #)	Transfer	Aniline (µM)	Transfer from[a]	NH_4^+ (mM)	NO_3^- (mM)	reducing agents (mM)	Medium Ref.	Day	Activity
1 (#134-1 to #134-3)	1st	500	#134	10	32.6	na[b]	This study	1254[g]	+ + +[f]
2 (#133-1 to #133-3)	1st	500 to 1500	#133	10	32.6	0.9[c]	Travkin et al., 2002	755	− − −
3 (#133-4 to #133-6)	1st	500 to 1500	#133	na[c]	32.6	0.9[e]	This study	755	− − −
4 (#133-7 to #133-9)	1st	500 to 1500	#133	na[c]	32.6	na[b]	Kahng et al., 2000	755	− + −
5 (#135-1 to #135-3)	1st	500	#135	10	32.6	na[b]	Kahng et al., 2000	335	− − −
1 (#134-1A to #134-1F)	2nd	500	#134-1	10	32.6	0.9[e]	This study	76[h]	+ + +
2 (#134-3A to #134-3C)	2nd	500	#134-3	10	32.6	0.9[e]	This study	492	− − −
1 (#134-1A-1 to #134-1A-3)	3rd	500	#134-1A	10	32.6	0.9[e]	This study	233[i]	− − −

[a] *Bottle numbers #133, 134, 135 refer to triplicates #1, 2 and 3 for the nitrate-amended aniline microcosms from the lightly contaminated freshwater canal location (Figures 4.1 and 4.2). #133-1, 133-2, etc. refer to transfer from each original microcosm.*

[b] *Not amended with reducing agents.*

[c] *Amended with 0.45 mM Na_2S and 0.45 mM cysteine.*

[d] *Not amended with ammonia source.*

[e] *Amended with 0.9 mM Na_2S but without cysteine.*

[f] *+++: all triplicates show loss of aniline. −−−: no triplicate showed activity.*

[g] *The enrichments were set up from lightly contaminated freshwater canal microcosm on day 1254.*

[h] *The second transfer enrichments were set up from the enrichment on day 76.*

[i] *The third transfer enrichments were set up from the second transfer enrichments on day 233.*

Table 3. 4. Universal bacterial primers used for PCR-DGGE analysis.

Primer	Sequence (5'$\rightarrow$ 3')	Ref.
Bac338f-GC	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCG C-CCCCGCCCTCCTACGGGAGGCAGCAG	(Nakatsu, Torsvik, and Øvreås 2000)
Bac338f	CTCCTACGGGAGGCAGCAG	Nakatsu, Torsvik, and Øvreås 2000)
Univ519r	ATTACCGCGGCTGCTGG	Nakatsu, Torsvik, and Øvreås 2000)

Table 3. 5. Summary of results for degradation of aniline or PCA from day 0 to day 340, initially amended with approximately 100 µM of aniline or PCA.

Lightly contaminated groundwater aquifer location									
Aniline					*Para*-chloroaniline				
Aero.[a]	Nitrate	Fe(III)	Sulf.	Meth.[c]	Aero.	Nitrate	Fe(III)	Sulf.	Meth.
+ + +[b]	+ + +	+ + +	+ + +	+ −	− − −	+ + +	− − −	− − −	− − −
GroundwaterAquifer Highly Contaminated location									
Aniline					*Para*-chloroaniline				
Aero.	Nitrate	Fe(III)	Sulf.	Meth.	Aero.	Nitrate	Fe(III)	Sulf.	Meth.
− − −	− − −	+ + +	+ − −	+ + +	− − −	− − −	− − −	− − −	− − −

Lightly contaminated freshwater canal location							
Aniline				*Para*-chloroaniline			
Nitrate	Fe(III)	Sulf.	Meth.	Nitrate	Fe(III)	Sulf.	Meth.
+ + +	+ + +	+ + +	+ + +	+ + +	− − −	− − −	− − −
Highly contaminated freshwater canal location							
Aniline				*Para*-chloroaniline			
Nitrate	Fe(III)	Sulf.	Meth.	Nitrate	Fe(III)	Sulf.	Meth.
+ + +	− − −	+ + +	+ + +	− − −	− − −	− − −	− − −

[a]*Aerobic treatments discontinued after 120 days.*

[b]*+ + +, all triplicates show loss of substrate; + + −, two of three triplicates show loss of substrate; + − −, one of three triplicates show loss of substrate; and − − − none of the triplicates show activity.*

[c]*One replicate was not spiked with aniline.*

Table 3. 6. Summary of results for degradation of aniline or PCA from day 340 to day 1200, concentration of aniline or PCA was increased to approximately 1500 µM.

Lightly contaminated groundwater aquifer location									
Aniline					*Para*-chloroaniline				
Aero.	Nitrate	Fe(III)	Sulf.	Meth.	Aero.	Nitrate	Fe(III)	Sulf.	Meth.
NA[a]	– –[b]	+ – –	– – –	– – –	NA	+ + –	– – –	– – –	– – –
Highly contaminated groundwater aquifer location									
Aniline					*Para*-chloroaniline				
Aero.	Nitrate	Fe(III)	Sulf.	Meth.	Aero.	Nitrate	Fe(III)	Sulf.	Meth.
NA	– – –	– – –	+ – –	+ – –	NA	– – –	– – –	– – –	– –[c]
Lightly contaminated freshwater canal location									
Aniline					*Para*-chloroaniline				
Nitrate		Fe(III)	Sulf.	Meth.	Nitrate		Fe(III)	Sulf.	Meth.
+ + +		+ – –	+ + +	+ + +	– – –		+ – –	– + –	+ + +
Highly contaminated freshwater canal location									
Aniline					*Para*-chloroaniline				
Nitrate		Fe(III)	Sulfate	Methanogenic	Nitrate		Fe(III)	Sulfate	Meth.
– + –		– – +	– – +	– + +	– – –		– – –	+ – –	+ + –

[a]*NA: aerobic treatments discontinued after 120 days.*

[b]*One triplicate sacrificed for transfer.*

[c]*One triplicate broken.*

Table 3. 7. Summary of methane production for groundwater aquifer microcosms (aniline). Average methane concentration in μM, standard deviation in μM.

Lightly contaminated groundwater aquifer location					
Nitrate[a]	Fe(III)[b]	Fe(III) control[b]	Sulfate[a]	Anaerobic control[a]	Methanogenic[a]
0.6±0.0[c]	0.5±0.0	0.5±0.1	0.7±0.1	1.1±0.8	201±295
– – –[d]	– – –	– – –	– – –	– –[e]	+ + +
Highly contaminated groundwater aquifer location					
Nitrate[a]	Fe(III)[b]	Fe(III) control[b]	Sulfate[a]	Anaerobic control[a]	Methanogenic[a]
4.6±1.3	1.0±0.2	1.9±0.3	2.5±2.1	2.2±0.3	3.8±2.0
+ + +	– – –	– – –	– – +	– – –	+ + –

[a]*Samples were taken on 04/23/08, day 376 (Calculated from first set up date).*

[b]*Samples were taken on 08/21/08, day 366 (Fe(III)-amended treatment were set up a few weeks later than other treatments for this site).*

[c]*Average methane concentration in μM ± standard deviation in μM.*

[d]*+ + +, all triplicates show methane concentration >3.0 μM; + + –, two of three triplicates show methane concentration >3.0 μM; + ––, one of three triplicates show methane concentration >3.0 μM.*

[e]*One of the triplicates was broken.*

Table 3. 8. Summary of methane production for groundwater aquifer microcosms (PCA).

Average methane concentration in μM, standard deviation in μM.

Lightly contaminated groundwater aquifer location					
Nitrate [a]	Fe(III) [b]	Fe(III) control [b]	Sulfate [a]	Anaerobic control [a]	Methanogenic [a]
0.5±0.0	0.6±0.2	0.6±0.1	0.5±0.0	0.6±0.1	317±258,
− − −	− − −	− − −	− − −	− − −	+ + +
Highly contaminated groundwater aquifer location					
Nitrate [a]	Fe(III) [b]	Fe(III) control [b]	Sulfate [a]	Anaerobic control [a]	Methanogenic [a]
1.8±0.9	3.2±2.8	1.7±1.2	2.0±0.5	1.3±0.5	1.9±0.6
− − −	− − +	− − −	− − −	− − −	− − [c]

[a] *Samples were taken on 08/21/08, day 496 (Calculated from first set up date).*

[b] *Samples were taken on 08/14/08, day 357 (Fe(III)-amended treatment were set up a few weeks later than other treatments for this site).*

[c] *Average methane concentration in μM ± standard deviation in μM.*

[d] *+ + +, all triplicates show methane concentration >3.0 μM; + + −, two of three triplicates show methane concentration >3.0 μM; + −−, one of three triplicates show methane concentration >3.0 μM.*

[c] *One of the triplicates was broken.*

Table 3. 9. Summary of methane production for freshwater canal microcosms (aniline).

Average methane concentration in μM, standard deviation in μM.

Lightly contaminated freshwater canal location [a]					
Nitrate	Fe(III)	Fe(III) control	Sulfate	Anaerobic control	Methanogenic
5.3±7.6, + − −	16.9±14.9, + + −	9.2±0.5, + + +	122±123, + + +	427±725, + + +	572±419, + + +
Highly contaminated freshwater canal Location [a]					
Nitrate	Fe(III)	Fe(III) control	Sulfate	Anaerobic control	Methanogenic
0.8±0.0, − − −	1.4±1.3, − − −	0.6±0.1, − − −	0.8±0.3, − − −	3.8±4.1, + − −	1470±2167, − + +

[a] *Samples were taken on 03/25/08, day 217 (Freshwater canal microcosms were set up later than groundwater sites).*

[c]*Average methane concentration in μM ± standard deviation in μM,*

[d]*+ + +, all triplicates show methane concentration >3.0 μM; + + −, two of three triplicates show methane concentration >3.0 μM; + −−, one of three triplicates show methane concentration >3.0 μM.*

Table 3. 10. Summary of methane production for freshwater canal microcosms (PCA).

Average methane concentration in μM, standard deviation in μM.

Lightly contaminated freshwater canal location [a]					
Nitrate	Fe(III)	Fe(III) control	Sulfate	Anaerobic control	Methanogenic
2.8±3.5, + − −	130±204, + + +	2.9±0.9, + − +	734±1195, + + +	345±546.2, + + +	1098±532, + + +
Highly contaminated freshwater canal Location [a]					
Nitrate	Fe(III)	Fe(III) control	Sulfate	Anaerobic control	Methanogenic
1.8±1.0, − − −	13.4±17.1, + − +	0.6±0.1, − − −	201±343, + − +	0.9±0.4, − − −	111±105, + + +

[a] *Samples were taken on 08/14/08, day 357 (Freshwater canal microcosms were set up later than groundwater sites).*

[c]*Average methane concentration in μM ± standard deviation in μM,*

[d]*+ + +, all triplicates show methane concentration >3.0 μM; + + −, two of three triplicates show methane concentration >3.0 μM; + −−, one of three triplicates show methane concentration >3.0 μM.*

Table 3. 11. Sequences obtained with PCR primers 338fGC and 519r from DGGE of

aniline-amended freshwater canal sediment microcosm samples.

Band name	Length (bp)	PCR products, sequence obtained
14	153	GCCGCCCCCGCCCTCCTACGGGAGGCAGCAGTGGGGAATCTTG GACAATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGGGAA GAAGGCCTTCGGGTTGTAAACCACTTTTGGCGGGGGCGAAATA TCGAATATTAATACCATTCGGTGA
17	147	CCCCCGCCCTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACA ATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGAGTGATGAA GGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACACCCG AGAGAGGATCTCGGACTGA
18	151	CCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACGGGAGGCAG CAGGGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCCA TGCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTG TACGGAACGAAAAGGTCTTTT
19	155	CGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACGG GAGGCAGCAGGAAGGAATCTTGGGCAATGGGCGAAAGCCTGA CCCAGCAACGCCGCGTGAGGGATGAAGGCTTTCGGGTTGTAAA CCTCTTTTCACAGGGAAGAATAATGAC
20	155	GCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTAC GGGAGGCAGCAGTGGGGAATATTGCGCAATAGGGGAAACCCT GACGCAGCGACGCCGCGTGGAGGATGAAGGTCTTCGGGTTGTA AACTCCTTTTCTCAGGGAAAATTATG
24	142	CGTCCCGCCGCCCCCGCCCTCCTACGGGAGGCAGCAGTGGGGA ATTTTGGACAATGGGCGCAAGCCTGATCCAGCCATTCCGCGTG CAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAAC GAAAAGGCTCTCT
25	136	GCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTAC GGGAGGCAGCAGTGGGGAATCTTCCGCAATGGGCGAAAGCCT GACGGAGCAACGCCGCGTGAGTGATGAAGGCCTTCGGGTTGTA AAACTCT

Table 3. 12. Reference sequences, similarity, accession number and isolation source for

matches to sequences obtained with primers 338fGC and 519r from DGGE

of aniline-amended freshwater canal sediment microcosm samples.

DGGE band name	Close relative	Similarity (%)	Accession	Reference	Isolation Source
14	*Thiomonas* sp. Ynys3	100	AF387303.1	(Chen et al. 2004)	NA
17	*Ammoniphilus oxalivorans*	100	NR_026433.1	(Zaitsev et al. 1998)	Aerobic ammonium-dependent obligately oxalotrophic bacteria
18	*Variovorax ginsengisoli*	99	FR682712.1	(Im et al. 2010)	Denitrifying bacterium isolated from soil of a ginseng field
19	*Dehalococcoides mccartyi strain* 195	99	AF004928.2	(Maymo-Gatell et al. 1997)	Dechlorinates tetrachloroethene to ethene
20	uncultured *Thermodesulfovibrio* sp.	99	FJ902311.1	Unpublished	(sulfate reducing bacteria) biomat in a rock outcrop
24	uncultured *Xylophilus* sp.	100	AM936163.1	(Militon et al. 2010)	pilot-scale bioremediation process of a hydrocarbon-contaminated soil
25	*Desulfotomaculum* sp. 175	100	AF295656.1	(Detmers et al. 2001)	Sulfate reduction

Table 3. 13. Sequences obtained for PCR-DGGE with primers 338fGC and 519r from

PCA-amended groundwater aquifer and freshwater canal sediment

microcosm bacterial community analysis.

Band name	Length (bp)	PCR products, sequence obtained
1	162	CCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACGGGAGGCAG CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCAA TGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTT ATCAGGAGCGAAATACCACGGGTTAATACCCTA
6	150	CGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACGGGAG GCAGCAGGGGGGAATCTTCCGCAATGGGCGAAAGCCTGACGG AGCAACGCCGCGTGAGTGAGGAAGGCCTTCGGGTCGTAAAACT CTGTCTTCAGGGAAGAAAAAAA
7	157	GCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACGGG AGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGAT GCAGCAACGCCGCGTGAGTGATGACGGTCTTCGGATTGTAAAA CTCTGTCTTCAGGGACGATAATGACGTAC
8	155	CCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACG GGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTG ATGCAGCAACGCCGCGTGAGTGATGAAGGCCTTCGGGTTGTAA AGCTCTGTCTTTTGGGACGATAATGA
9	128	CCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACGGGAGGCAG CAGGGGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCA ACGCCGCGTGAATGAAGAAGGCCTTCGGGTTGTAAAATTCTGT
10	127	CCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACGGGAGGCAG CAGTGGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCG ACGCCGCGTGAGCGATGAAGGCCTTAGGGTTGTAAAGCTCTG
12	189	GCCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACGGGAGGCA GCAGTGGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGC AACGCCGCGTGAGTGATGAAGGTCTTCGGATTGTAAAACTCTG TC

Table 3. 14. Reference sequence, similarity, accession number and isolation source for matches to sequences obtained with primers 338fGC and 519r from PCR-DGGE of PCA-amended groundwater aquifer and freshwater canal sediment microcosm bacterial community analysis.

DGGE band name	Close relative	Similarity (%)	Accession	Reference	Isolation Source
1	*Rhodanobacter* sp. 3LS1	100	FJ851445.1	(Green et al. 2010)	Denitrifying bacterium; capable of using nitrate as a terminal electron acceptor; isolated using lactate as electron donor and carbon source
6	methanogenic bacterium enrichment culture clone	99	FJ802166.1	(van der Zaan et al. 2009)	Ebro River Delta sediment
7	*Clostridium* sp. C5S17	99	AB539906.1	Submitted	rice paddy field soil
8	uncultured *Clostridium* sp.	100	FR774831.1	Submitted	Soil
9	uncultured *Dehalobacter* sp.	97	FM204994.1	(Marzorati et al. 2010)	1,2-dichloroethane contaminated aquifer
10	uncultured *Pelospora* sp.	100	DQ173910.1	(Chauhan and Ogram 2006)	Wetland
12	*Clostridium crotonatovorans*	100	AY742899.1	Submitted	Anaerobic granular sludge
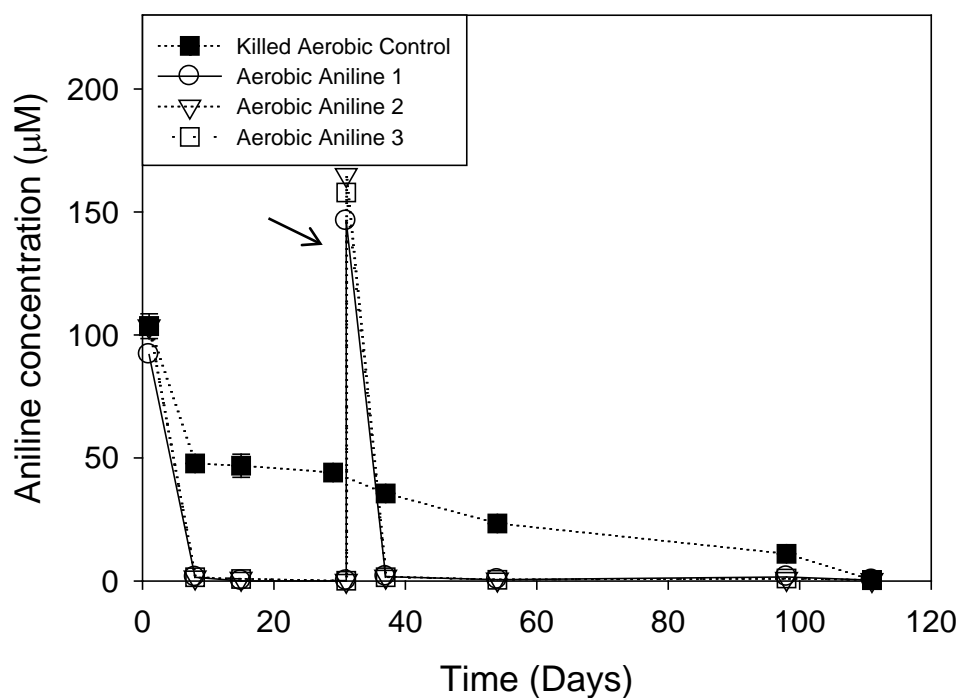
Figure 3. 1. Aniline in aerobic and killed aerobic aquifer microcosms from the lightly

contaminated groundwater aquifer location, day 0 to 120. For the killed

control, symbols are averages of triplicates and error bars represent one

standard deviation. The arrow indicates the re-amendment of substrate.
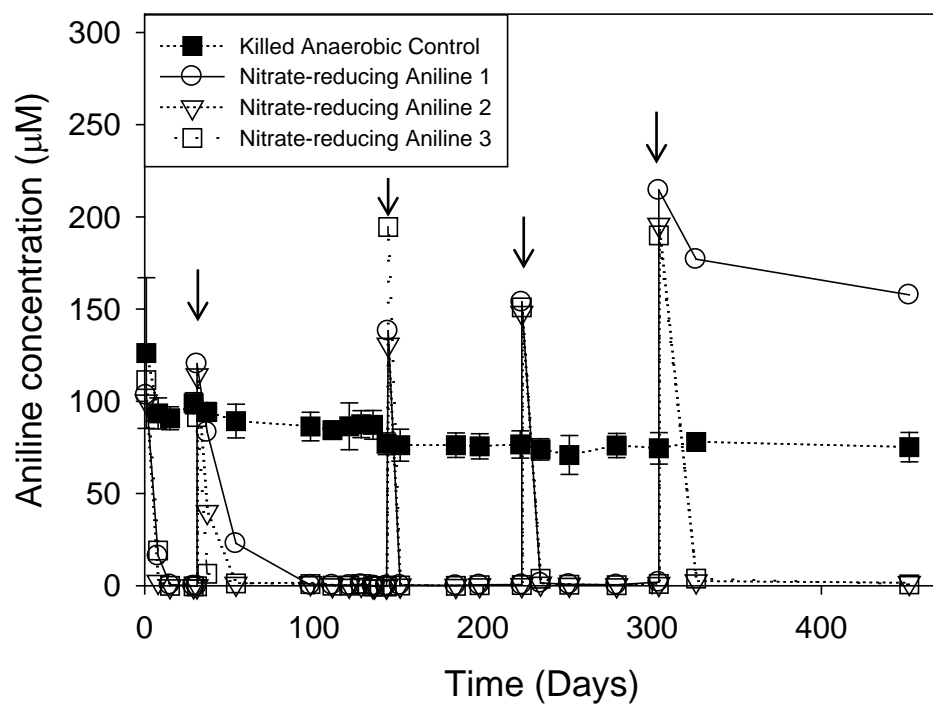
Figure 3. 2. Aniline in nitrate-amended and killed anaerobic aquifer microcosms from

the lightly contaminated groundwater aquifer location, day 0 to 450. For the

killed control, symbols are averages of triplicates and error bars represent one

standard deviation. The arrows indicate the re-amendment of substrate.
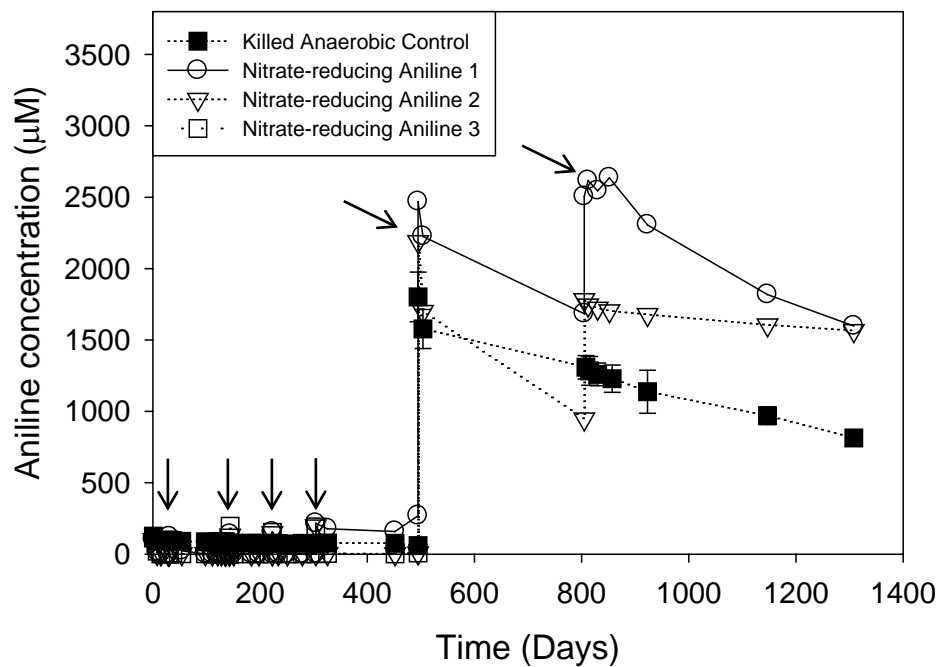
Figure 3. 3. Aniline in nitrate-amended and killed anaerobic aquifer microcosms from

the lightly contaminated groundwater aquifer location, day 0 to 1350. For the

killed control, symbols are averages of triplicates and error bars represent one

standard deviation. The arrows indicate the re-amendment of substrate.
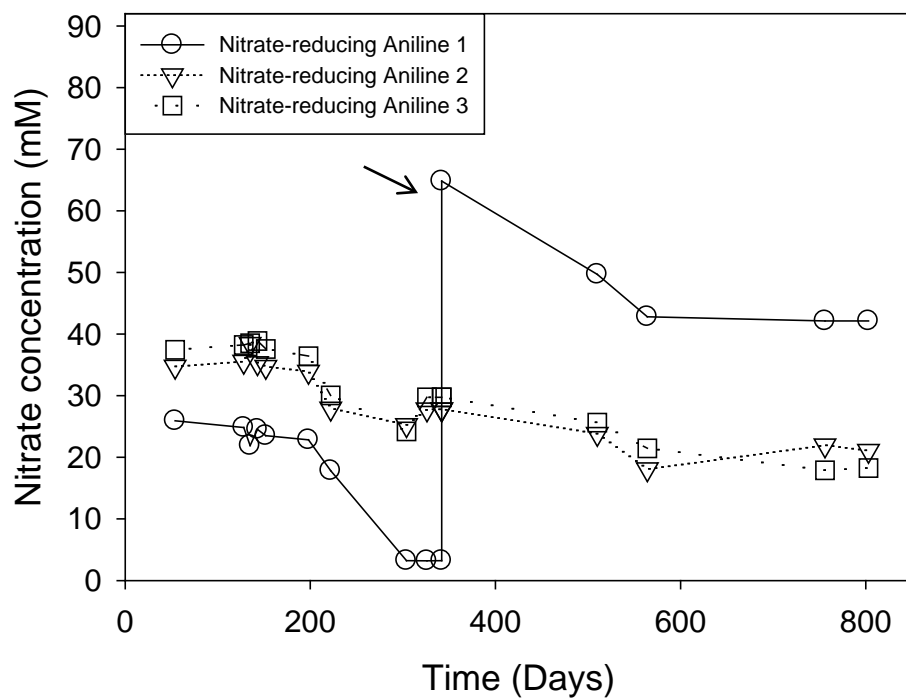
Figure 3. 4. Nitrate in nitrate-amended microcosms from the lightly contaminated groundwater aquifer location, day 0 to 800. The arrow indicates the re-amendment of nitrate.
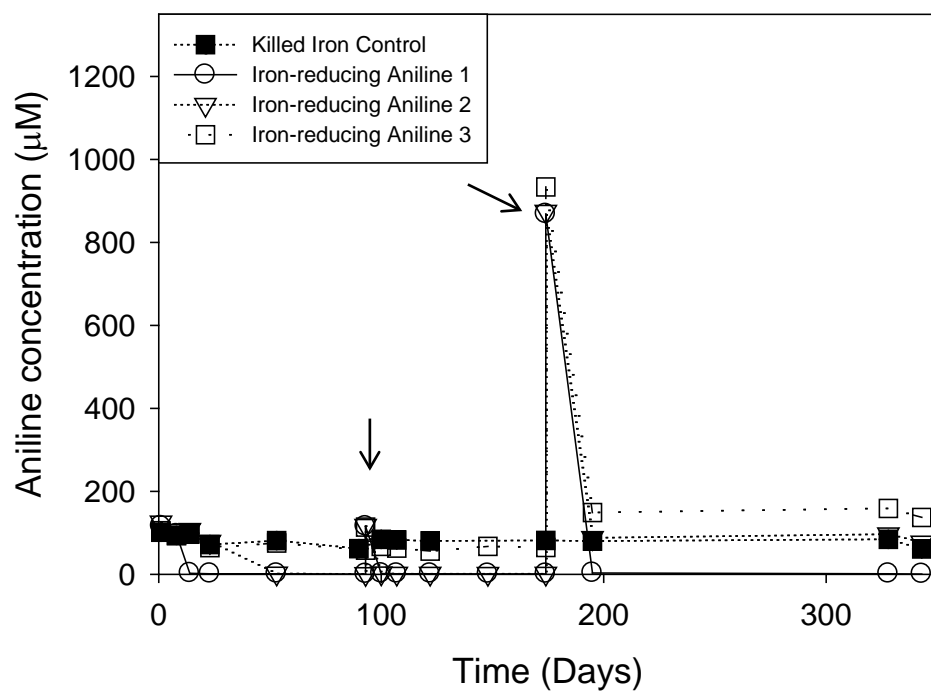
Figure 3. 5. Aniline in Fe(III)- amended and killed Fe(III)- amended microcosms from

the lightly contaminated groundwater aquifer location, day 0 to 350. For the

killed control, symbols are averages of triplicates and error bars represent one

standard deviation. The arrow indicates the re-amendment of substrate.
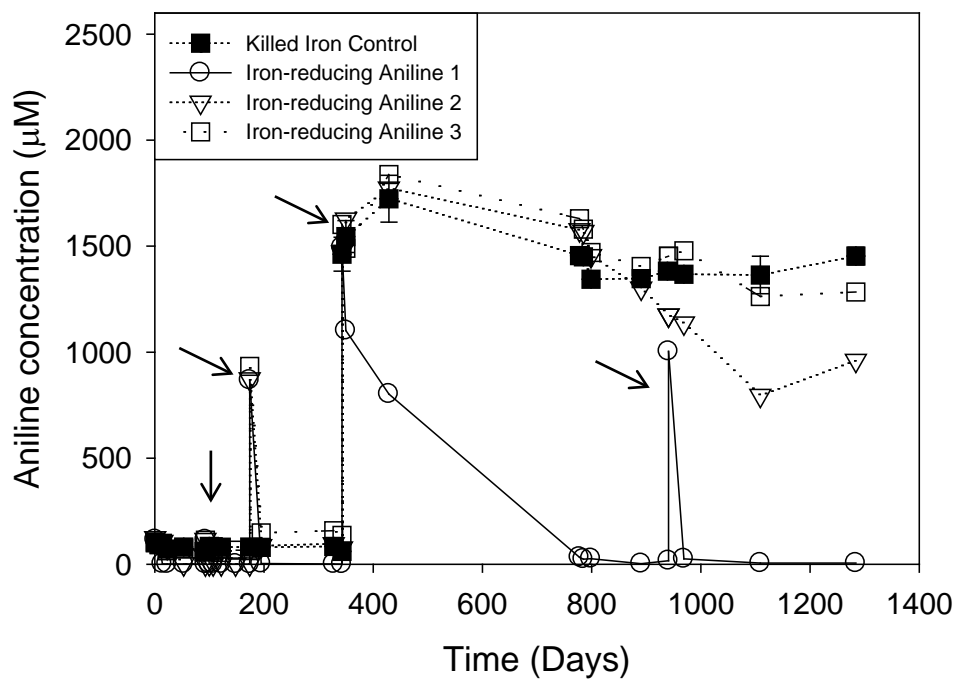
Figure 3. 6. Aniline in Fe(III)- amended and killed Fe(III) -amended microcosms from

the lightly contaminated groundwater aquifer location, day 0 to 1350. For the

killed control, symbols are averages of triplicates and error bars represent one

standard deviation. The arrows indicate the re-amendment of substrate.
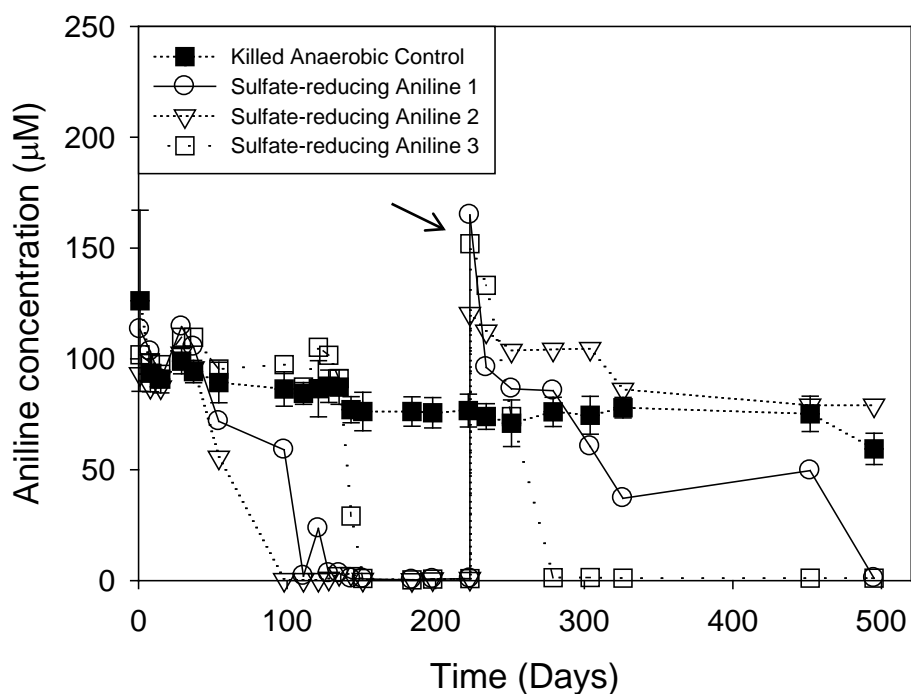
Figure 3. 7. Aniline in sulfate-amended and killed anaerobic aquifer microcosms from

the lightly contaminated groundwater aquifer location, day 0 to 500. For the

killed control, symbols are averages of triplicates and error bars represent one

standard deviation. The arrow indicates the re-amendment of substrate.
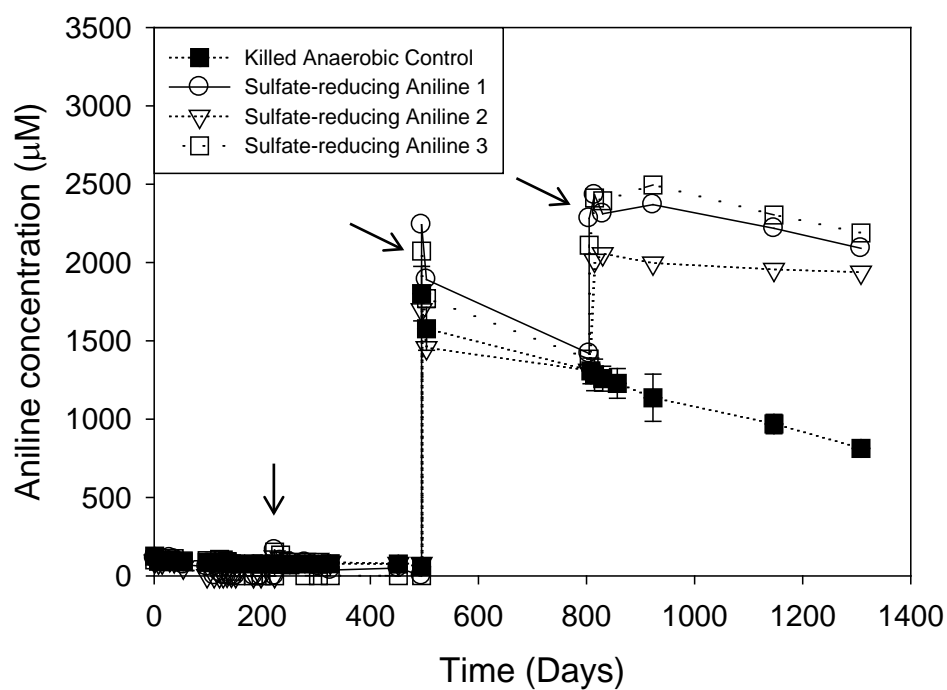
Figure 3. 8. Aniline in sulfate-amended and killed anaerobic aquifer microcosms from

the lightly contaminated groundwater aquifer location, day 0 to 1350. For the

killed control, symbols are averages of triplicates and error bars represent one

standard deviation. The arrows indicate the re-amendment of substrate.
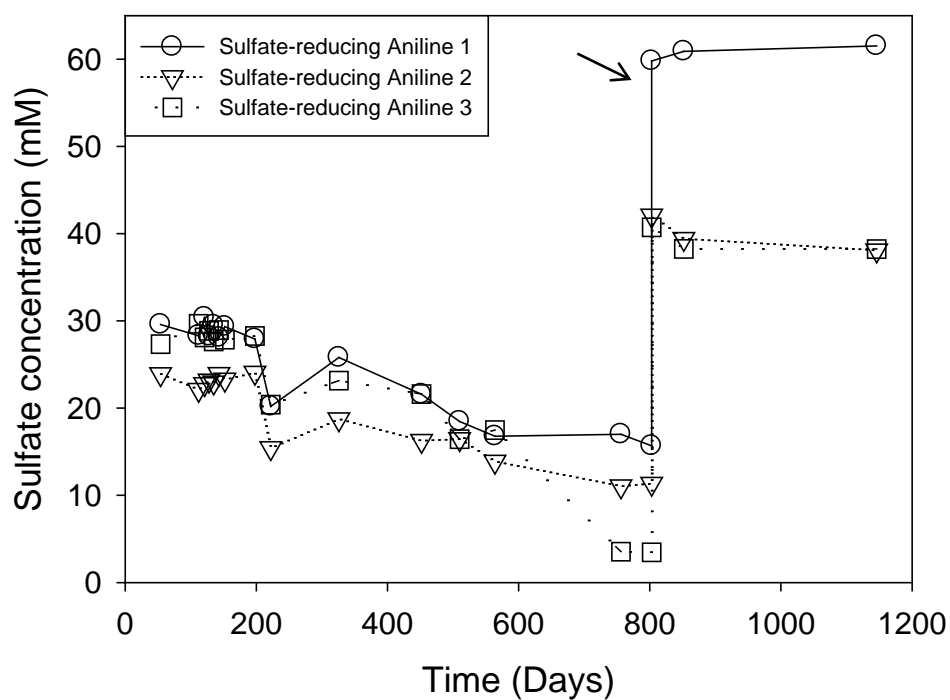
Figure 3. 9. Sulfate in sulfate-amended microcosms from the lightly contaminated

groundwater aquifer location, day 0 to 1146. The arrow indicates the re-

amendment of sulfate.
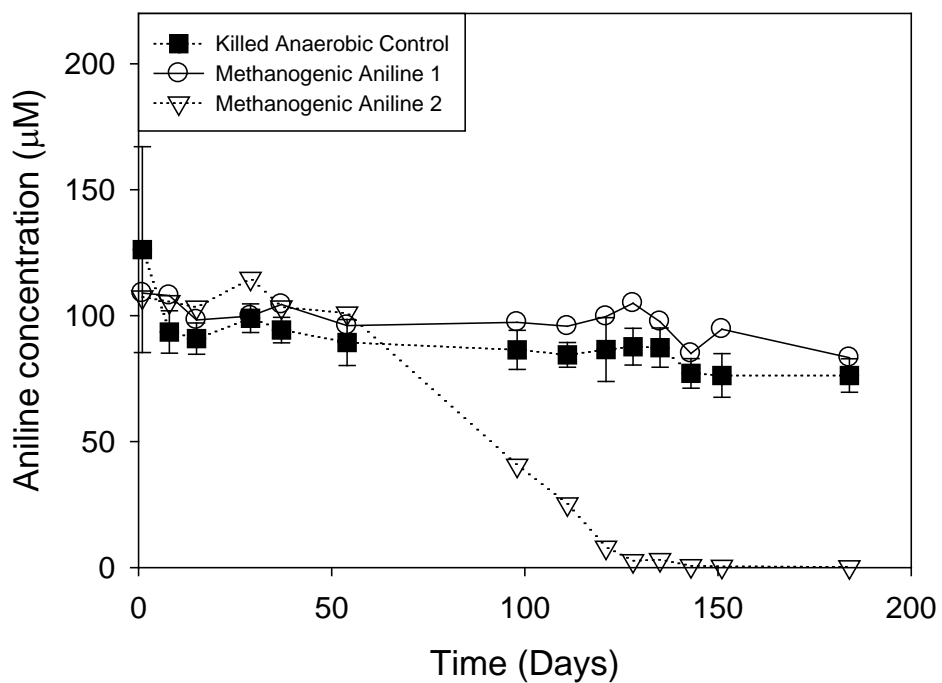
Figure 3. 10. Aniline in methanogenic and killed anaerobic aquifer microcosms from the

lightly contaminated groundwater aquifer location, day 0 to 200. For the

killed control, symbols are averages of triplicates and error bars represent one

standard deviation.
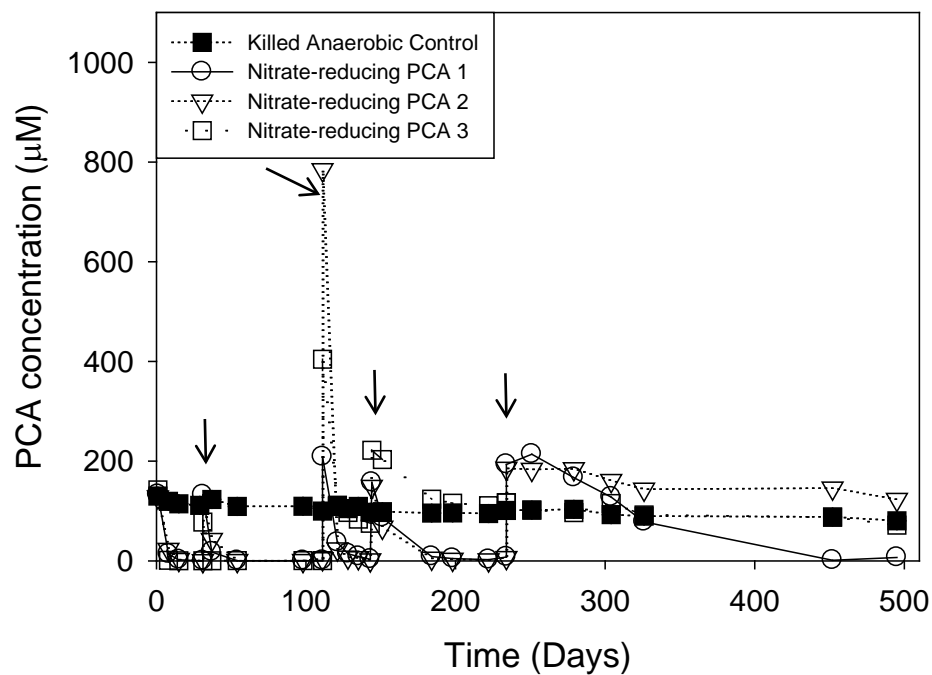
Figure 3. 11. PCA in nitrate-amended and killed aquifer microcosms from the lightly

contaminated groundwater aquifer location, day 0 to 300. Symbols are

averages of triplicates and error bars represent one standard deviation. The

arrows indicate the re-amendment of substrate.
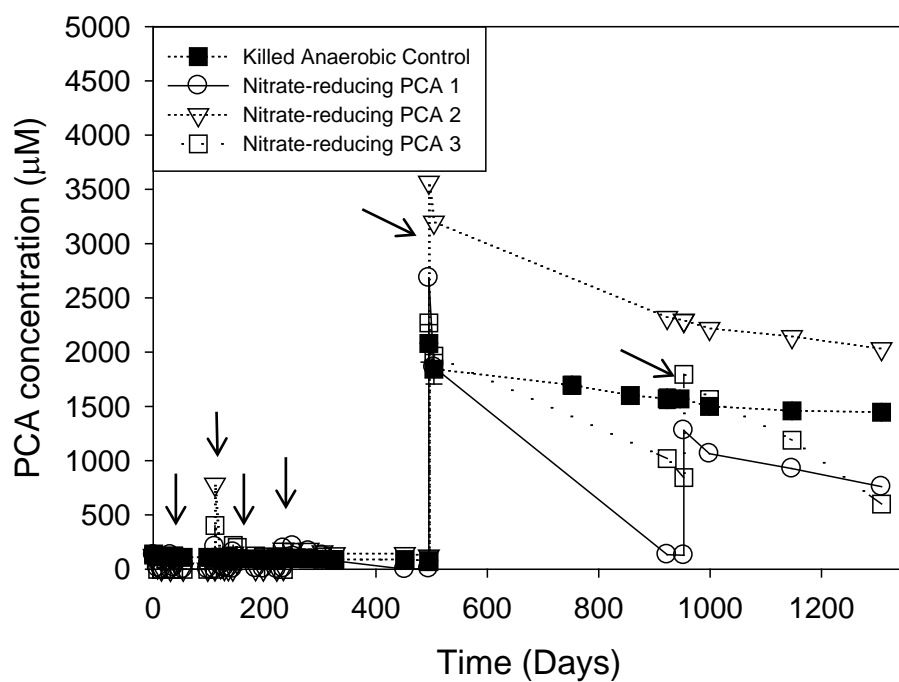
Figure 3. 12. PCA in nitrate-amended and killed aquifer microcosms from the lightly contaminated groundwater aquifer location, day 0 to 1300. Symbols are averages of triplicates and error bars represent one standard deviation. The arrows indicate the re-amendment of substrate.
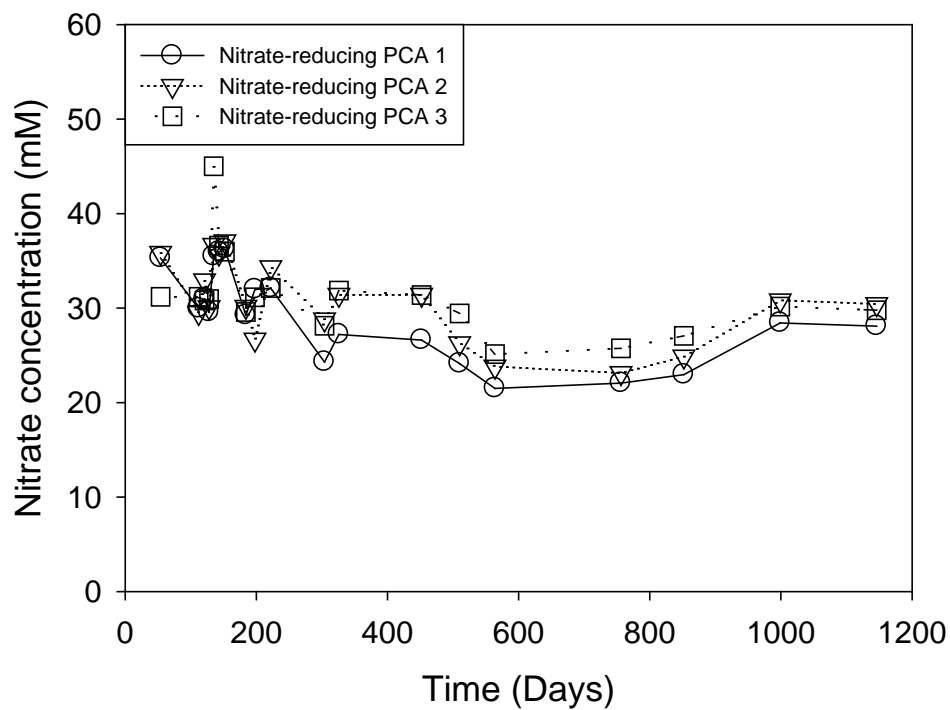
Figure 3. 13. Nitrate concentration in PCA-spiked, nitrate-amended aquifer microcosms from the lightly contaminated groundwater aquifer location, day 0 to 1146.
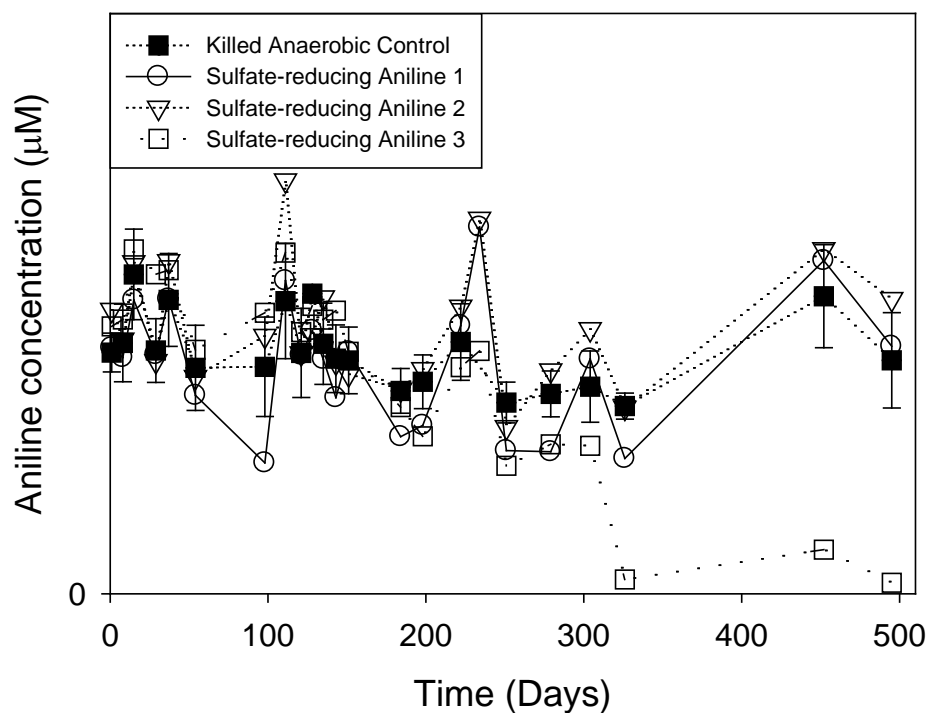
Figure 3. 14. Aniline in sulfate-amended and killed anaerobic aquifer microcosms from

the highly contaminated groundwater aquifer location, day 0 to 500. Symbols

are averages of triplicates and error bars represent one standard deviation.
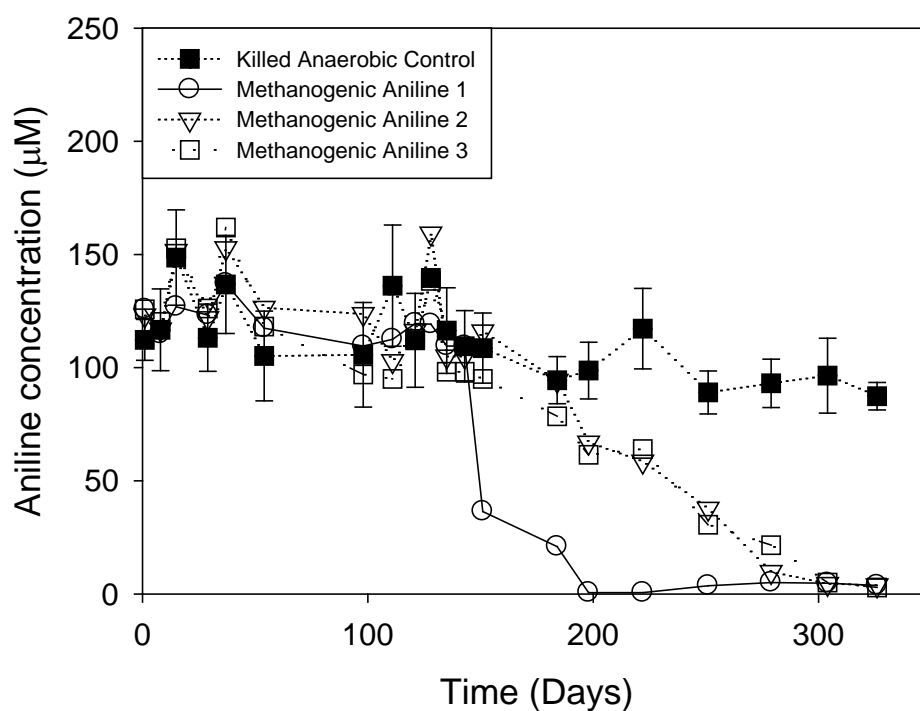
Figure 3. 15. Aniline in methanogenic and killed anaerobic aquifer microcosms from the

highly contaminated groundwater aquifer location, day 0 to 400. For the

killed controls, symbols are averages of triplicates and error bars represent

one standard deviation.
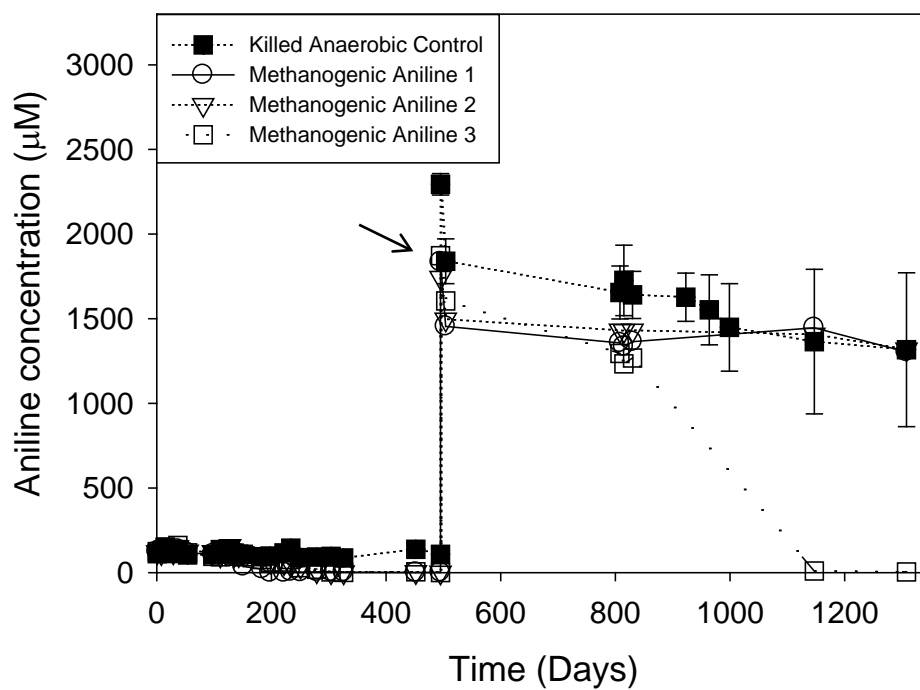
Figure 3. 16. Aniline in methanogenic and killed anaerobic aquifer microcosms from the

highly contaminated groundwater aquifer location, day 0 to 1400. For the

killed controls, symbols are averages of triplicates and error bars represent

one standard deviation. The arrow indicates the re-amendment of substrate.
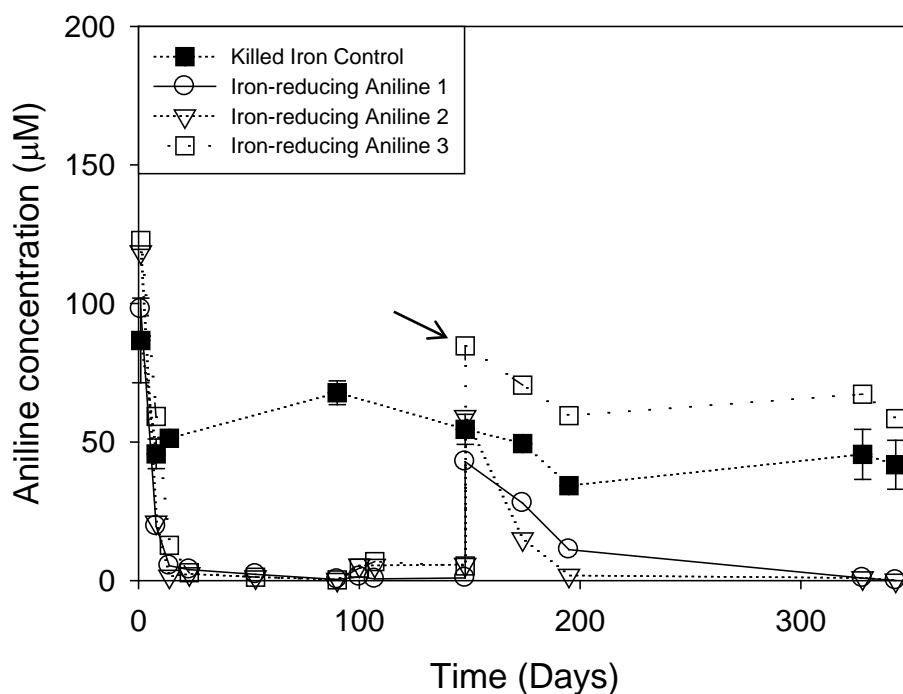
Figure 3. 17. Aniline in Fe(III)-amended and killed Fe(III)-amended sediment

microcosms from the the lightly contaminated freshwater canal location, day

0 to 350. For the killed control symbols are averages of triplicates and error

bars represent one standard deviation. The arrow indicates the re-amendment

of substrate.
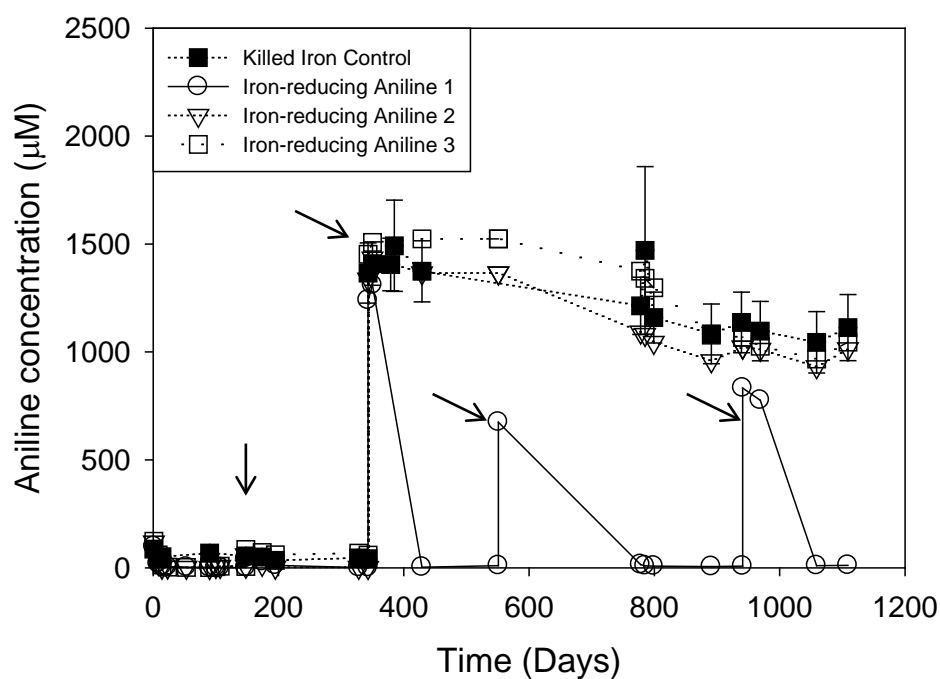
Figure 3. 18. Aniline in Fe(III)-amended and killed Fe(III)-amended sediment

microcosms from the lightly contaminated freshwater canal location, day 0 to

1200. For the killed control symbols are averages of triplicates and error bars

represent one standard deviation. The arrows indicate the re-amendment of

substrate.
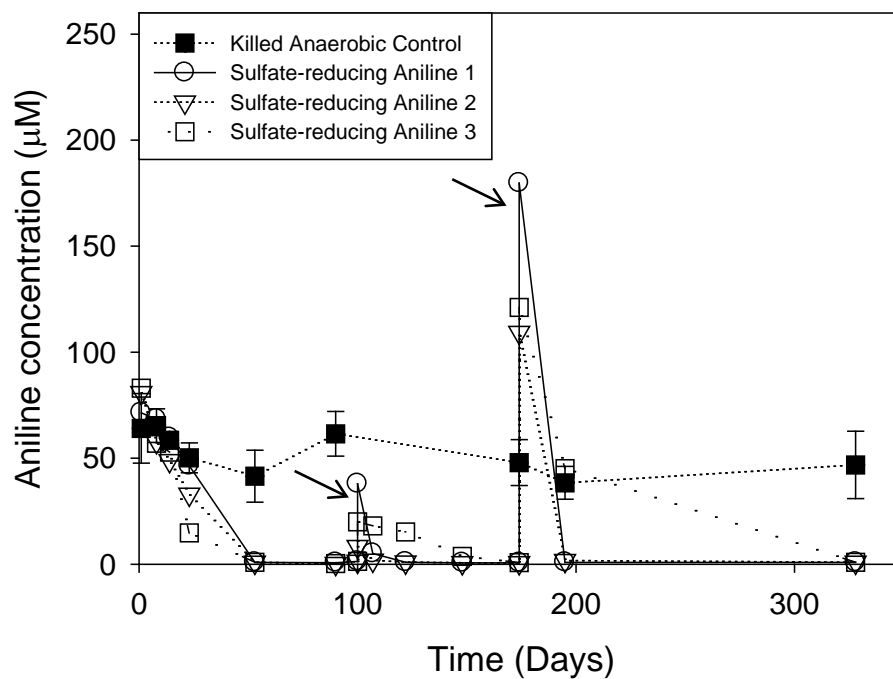
Figure 3. 19. Aniline in sulfate-amended and killed anaerobic sediment microcosms from

the lightly contaminated freshwater canal location, day 0 to 350. Symbols are

averages of triplicates and error bars represent one standard deviation. For

the killed control symbols are averages of triplicates and error bars represent

one standard deviation. The arrows indicate the re-amendment of substrate.
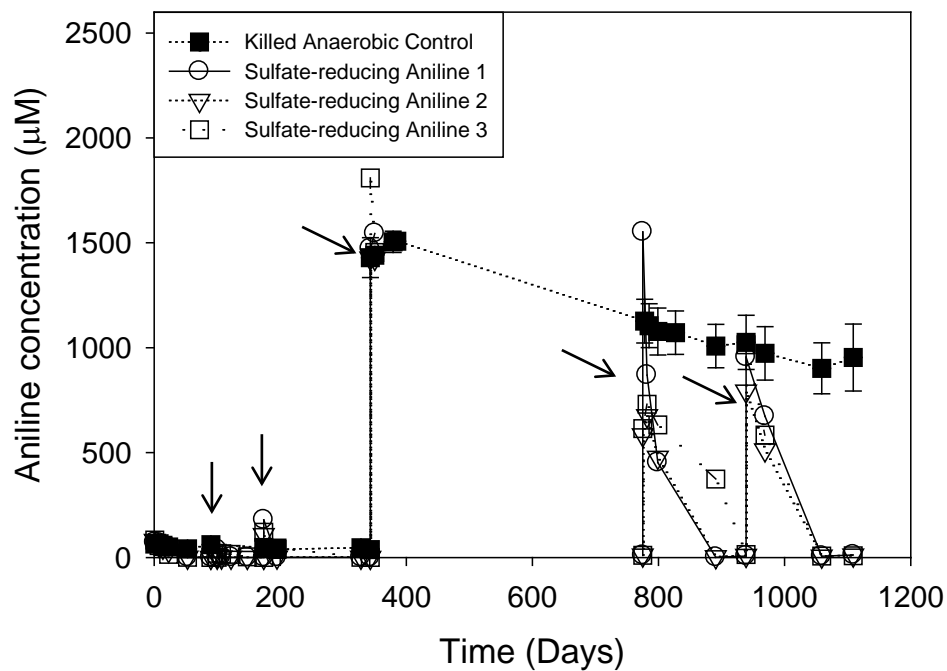
Figure 3. 20. Aniline in sulfate-amended and killed anaerobic sediment microcosms from

the lightly contaminated freshwater canal location, day 0 to 1200. Symbols

are averages of triplicates and error bars represent one standard deviation.

The arrows indicate the re-amendment of substrate.
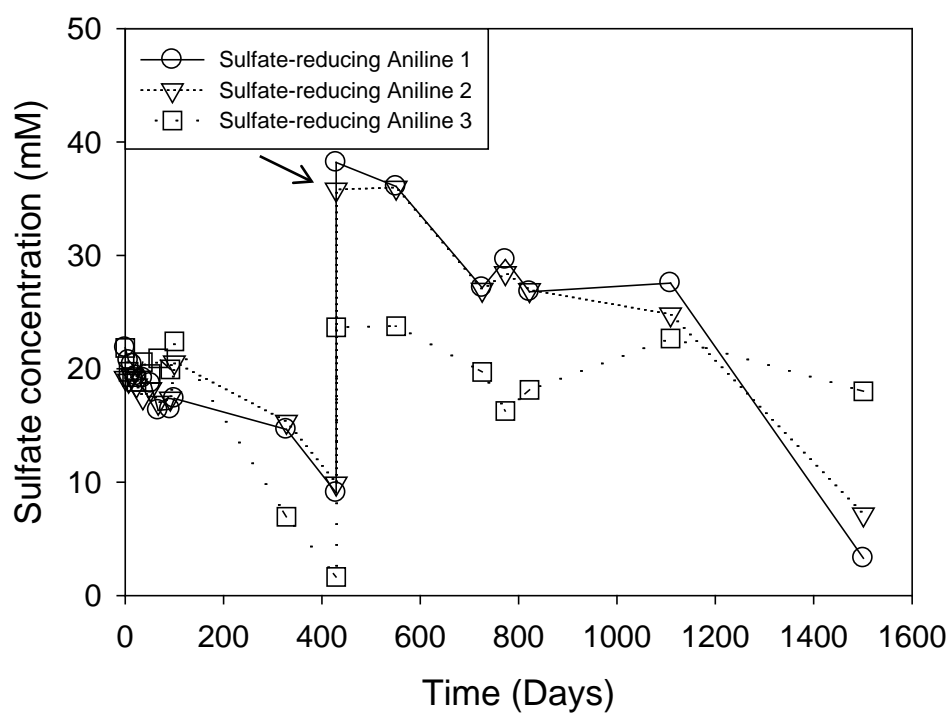
Figure 3. 21. Sulfate concentration in aniline-sulfate-amended sediment microcosms

from the lightly contaminated freshwater canal location. The arrow indicates

the re-amendment of sulfate.
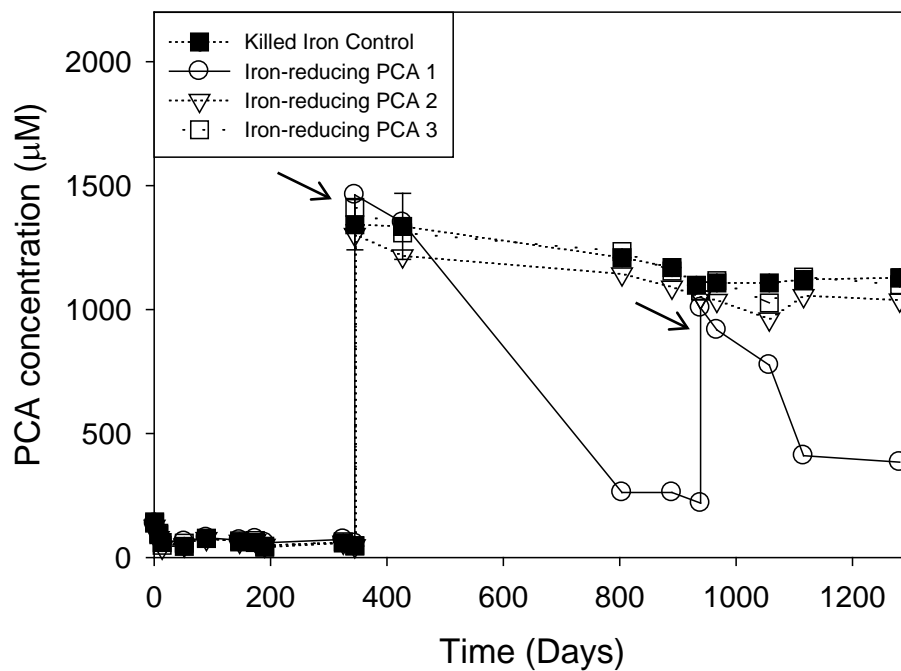
Figure 3. 22. PCA in Fe(III)-amended and killed Fe(III)-amended sediment microcosms

from the lightly contaminated freshwater canal location, day 0 to 1300.

Symbols are averages of triplicates and error bars represent one standard

deviation. The arrow indicates the re-amendment of substrate.
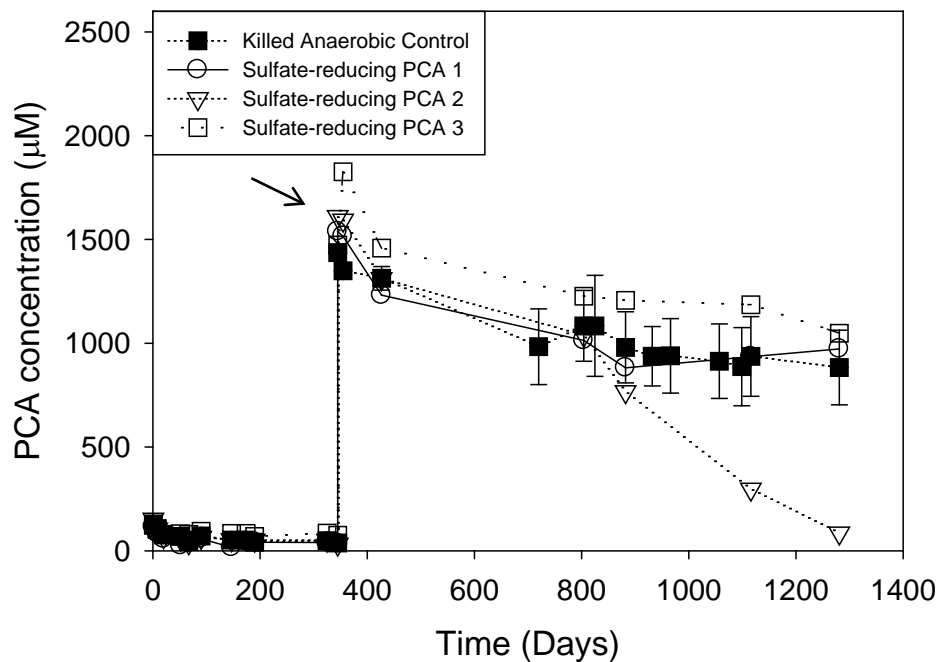
Figure 3. 23. PCA in sulfate-amended and killed anaerobic sediment microcosms from

the lightly contaminated freshwater canal location, day 0 to 1300. For the

killed control symbols are averages of triplicates and error bars represent one

standard deviation. The arrow indicates the re-amendment of substrate.
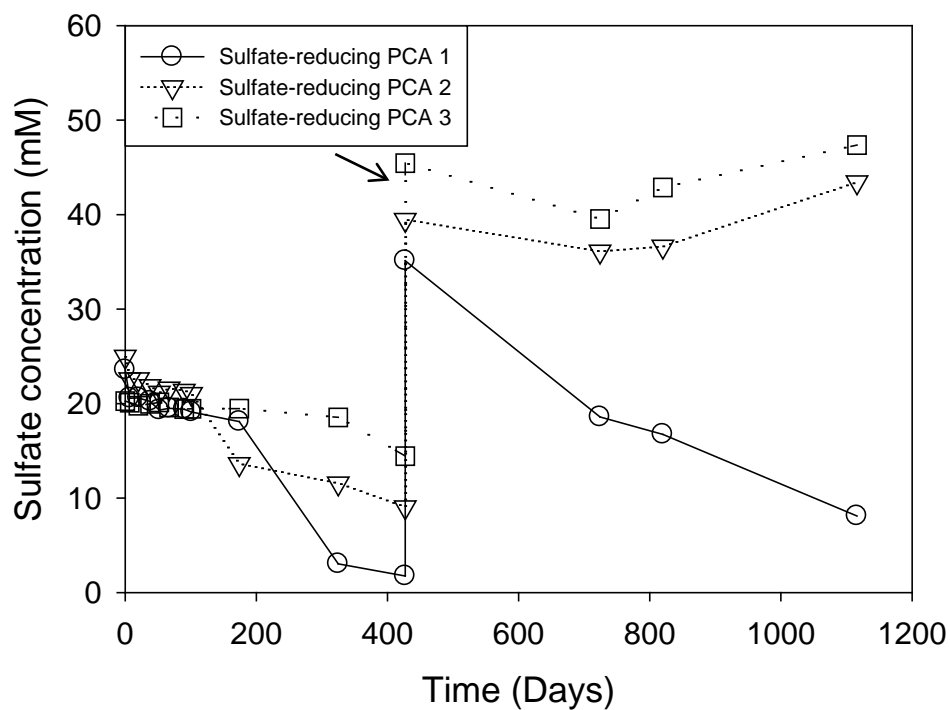
Figure 3. 24. Sulfate concentration in PCA spiked, sulfate-amended sediment

microcosms from the lightly contaminated freshwater canal location. The

arrow indicates the re-amendment of sulfate.
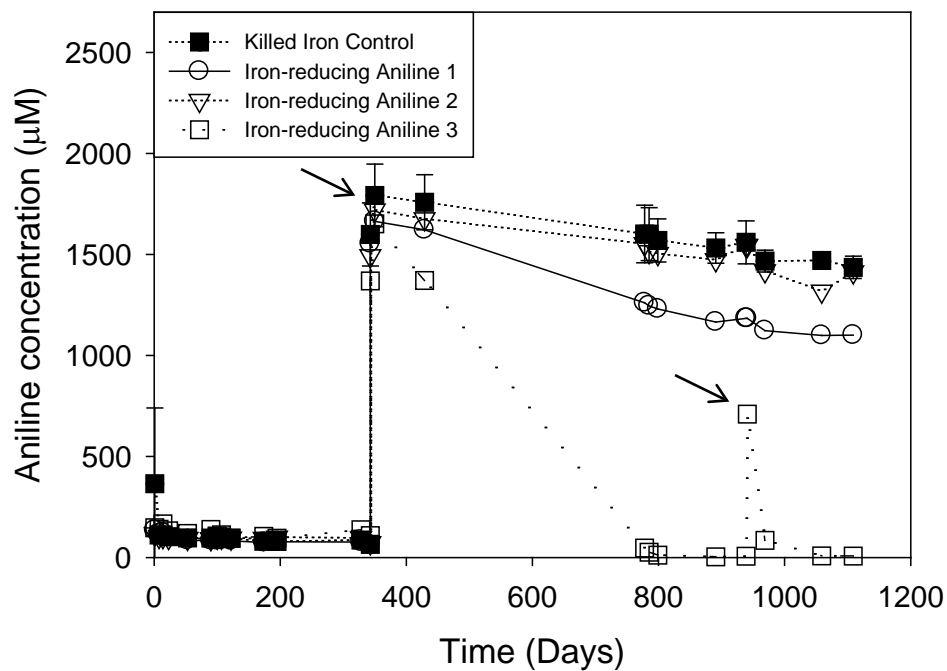
Figure 3. 25. Aniline in Fe(III)-amended and killed Fe(III)-amended sediment

microcosms from the highly contaminated freshwater canal location, day 0 to

1100. Symbols are averages of triplicates and error bars represent one

standard deviation. The arrow indicates the re-amendment of substrate.
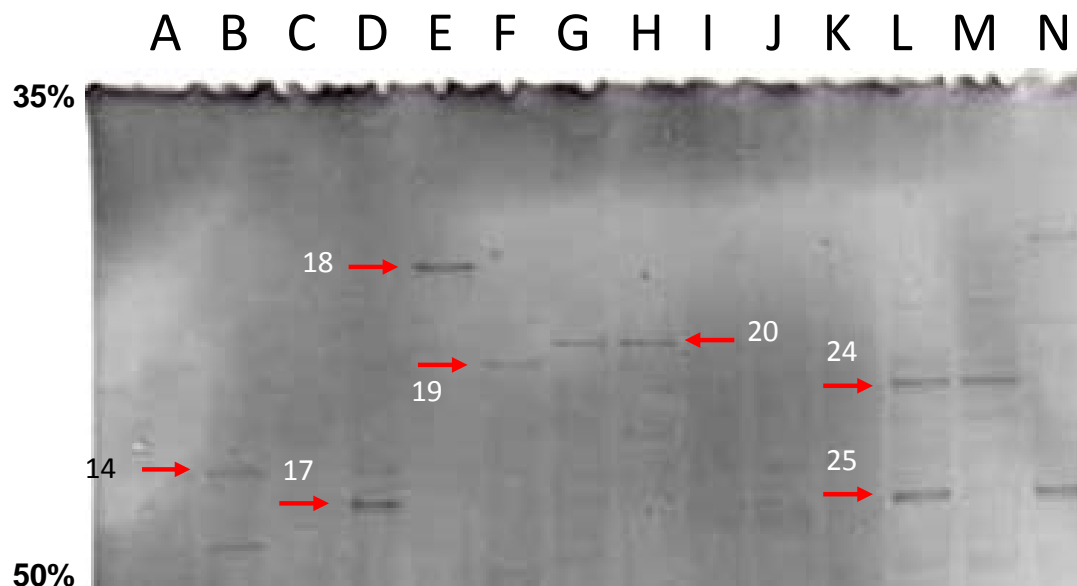
Figure 3. 26. DGGE of aniline-amended freshwater canal sediment microcosm samples.

DGGE analysis is of PCR products for conserved 16S rRNA gene. Blank was labeled as A; other samples were as follows: B – Highly contaminated freshwater canal sediment (sulfate–amended) day 939; C, D, E – Highly contaminated freshwater canal sediment (nitrate-amended) day 939; F, G, H – Lightly contaminated freshwater canal sediment (sulfate–amended) day 939; I, J, K – Highly contaminated freshwater canal sediment (methanogenic) day 1011; L, M, N– Lightly contaminated freshwater canal sediment (nitrate-amended) day 969.
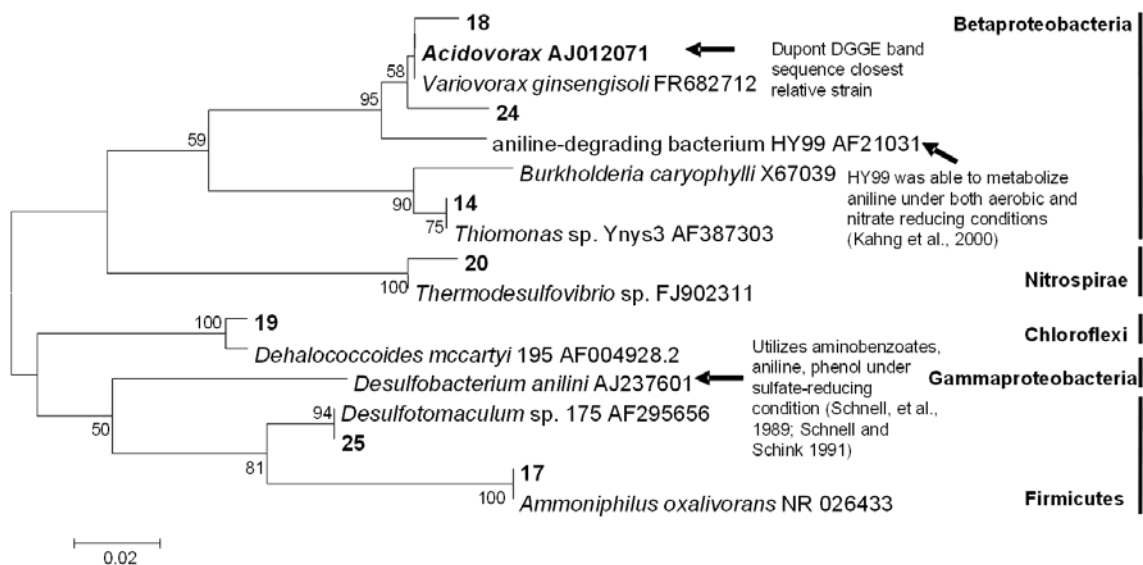
Figure 3. 27. Phylogenetic relationships of 16S rRNA gene sequences obtained with

primers 338fGC and 519r from PCR-DGGE of aniline-amended freshwater

canal sediment microcosm samples (Figure 3.26).
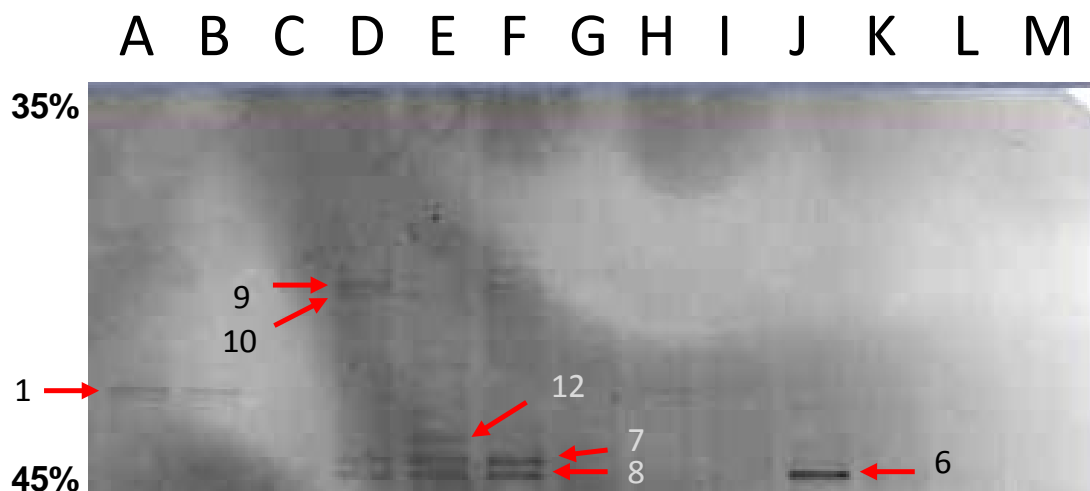
Figure 3. 28. DGGE of PCA-amended sediment microcosm samples. DGGE analysis is

of PCR products for conserved 16S rRNA gene. Blank was labeled as M;

other samples were as follows: A, B, C – Lightly contaminated groundwater

aquifer sediment (nitrate-amended) day 946; D, E, F – Lightly contaminated

freshwater canal sediment (methanogenic) day 1046; G, H, I – Lightly

contaminated freshwater canal sediment (Fe(III)-amended) day 932; J, K, L –

Highly contaminated freshwater canal sediment (methanogenic) day 932.
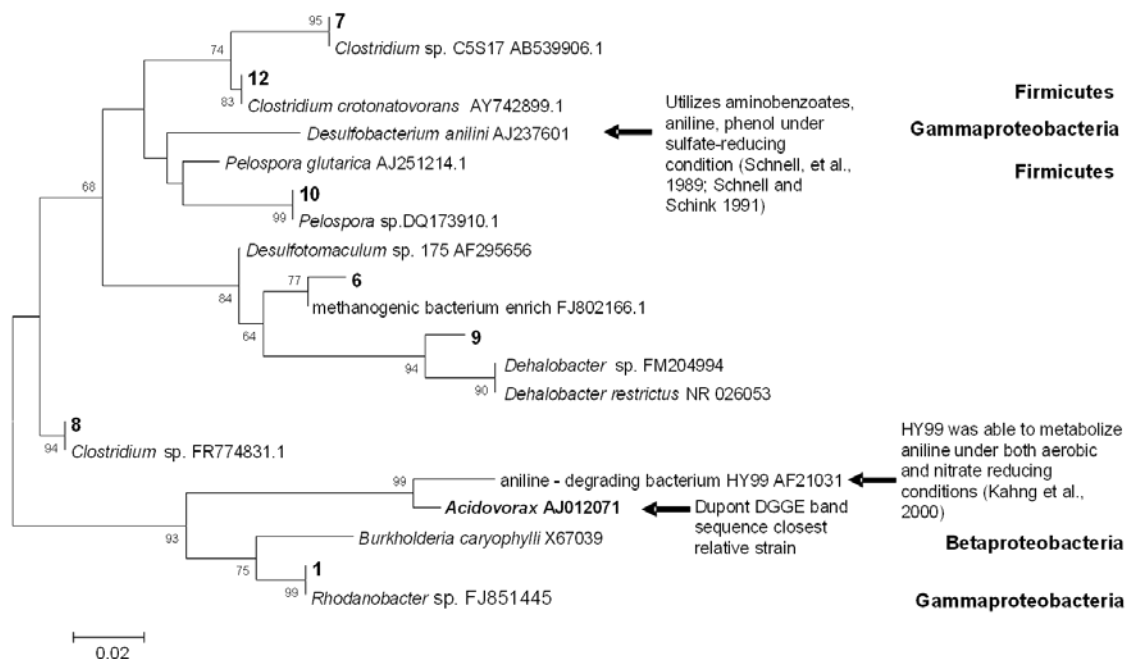
Figure 3. 29. Phylogenetic relationships between 16S rRNA gene sequences of

phylotypes obtained with primers 338fGC and 519r from PCR-DGGE of

PCA-amended sediment microcosms (Figure 3.28)

Chapter 4   Biotransformation of Aniline under Nitrate-Amended and Methanogenic Conditions in the Sediments of an Industrial Site

4.1 Abstract

Aniline is a significant environmental contaminant associated with chemical production facilities. Biotransformation of aniline was investigated in microcosms prepared from sediments obtained from a lightly contaminated location of a freshwater canal that borders a large chemical manufacturing site. The sediments were contaminated with aniline in addition to other contaminants such as *para*-chloroaniline, benzene, dichlorobenzene and PAHs.

Loss of aniline under nitrate-amended and methanogenic conditions was observed in microcosms. Further, nitrate loss was observed in nitrate-amended microcosms and substantial methane production was observed in methanogenic microcosms, indicating that the expected redox conditions were established. Nitrate-amended enrichment cultures were initiated by inoculating fresh medium with 10% (vol/vol) active sediment

microcosms. Only some of the enrichments that were established were active. From active first enrichments, a second enrichment also showed activity, but the rate of aniline loss slowed as additional aniline was added.

Partial mineralization of aniline was confirmed by release of $^{14}CO_2$ from ^{14}C-labled aniline added to sediment slurry microcosms and to first transfer enrichments established under nitrate-amended conditions. Microcosms amended with 50 μM aniline and 0.03 μM ^{14}C-aniline produced $^{14}CO_2$ in short-term incubations of up to 22 d and 5.7% of the label was recovered as $^{14}CO_2$. The recovery of $^{14}CO_2$ from an enriched culture from freshwater canal sediment amended with 1300 μM aniline and 0.03 μM ^{14}C-aniline was 4.8% 69 days after amendment with ^{14}C-aniline. These results demonstrate that only a small portion of ^{14}C-aniline was apparently mineralized in these systems. Despite the incomplete mineralization, no expected anaerobic degradation metabolites including amino-benzoate, benzoate or phenol were detected under either condition in the microcosms. The loss of aniline accounted for only some of the loss of nitrate that occurred according to stoichiometric balances. This indicated that additional xenobiotic or natural organic matter was degraded. However, the loss of aniline in methanogenic microcosms produced only a portion of the theoretically expected methane, suggesting that aniline disappearance did not result in complete degradation to methane and carbon dioxide.

Bacterial community analysis of original sediment, microcosms and enrichments using polymerase chain reaction coupled to denaturing gradient gel electrophoresis (PCR-DGGE) detected Proteobacteria and Firmicutes as the dominant phyla. Phylogenetic

analyses revealed that the dominant phylotypes fell into the same cluster with *Variovorax* sp., closely related to *Acidovorax caeni*, which is a known denitrifying species. The most significant finding from the community analysis is the dominance of a phylotype closely related to *Magnetospirillum* in the aniline-nitrate enrichments. *Magnetospirillum* were reported to reduce nitrate and contain functional genes for degradation of aromatic hydrocarbons.

4.2 Introduction

Aniline has been used on a large scale for many decades as a precursor for industrial synthesis of dyes, pesticides, pharmaceuticals and other compounds (Travis 1997). Aniline contamination was shown to result from improper disposal to the environment (Travis 1997), or when nitrobenzenes are reduced to aniline derivatives under anoxic conditions (Spain 1995). Aniline has carcinogenic and genotoxic properties (Bomhard and Herbold 2005) which make it a high priority pollutant.

Bacterial biotransformation of aniline occurs under both aerobic and anoxic conditions. Aniline can be rapidly metabolized via the catechol pathway under aerobic conditions by a variety of bacterial species (Bachofer et al., 1975; Zeyer and Kearney 1982; Latorre et al., 1984; Spain 1995; Liu et al. 2002).

Degradation of aniline also occurs under anoxic conditions. *Desulfobacterium anilini* Ani 1, originally isolated from marine sediment from the North Sea, utilizes aniline under sulfate-reducing conditions. Degradation of aniline proceeds via 4-aminobenzoate then through CoA-thioesterification and reductive deamination of 4-aminobenzoyl-CoA to benzoyl-CoA (Schnell et al., 1989; Schnell and Schink 1991). Strain HY99 (Kahng et al., 2000), isolated from Korean orchard soils, was reported to degrade aniline under both aerobic and nitrate-reducing conditions. Evidence of degradation under nitrate-reducing conditions was by detection of 4-aminobenzoate by gas chromatography-mass spectrometry in the culture fluid (Kahng et al., 2000) and by detection of nitrite as a denitrification product. HY99 is closely related to *Delftia acidovorans* an aerobic aniline degrader (originally described by Loidle et al. 1990).

Only these two reports exist of specifically identified anaerobic aniline degraders. However, there are several reports of biologically-linked aniline degradation in sediments and sludges by unidentified microbes. Aniline loss was observed under nitrate-reducing conditions in sediment from Newtown Creek, a estuarine channel located in New York, NY, USA, and anaerobic digester sludge from the Berkeley Heights wastewater treatment plant in Berkeley, NJ, USA. No aniline loss was observed under methanogenic conditions after 217 days in Newtown Creek sediments (De et al., 1994). Aniline loss in Hudson River sediments was observed under Fe(III)-amended conditions within 65 days, but the activity was not sustained by refeeding (Kazumi et al. 1995a). Thus, the extent of knowledge about aniline degrading bacteria that are active under anoxic conditions is limited.

The overall goal of this study was to investigate biodegradation of aniline under anoxic conditions in contaminated freshwater sediment from a canal at a large chemical manufacturing site. Aniline is one of many contaminants at the site. This research was intended to strengthen understanding of fate of aniline under reduced conditions and to identify bacterial community members in active aniline-degrading systems.

4.3 Materials and Methods

4.3.1 Chemicals and reagents

Aniline (99.5+%, A.C.S. Reagent) was purchased from Sigma-Aldrich (St. Louis, MO). Aminobenzoic acid (≥99%), diphenylamine (≥99%), benzoic acid (≥99.5%) and azobenzene (99%) were purchased from Sigma-Aldrich (St. Louis, MO). Phenol and acetonitrile (certified A.C.S.) was obtained from Fisher Scientific (Pittsburgh, PA). Aniline [^{14}C] hydrochloride (solid), 50-60 mCi/mmol, 250 μCi (1.85-29.6 GBq/mmol) was obtained from American Radiolabeled Chemicals, Inc. (Saint Louis, MO). Phenethylamine (≥99.5%, GC, Fluka Analytical, Albany, NY), methanol (≥99.8 %, certified A.C.S., Fisher Scientific, Pittsburgh, PA) and ScintiVerse™ Cocktail

(Scintanalyzed™, Fisher Scientific, Pittsburgh, PA) were used during $^{14}CO_2$ evolution studies.

4.3.2 Sediment microcosm set up

Lightly and highly contaminated sediment, along with canal water was obtained from a freshwater canal adjacent to an industrial site in New Jersey in January of 2007. An additional sampling from the lightly contaminated location was performed in April of 2011. Canal sediments obtained in January of 2007 were contaminated under the influence of groundwater flow from an aquifer adjacent to the canal beneath a dyes manufacturing area. At the more highly contaminated locations, the canal sediments were reported to also contain para-chloroaniline, benzene, monochlorobenzene, dichlorobenzene, PAHs, dyes and other contaminants, in addition to aniline. Three 5 ft × 3.2 in. in diameter sediment cores in acetate sleeves were provided from both locations in the canal during the 2007 sampling event. The cores were cut into sections, labeled, capped and placed on ice for shipment overnight to Rutgers University. Canal water was provided in a 10 L polyethylene carboy. A grab sample of approximately 2 L of sediment from the depth interval 0 to 0.5 ft and 10 L of canal water from the bottom of the water column were obtained in April, 2011 by ponar recovery. Materials were shipped overnight on ice to Rutgers University. All materials were stored at 4 °C for less than one month until use. For complete site description and sample locations, see Chapter 1, Section 1.2.

The sediment cores were composited under anoxic conditions using sterile instruments in a disposable glove bag (Cole Parmer Instrument Company, Veron Hills, IL) under a

sterile nitrogen gas purge. After compositing, three 15 mL samples were removed per location and placed in 15 mL polypropylene tubes at -80 °C for later DNA extraction and microbial molecular analysis. The remainder of the material was packed into sterile glass jars, and along with canal water, was used to set up sediment slurry microcosms.

Canal water was purged with sterile, anoxic nitrogen prior to use. Jars of composited sediment, site water, and autoclaved serum bottles and other material were placed inside the disposable glove bag in a chemical fume hood and were purged with sterile nitrogen for 2 hours prior to opening the sediment. Microcosms were set up in volumes of 100 mL of sediment slurry (20% composited canal sediment and 80% canal water (volume:volume)) in sterile 160 mL serum bottles. KNO_3 (30 mM) was added to establish nitrate-amended conditions. For methanogenic microcosms no alternate electron acceptor was added (other than bicarbonate) so that methanogenic conditions would be promoted. The methods followed those previously published for establishing sediment slurry microcosms (Monserrate and Haggblom 1997). The serum bottles were capped with Teflon™-coated butyl rubber stoppers and crimped with aluminum seals under a nitrogen headspace. The killed anaerobic controls were prepared identically to the methanogenic live treatment and then were autoclaved at 121 °C for 30 min on each of three consecutive days. 100 μM of aniline and 0.5 mg/L resazurin were added from anoxic, sterile stocks to each serum bottle based upon the final volume of 100 mL. All microcosms were incubated at 28 °C, in the dark, without shaking. These original microcosms had a pH near neutral.

4.3.3 Enrichment set up

Enrichment cultures were established by transferring microcosm slurry to fresh minimal

medium in 60-mL serum bottles (containing 36 mL of medium) using previously

described procedures (Monserrate and Haggblom 1997).Transfers were inoculated with

10% (vol/vol) of slurry from active microcosms using a sterile, N_2 flushed plastic syringe

with an 18 G stainless steel needle. Enrichments were incubated at room temperature

without agitation. Denitrifying minimal medium was prepared as described previously

(Kazumi et al. 1995a). The medium contained: 32.6 mM KNO_3, 17.4 mM KCl, 1.5 mM

KH_2PO_4, 20 mM NaCl, 9.3 mM NH_4Cl, 0.7 mM $CaCl_2 \cdot 2H_2O$, 0.9 mM $MgCl_2 \cdot 6H_2O$,

29.8 mM $NaHCO_3$, and 0.004 mM resazurin sodium salt. Trace salts and vitamin stock

solution were also added to achieve a final concentration in the medium as follows: 0.4

µM d-biotin, 0.2 µM folic acid, 2.4 µM pyridoxine hydrochloride, 0.7 µM thiamine

hydrochloride, 0.7 µM riboflavin, 2.0 µM nicotinic acid, 0.5 µM DL-calcium

pantothenate, 0.04 µM vitamin B12, 1.8 µM *p*-aminobenzoic acid, 1.2 µM lipoic acid,

1.3 µM 1,4-naphthaquinone, 4.1 µM nicotinamide, 0.1 µM hemin, 21.4 µM

$MnCl_2 \cdot 6H_2O$, 8.1 µM H_3BO_3, 3.7 µM $ZnCl_2$, 2.1 µM $CoCl_2 \cdot 6H_2O$, 1.9 µM

$NiCl_2 \cdot 6H_2O$, 2.2 µM $CuCl_2 \cdot 2H_2O$, 0.5 µM $NaMoO_4 \cdot 2H_2O$, 7.5 µM $FeCl_2 \cdot 4H_2O$,

0.02 µM Na_2SeO_3 and 0.03 µM Na_2WO_4.

The denitrifying medium first transfer enrichments were established according to

recommendations in previous studies regarding addition of nitrogen sources and

reductancts as shown in Table 4.1. Different media recipes were used to improve

transferring success. Transfer sets 1, 2 and 5 used denitrifying medium containing

NH_4Cl, 0.5 g/L as a nitrogen source. Transfer sets 3 and 4 did not add additional reduced nitrogen, but rather the nitrogen sources were nitrate (added as the terminal electron acceptor) and the nitrogen contained in aniline itself. The second and third sets of denitrifying medium contained either cysteine or sodium sulfide at 0.9 mM as reducing agents to ensure that a reduced redox condition was maintained. For media preparation, KCl, KH_2PO_4, NaCl, NH_4Cl, $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, and KNO_3 were first added to a pressure-resistant 1-L bottle along with a few mLs less than 1 liter of DI water. Media was then purged with N_2 through a fine diffusing stone for 30 to 45 min then switched to purge with $70\%N_2/30\%CO_2$ and $NaHCO_3$ was added. After 15 min, the bottle was sealed and a purged 60 mL syringe with a 1.5 in. needle was used to remove 180 mL of gas from the media bottle. The bottle was placed in a tub with a few inches of water and autoclaved for 30 minutes. A sterile and purged syringe with 1.5 inch needle was used to add scrubbed $70\%N_2/30\%CO_2$ until the bottle was re-pressurized. Resazurin stock solution and trace salts solution were added. Two mL media was removed with an anoxic, sterile syringe and used to confirm that the pH was neutral.

The second and third transfers were set up similarly to the first transfers (Table 4.1) except that 10% inoculum was added from an active transfer, rather than from an original microcosm.

4.3.4 Analytical methods

Microcosms were sampled for aniline and nitrate, initially weekly, and thereafter, periodically. For aniline analysis, one mL of well-mixed slurry was withdrawn from each serum bottle using a sterile 1 mL plastic syringe equipped with an 18 gauge stainless steel

needle that had been pre-flushed with sterile N_2/CO_2. Samples were placed in 1.5 mL

Eppendorf tubes and either frozen at -20 °C or extracted immediately. Extractions were

performed using slight modification of previously described methods (Struijs and Rogers

1989) by adding 1 mL of acetonitrile, mixing, and centrifuging at 10,000 rpm for 3 min.

The supernatant was removed using a plastic syringe, filtered through a 0.45 μm nylon

filter, and placed in a glass sample vial sealed with a Teflon™-backed butyl rubber

septum. Analysis was by an Agilent 1100 high performance liquid chromatography

(HPLC) system equipped with a diode array detector operating at 244 nm (Agilent

Technologies, Inc., Santa Clara, CA). Isocratic separations were made on a Luna 5μ

C18(2) 120 column (250 × 2 mm) (Phenomenex®, Torrance, CA). The column was held

at 40°C. A mobile phase water:acetonitrile (ACN) mixture (45:55 vol:vol) was supplied

at a flow rate of 0.33 mL min^{-1}. Aniline eluted at 2.8 min and was identified by

comparison of the retention time to a known standard. Aniline concentrations in samples

were quantified using a five point calibration standard with a range of 0.05 to 2 mM. The

detection limit was estimated to be approximately 0.15 μM.

Potential metabolites of aniline transformation were selected for analysis so that

biotransformation pathways could be investigated. Diphenylamine and azobenzene

standards prepared in acetonitrile were detected by the same analytical method as aniline,

and these potential metabolites of aniline transformation eluted at 9.6 and 6.1 min,

respectively. 4-aminobenzoate, benzoic acid and phenol standards were detected using a

solvent system of acetonitrile (ACN):0.1% acetic acid buffer (25:75) and supplied at a

flow rate of 0.35 mL min^{-1}. Analysis was by the Agilent 1100 high performance liquid

chromatography (HPLC) system (Agilent Technologies, Inc., Santa Clara, CA) equipped

with a diode array detector operating at 220, 230, 254, and 280 nm and the wavelength of the fluorescence detector (FLD) was operated at excitation wavelength (Ex) = 244 and emission wavelength (Em) = 419 nm. Isocratic separations were made on a HyperClone 5µ ODS (C18) 250 × 2.00 mm 5µ column (Phenomenex®, Torrance, CA).

Lactic, acetic, propionic and butyric acid standards were analyzed on a Beckman Coulter® System Gold^TM HPLC (Beckman-Coulter, Inc., Fullerton, CA) equipped with a Bio-Rad® Aminex HPX-87H organic acid analysis column (Bio-Rad Laboratories, Hercules, CA). UV absorption detection was operated at a wavelength of 210 nm. The column was held at 60 °C. Filtered 5.0 mM $H_2SO_4$was supplied at a flow rate of 0.6 mL min^{-1} as the eluent.

Extracted samples from certain time points collected from active microcosms were analyzed using the other methods described above to determine if the potential metabolites could be detected.

Headspace methane content was analyzed with a gas chromatography system (Agilent 6890N G1530N network GC system, Agilent Technologies, Inc., Santa Clara, CA) equipped with a flame ionization detector. The injection block was maintained at 250 °C, the detector heater was maintained at 250 °C and the oven was 150 °C. The column (Agilent 113-4332, GS-GasPro, capillary: 30 m x 0.32 mm I.D.) was used with helium carrier gas at a flow rate of 1.3 mL min^{-1}. The hydrogen flow to the flame jet was 40 mL min^{-1} and air flow to the detector was 45 mL min^{-1}. Under these conditions, methane was eluted at 1.6 min.

The nitrate samples were analyzed using a Dionex ICS-1000 (Dionex, Sunnyvale, CA) ion chromatography system equipped with an AS9-HC guard in column and an AG9-HC guard out column. The mobile phase was 9 mM Na_2CO_3 with a flowrate of 1 mL min^{-1}. Nitrate was quantified using a five-point calibration curve over the range from 0.5 to 5 mM. A 0.5-mL sample of slurry microcosm was removed using a sterile nitrogen-flushed syringe and filtered through a 0.45 μm filter. The filtrate was diluted 20 times in DI water and filtered through a 0.45 μm filter. The detection limit for nitrate was estimated to be approximately 30 μM.

The aniline concentrations and electron acceptor concentrations for each microcosm are presented individually when replicates behaved substantially differently from each other. When replicates behaved similarly, the average of the triplicates with error bars representing plus or minus one standard deviation are presented.

4.3.5 Stoichiometry of aniline degradation

To examine whether aniline degradation was coupled to reduction of the electron acceptor, predicted values based on stoichiometric equations were applied to compare to nitrate loss or methane evolution. Equations 4.1 and 4.2 assume that aniline is completely mineralized to CO_2 (Struijs and Rogers 1989) and ignores the production of cells.

Denitrifying:

$$C_6H_7N + 5.6NO_3^- + 1.2H_2O \rightarrow 6HCO_3^- + NH_3 + 0.4H^+ + 2.8N_2 \quad \text{(Equation 4.1)}$$

Methanogenic:

$$C_6H_7N + 7.5H_2O \rightarrow 2.5HCO_3^- + 3.5CH_4 + NH_3 + 2.5H^+ \quad \text{(Equation 4.2)}$$
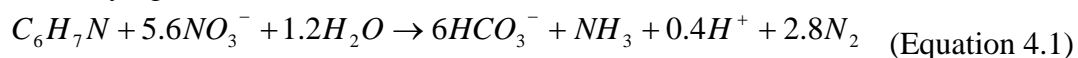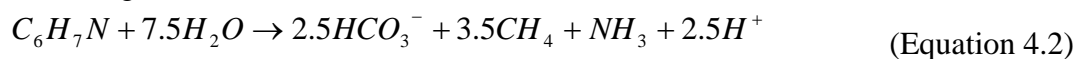
4.3.6 Protocol for detecting mineralization of of aniline using [^{14}C] aniline–amended microcosms and enrichments

^{14}C-aniline was fed to sediment microcosms and enrichments and ^{14}CO$_2$ evolved was captured to provide an estimate of the extent of mineralization of aniline. ^{14}CO$_2$ recovery tests were monitored by trapping ^{14}CO$_2$ evolved from ^{14}C-labeled aniline under nitrate-amended conditions from lightly contaminated freshwater canal sediment (sediment collected in 2011) microcosms and second-generation nitrate-amended enrichment cultures transferred from lightly contaminated freshwater canal sediment (sediment collected in 2007).

The recovery of aniline as CO$_2$ (*R%*) was estimated by the relationship shown in Equation 4.3.

$$R\% = \frac{^{14}CO_{2-treatment} - ^{14}CO_{2-control}}{[^{14}C]_{aniline-\deg raded}}$$

(Equation 4.3)

where:

^{14}C aniline degraded = the amount of ^{14}C-aniline estimated to have been degraded in live treatments based on monitoring aniline loss activity in replicate microcosms or enrichments (HPLC controls);

^{14}CO$_2$ treatment = the amount of radioactivity recovered as CO$_2$ from live systems;

$^{14}CO_2$ *control* = the amount of radioactivity recovered as CO_2 from autoclaved or abiotic control systems.

To prepare stock solutions, a glass vial containing 250 μCi aniline [^{14}C] hydrochloride powder (solid) (American Radiolabeled Chemicals, Inc., Saint Louis, MO) was centrifuged briefly before opening. A 3 mL syringe was used to add 1 mL sterile DI water and to then transfer the dissolved aniline [^{14}C] hydrochloride solution to a sterile and anoxic serum bottle capped with a gray butyl Teflon®-lined septum. The glass vial was subsequently rinsed with sterile DI water three times, and the rinse water was pooled with the dissolved aniline [^{14}C] hydrochloride solution. After completing the process, the aniline [^{14}C] hydrochloride was dissolved in a total volume of 2.2 mL DI water resulting in a solution concentration of 2 mM [^{14}C] aniline (denoted the "first" stock solution). The first stock solution was then diluted 500-fold providing a 4 μM [^{14}C] aniline "second" stock solution.

The first and second stock solution activities were checked by counting with 3 mL ScintiVerse cocktail (Table 4.2). The stock solutions were stored sealed at -20 °C. Table 4.3 shows the initial amount of [^{14}C] aniline added and the final concentration in the microcosms or enrichments used for the mineralization experiments.

4.3.6.1 Microcosm experiment with ^{14}C aniline

Triplicate live treatments and triplicate live HPLC control microcosms (for routine monitoring of aniline loss over time) were set up with 8 mL of sediment and 32 mL of canal water (See Section 4.3.1 Sediment microcosm set up). Triplicate live treatment

microcosms were amended with 50 μM of aniline (July 11, 2011) and 0.01 μM uniformly labeled ^{14}C-aniline (July 12, 2011). Triplicate control microcosms were amended with 50 μM of aniline (July 11, 2011) only (Table 4.4). Control microcosms for HPLC analysis were sampled weekly and the samples were tested by HPLC to determine the extent of aniline degradation (see Section 4.3.4 Analytical methods). The concentration of nitrate was monitored by Ion Chromatography (see Section 4.3.4 Analytical methods). After 22 days of incubation at room temperature, two of the triplicate live treatment microcosms were flushed with air for 30 min through a stripping chain to capture any evolved ^{14}CO$_2$. The stripping chain was assembled by connecting an air gassing station, the reaction serum bottle (via an input and output needle through the septum) and alkaline trap vials. The trap vials were filled with a mixture of 3 mL phenethylamine, 3 mL methanol and 6 mL ScintiVerse cocktail that selectively trapped CO$_2$ using previously described methods (Hines et al. 2006). The ^{14}C present was then determined by scintillation counting of the radioactivity that had been trapped. Samples were counted on a Beckman model LS 6500 scintillation counter (Beckman Instruments, Brea, CA). After 58 days of incubation the remaining live treatment microcosm was flushed with air and samples were counted along with the standard on Sep 8, 2011.

4.3.6.2 Enrichment experiment with ^{14}C aniline

Six replicates of a second-generation nitrate-amended enrichment culture (two live replicates, two autoclaved killed-controls and two live HPLC controls each containing 40 mL of culture) were amended with 500 μmol of aniline on day 0 and later re-amended with approximately1500 μmol of aniline (see Section 4.3.3 Enrichment set up). After 275

days incubation with aniline (Jan. 12, 2012), the two live replicates were amended with 0.01 μM uniformly labeled ^{14}C-aniline (Table 4.4). After 69 days incubation with ^{14}C-aniline, the $^{14}CO_2$ content in active treatment enrichments and the killed controls were determined in a manner similar to that described for the microcosms. The enrichments were flushed with air and the effluent stream was bubbled through a mixture of 3 mL phenethylamine, 3 mL methanol and 6 mL ScintiVerse cocktail for capturing CO_2. After flushing, a one mL liquid sample was taken from each culture and killed control. The ^{14}C remaining in the liquid was estimated by accounting for the volume of the remaining culture and using the scintillation counter reading of the one mL sample of the culture mixing with the 3 mL phenethylamine, 3 mL methanol and 6 mL ScintiVerse cocktail to find activity at the end of the incubation period. A mixture of 3 mL phenethylamine, 3 mL methanol and 6 mL ScintiVerse cocktail was also counted as a blank. The overall protocol is shown in Table 4.4.

4.3.7 DNA extraction and PCR amplification of 16S rRNA genes

Total genomic DNA extraction was performed with the PowerSoil^{TM} DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions, from a 1 mL slurry sample from sediment microcosms or enrichment cultures, or a 0.25 to 1 g background composited sediment sample which had been stored at -80 °C. DNA was eluted in 50 µL of PowerSoil^{TM} Solution C6.

The polymerase chain reaction (PCR) products were generated with Bac338f-GC and Univ519r primers (Table 4.5), resulting in expected 200 bp DNA fragment of amplified bacterial 16S rRNA genes. Each 50 µL PCR reaction mixture contained the following: 1x

PCR buffer (all reagents were obtained from USB Corp., Cleveland, OH, USA), 2.5 mM of $MgCl_2$, 20 nmol of deoxynucleoside triphosphate, 10 pmol of each PCR primer (all primers were obtained from Integrated DNA Technology, Coralville, IA, USA), 1.5 U of Taq polymerase, and 4 µL of template DNA. 10 pmol $µL^{-1}$ primers were made from 100 pmol $µL^{-1}$ original stock by dilution in PCR quality water. The thermocycling program was as follows: initial denaturation at 94°C for 5 minutes, then 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds (30 cycles), and a final elongation at 72 °C for 7 minutes. Final hold was set at 4 °C for an indefinite time. The expected PCR amplicon sizes were verified by running on a 1.5 % agarose gel. A blank (negative control) using DI water as template was analyzed with each set of samples.

4.3.8 DGGE and sequence analysis of bands excised from DGGE gels

The PCR products were subjected to denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993) using 8% polyacrylamide gels with a 40 to 70% urea-formamide gradient to resolve DNA fragments of different bacterial phylotypes. Amplified DNA products were electrophorized for 300 min at 150 V and 60 °C using a DCode mutation detection system (Bio-Rad Laboratories, Hercules, CA). DNA was visualized after 0.1% ethidium bromide staining by UV transillumination on a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA). Each prominent band detected in the DGGE gel was excised using a sterile razor blade and placed in 20 µL of sterilized DI water overnight at 4 °C. The DNA eluted from the gel to the DI water was then used as template DNA and re-amplified using the Bac338f and Univ519r primer set (Table 4.5). The resulting amplicons were verified on a 1.5 % agarose gel and purified with an

UltraClean PCR Clean-up kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Purified PCR products were then sequenced by Genewiz (Genewiz Inc., South Plainfield, NJ).

4.3.9 Construction of phylogenetic trees

The 16S rRNA gene fragments were analyzed and compared to sequences in the GenBank database by BLAST® (BlastN, National Center for Biotechnology Information Database; http://www.ncbi.nlm.nih.gov). Phylogenetic trees were constructed from unique sequences obtained from the DGGE analysis and closely related sequences of isolates or environmental phylotypes selected from the GenBank® database by Mega (www.megasoftware.net). Bootstrap values were determined from 100 iterations of the maximum likelihood calculation.

4.4 Results

4.4.1 Background concentrations

After set up of microcosms (day 0) and before spiking with aniline stock solutions (day 1), samples were taken from three random microcosms from each location (lightly and heavily contaminated) and analyzed for background aniline concentrations. The

concentrations of aniline in freshwater canal materials were near the detection limit of the HPLC method (0.15 μM).

4.4.2 Biological microcosm results

Results from the nitrate-amended-aniline and methanogenic-aniline microcosms prepared using the lightly contaminated freshwater canal sediments are shown in Figure 4.1 through 4.5. Loss of aniline with 14 days lag time was observed under nitrate-amended conditions in all triplicate microcosms (Figure 4.1).

There was no loss of aniline in the killed anaerobic controls in the first sampling interval between days 1 and 8. However, there was an average loss of 41% of the original added aniline from the killed anaerobic controls over 195 days. The nitrate-amended microcosms were re-amended with aniline on days 100 and 174 with subsequent loss of the added aniline.

Nitrate concentrations in the microcosms are shown in Figures 4.5 and 4.6. The loss of nitrate confirmed its use as an electron acceptor in the sediments. The nitrate-amended microcosms were re-amended with nitrate on day 429 and 969 (Figure 4.6). According to the stoichiometry shown in Eq. 4.3, amendment of aniline on day 0 and its subsequent biodegradation would have consumed approximately 0.52 mM nitrate. Nitrate concentration data showed approximately 14.3 mM nitrate loss between days 0 and day 52. This additional substantial loss of nitrate may have been linked to biodegradation of other contaminants in the sediments or in biodegradation of natural organic matter.

The microcosms were re-amended with approximately 1500 µM aniline for the fourth to seventh amendment events (Figure 4.2). Loss of aniline in triplicates was noted compared to anaerobic killed controls at each amendment between days 343 to 969.

There was only 5 µM methane accumulation in aniline-nitrate-amended freshwater canal sediment microcosms headspaces by day 217 (Table 3.7). No benzoic acid, phenol, 4-aminobenzoate, diphenylamine or azobenzene were detected by HPLC (or their concentrations were lower than 5 µM). No lactic acid, acetic acid, propionic acid or butyric acid were detected (detection limit 0.5 µM).

In methanogenic microcosms prepared with material from the lightly contaminated freshwater canal location, loss was noted in two of the triplicates after 50 days and after a lag of 90 days, loss in the third triplicate was observed (Figure 4.3). These microcosms had the same set up as the nitrate-amended treatment but with no added nitrate. Microcosms were re-amended with approximately 1500 µM aniline for the second and third amendments (Figure 4.4). Loss of aniline in all triplicates was noted after re-amendment. There was no sampling time point between day 350 and 778.

Average methane accumulation in these microcosms was 572 µM by day 217 (Table 3.7). According to the stoichiometry in Equation 4.2, an average of 900 µM aniline (three amendments by day 217) could be completed mineralized and produce an average of 3150 µM methane in each triplicate microcosm (ignoring the production of cells). This indicates that aniline might not be completely mineralized to methane and carbon dioxide. Further, part of the carbon source was consumed in cell production.

4.4.3 Nitrate-amended enrichment results

The active microcosms were re-amended with aniline over many days, and transfer

enrichment cultures were initiated. The results from all attempted enrichments are shown

in Table 4.1. The aniline concentration data for enrichments where there was little or no

activity (or which were not studied further) are shown in Figures B.53, B.57, B.58, B.59

and B.60. The results from active nitrate-amended-aniline enrichment results transferred

from the lightly contaminated freshwater canal sediment microcosms are shown in Figure

4.7 and 4.8. Loss of aniline without lag was observed in all triplicate enrichments (Figure

4.7). No benzoic acid, phenol, 4-aminobenzoate, diphenylamine or azobenzene were

detected as metabolites or the concentrations were lower than 5 μM.

The nitrate-amended enrichments were re-amended with aniline on day 27, 57, 75 and

118, with subsequent loss of the added aniline. There was no aniline loss in the nitrate-

amended medium blank. Nitrate was present in excess (data not shown) in all

enrichments and its availability could not have limited aniline degradation. There was 15

mM nitrate loss by day 75 in the first transfer enrichments, corresponding to 2.7 mM

aniline. According to the stoichiometry in Equation 4.1, an average of 2.7 mM aniline

could be completed mineralized and consume an average of 15.1 mM nitrate in each

triplicate microcosm. This indicates that aniline might have been completely mineralized.

The second transfers were inoculated with 10% (vol/vol) of slurry from the first transfer

microcosm triplicate #1 (#134-1, day 106). Loss of aniline with 26 to 233 days lag was

observed in all six second generation nitrate-amended enrichments (Figure 4.8). The

second enrichment transfers #134-1A, 134-1B, 134-1C were re-amended with aniline on

day 177; the second enrichment transfers #134-1D, 134-1E, 134-1F were re-amended with aniline on day 218. Five sets of third generation transfers were inoculated with 10% (vol/vol) of slurry from the second transfer microcosm triplicate #1 (#134-1A, day 233) (Table 4.1). No loss of aniline was observed in any of the third nitrate-amended transfers (data not shown) after 100 days.

4.4.4 Diversity of bacteria in the nitrate-amended microcosms and enrichment consortia

Images of DGGE gels of amplified16S rRNA genes from genomic DNA isolated from canal sediment, nitrate-amended microcosms and nitrate-amended enrichments (Figures 4.9 and 4.10) showed visible bands and suggested a broad diversity within the domain bacteria. No dominant DGGE bands were observed (nor were DNA sequences obtained) in the original sediment (canal native sediment sample was labeled as B in Figure 4.9). The sequences from the lightly contaminated freshwater canal location inoculated with nitrate and aniline shows bands labeled 134-1, 134-2 and 135-2 as the dominant DGGE bands from the nitrate-amended microcosms on day 1342. Sequences (<160 bp) (Table 4.6) were compared by BLAST® and matched by similarity to sequences existing in the GenBank® database (Table 4.7). A neighbor-joining bootstrap generated phylogenetic tree of the sequences obtained from DGGE is shown in Figure 4.11. Phylogenetic analysis of the 16S rRNA gene sequences obtained with primers 338fGC and 519r from DGGE suggested a broad diversity within the bacterial phyla β, δ, γ-Proteobacteria, Chloroflexi and Firmicutes.

Included in the β-Proteobacteria are DGGE bands 133-4, 134-1,134-2 from nitrate-amended microcosms and DGGE bands 1 and 3 from nitrate-amended enrichments. The reference species *Ralstonia*, *Burkholderia* sp., *Delftia acidovorans*, aniline-degrading bacterium HY99, *Acidovorax* and a *Variovorax* sp. are included in the tree. *Acidovorax* AJ012071 is the closest relative of a DGGE band sequence provided by researchers investigating the *in situ* microbial community at the chemical manufacturing site using BioTrap® Samplers installed in groundwater monitoring wells near the canal [Drs. Eleanor Jennings and Elizabeth-Erin Mack, personal communication]. Aniline-degrading bacterium HY99 (Kahng et al., 2000) is known as a denitrifying species, which was identified as most similar to *Delftia acidovorans*. *Delftia acidovorans* can degrade aniline only under aerobic conditions (originally described by Loidle et al. 1990), but strain HY99 has been reported to degrade aniline under both aerobic and nitrate-reducing conditions. By comparing with reference species, DGGE band 133-4, 134-1 and band 1 are shown to fall into a cluster with *Variovorax* sp. Bacteria of the genus *Variovorax* are common community members isolated from soil. For example, *Variovorax ginsengisoli* sp. is a denitrifying bacterium isolated from soil of a ginseng field (Im et al. 2010). The sequence of band 134-1 had 100% similarity (Table 4.7) with an uncultured *Variovorax* recovered from a pilot scale system remediating hydrocarbon-contaminated soil (Militon et al. 2010). DGGE band 1 not only has 100% similarity with *Variovorax* sp. but also has 98% similarity with *Acidovorax caeni*, which is also known as denitrifying species (Heylen et al., 2008). DGGE band 134-2 falls into the same cluster with *Ralstonia* and *Burkholderia* sp. The sequence of band 134-2 was 98% similar to that of *Burkholderia ubonensis* (Tayeb et al. 2008). *Burkholderia* sp. strain JS667 (See Chapter 2.2) was able

to convert diphenylamine to aniline and catechol, which were further biodegraded by the well-established aniline aerobic degradation pathway (Shin and Spain 2009). *Burkholderia xenovorans* LB400 is a well-known polychlorinated biphenyl (PCBs) degrader and is able to degrade benzoate via both catechol and benzoyl-CoA pathways (Denef et al. 2006). *Variovorax, Acidovorax, Delftia*, *Burkholderia* and *Ralstonia* all belong to the order Burkholderiales, in the Betaproteobacteria.

DGGE bands 135-2 and 6 fall within the Firmicutes. Band 135-2 had 100% similarity with Peptococcaceae bacterium enrichment culture 13Fcon03, a toluene degrader identified from sediment SIP enrichments amended with ferric iron (Pilloni et al. 2011). The band 6 phylotype is also closely related to an Fe(III)-reducing Peptococcaceae bacterium enrichment culture. Members of the family Peptococcaceae have also been identified in nitrate- (van der Zaan et al. 2012) or sulfate-reducing conditions (Alazard et al. 2010; van der Zaan et al. 2012).

Within the Deltaproteobacteria the phylotype represented by DGGE band 134-3 from nitrate-amended microcosms clustered with the reference species *Desulfovibrio piger* and *Desulfobacterium anilini*. Phylotype 134-3 showed 94% similarity with an uncultured Desulfuromonadaceae bacterium and was included in the same cluster with a sulfate reducer, *Desulfovibrio piger*, which oxidizes lactate and pyruvate incompletely to acetate (Loubinoux et al. 2002). *Desulfobacterium anilini* degrades aniline under sulfate-reducing conditions and is only one of two reported isolates that degrades aniline under anoxic conditions (Schnell et al., 1989; Schnell and Schink 1991) (See Chapter 2.2).

A nitrate-amended microcosm DGGE band 135-3, nitrate-amended enrichment DGGE

bands 7, 8, 9 and 10 and several reference spp. including *Sphingomonas*,

Magnetospirillum, *Azospirillum* and *Agrobacterium* sp. are shown to fall within the α-

Proteobacteria. DGGE bands 135-3 and 7 have 99% similarity with *Sphingomonas* sp.,

which is generally considered as an aerobic soil bacterial community member. However,

the strain *Sphingomonas oligophenolica* was reported to degrade phenol and to be able to

reduce nitrate to nitrite, although complete denitrification to nitrogen gas was not

detected (Ohta et al. 2004). Phylotypes represented by DGGE bands 8, 9 and 10 are

100% similar to a *Magnetospirillum* sp., which was reported to be able to degrade

vanillin under anoxic conditions according to the BLAST® report (Table 4.7). DGGE

bands 8, 9 and 10 also fall within the same cluster with *Azospirillum*, which is a nitrogen-

fixing genus but also recognized as denitrifying bacterial species (Tiedje 1982).

4.4.5 $^{14}CO_2$ *recovery during mineralization tests*

There was no detection of potential intermediates or metabolites during transformation of

aniline in denitrifying microcosms or enrichment cultures. Therefore, feeding with ^{14}C-

labeled aniline was used as a means to demonstrate mineralization of aniline in

microcosms and enrichments. Table 4.2 shows that the radioactivity of the second

aniline [^{14}C] hydrochloride stock solution (0.02 μCi/100 μL) used for the feeding

experiments, decreased over time. The reading was recorded in both DPM and CPM.

Triplicate HPLC control microcosms were amended with approximately 60 μM aniline

(Figure 4.12). Loss of aniline was noted between days 0 and 60 (data from the third

triplicate is not shown). Table 4.8 indicates that an average loss of 44.1% of the amended

aniline occurred in the HPLC control microcosms from day 0 to day 21, with a lag of only 10 days, indicating robust activity.

Separate triplicate treatments set up exctly as the HPLC controls were amended with approximately 60 µM aniline and 0.01 µM aniline [^{14}C] hydrochloride. Aniline was not monitored by HPLC for these treatments, but they were assumed to have similar biological activity and aniline removal as the HPLC controls. Two of the triplicate treatments were purged and ^{14}C activity was measured on day 22. The third triplicate treatment was purged and measured on day 58. Table 4.9 shows the captured ^{14}C activity reading in two (L2 and L3) of the triplicate microcosms on day 22. Microcosm L2 was acidified with 1 mL of 6N HCl before purging. The activity reading was not higher than that of L3, which was not acidified. The remaining ^{14}C enrichment culture experiments were therefore not acidified before purging and trapping.

Table 4.2 shows the ^{14}C-activity reading of the second aniline [^{14}C] hydrochloride stock solution on day 22 (Jul. 12, 2011). Assuming that the rate of loss of aniline-[^{14}C] and of aniline –[^{12}C] are the same, the ^{14}CO$_2$ recovery (*R%*) may be calculated from the average ^{14}C recovery from the gas phase according to Equation 4.3 as follows:

$$R\% = \frac{^{14}CO_{2-treatment} - ^{14}CO_{2-control}}{[^{14}C]aniline}$$

(Equation 4.3)

Thus, an average of 5.1% recovery of $^{14}CO_2$ was detected from two of the triplicate microcosms from the freshwater canal sediment microcosms on day 22. For the third live microcosm where activity was measured on day 58, only, 0.5% recovery of $^{14}CO_2$ was detected. This is in contrast to that measured for the two replicates on Day 22 (Table 4.9).

Six replicates of a second-transfer enrichment (containing 40 mL of culture) were amended with 500 μmol of aniline on day 0 and later re-amended with approximately 1500 μmol of aniline. Loss of aniline was noted between days 0 to 200 (Figure 4.13). Two of the most active enrichments were selected as HPLC controls (134-1B and 1D) and two were selected as live replicates for the ^{14}C aniline mineralization test (134-1E and 1F). Further, 134-1A and 1C were autoclaved three times on day 273-275 (Jan. 10 to 12, 2012) and served as killed controls.

After 275 days incubation with aniline (Jan. 12, 2012), the two replicates (134-1E and 1F) and two killed controls were amended with 0.01 μM uniformly labeled ^{14}C-aniline (Table 4.3). The on-going loss of aniline in the HPLC controls is shown in Figure 4.14. While one of the replicates 134-1D was robustly active, the other, 134-1B, exhibited only modest aniline loss. Complete loss of aniline was detected in 134-1D and little/no loss of aniline was detected in 134-1B by day 300.

After 69 days of additional incubation (March 21, 2012, day 344) with ^{14}C-aniline, the $^{14}CO_2$ content in live treatment enrichments and the killed controls was recovered by flushing with air. A water blank microcosm was also set up on March 21, 2012 and flushed with air to detect background activity. Table 4.10 shows the ^{14}C activity reading

in the blank, the two killed controls (134-1A and 1C), and the two live enrichments (134-1E and 1F) on day 344 (69 days after spiking with ^{14}C-aniline). The killed controls had a similar reading as the water blank.

Table 4.2 shows the radioactivity reading of the second aniline [^{14}C] hydrochloride stock solution, which decreased over time to 74766 DPM and 68141 CPM as measured on March 21, 2012.

Assuming a 100% loss of aniline (as observed in HPLC live control 134-1D), ^{14}CO$_2$ recovery ($R\%$) may be calculated as follows:

$$R\% = \frac{0.5 \times (1969.14 DPM + 5178.22 DPM) - 325.71}{74766.0 DPM} \times 100\% = 4.34\%$$

Assuming a 50% loss of aniline, ^{14}CO$_2$ recovery would be:

$$R\% = \frac{0.5 \times (1969.14 DPM + 5178.22 DPM) - 325.71}{74766.0 DPM \times 0.5} \times 100\% = 8.69\%$$

The overall recovery of ^{14}CO$_2$ for ^{14}C-aniline degradation under nitrate-amended conditions from freshwater canal sediment microcosms and enrichments from freshwater canal sediment microcosms was thus quite low and not indicative of unequivocal mineralization. This experiment did not provide enough evidence to confirm that aniline was biodegraded and completely mineralized under nitrate-amended conditions in site

sediment microcosms and enrichments. However, the $^{14}CO_2$ recovered from live

incubations was substantially higher than that of killed controls or blanks.

4.5 Discussion

The aim of this study was to assess the potential for biodegradation of aniline by

microbial communities in sediments from a freshwater canal under nitrate-amended and

methanogenic conditions and to identify biodegradation mechanisms and the bacteria

involved. Aniline loss was observed under nitrate-amended and methanogenic conditions

in microcosms prepared using sediments from the lightly contaminated location

compared to anaerobic killed controls. Positive results of aniline biotransformation were

observed in microcosms inoculated with both 500 µM and 1500 µM of aniline under

nitrate-amended and methanogenic conditions.

Average consumption of nitrate concomitant with the spike of aniline was 10 times

higher than the expected consumption estimated from theoretical redox reaction

stoichiometry (and neglecting cell growth). This may have been caused by the presence

of both spiked aniline and complex mixtures of other xenobiotic aromatic compounds in

the sediment. Two of the methanogenic triplicates showed loss of aniline in the first 50

days of incubation without any addition of electron acceptor and all of the nitrate-

amended triplicates showed loss of aniline in the first 50 days of incubation. Methanogenic and nitrate-amended microcosms amended with 1500 µM aniline during the second and third year incubation both exhbited loss of aniline. Loss of aniline in all triplicates was noted after re-amendment.

There was only 5 µM methane accumulation in aniline-nitrate-amended freshwater canal sediment microcosms by day 217 (Table 3.7) and nitrate loss was observed (Figure 4.5 and 4.6), suggesting nitrate-reducing conditions prevailed. Additionally, no volatile fatty acids, benzoate, phenol, 4-aminobenzoate, diphenylamine, azobenzene or any other major intermediate products were detected under nitrate-amended conditions from either freshwater canal sediment or enrichments. However, low recovery (approximately 5%) of $^{14}CO_2$ from ^{14}C-aniline degradation under nitrate-amended conditions from both freshwater canal sediment microcosms and enrichments was observed. Hence, it was not conclusively confirmed that aniline is biodegraded under nitrate-amended conditions. However, by comparing with reference species, the bacterial community members from aniline and nitrate-amended microcosms were closely related to nitrate-dependent and/or aromatic compound degrading strains including *Delftia acidovorans*, aniline-degrading bacterium HY99, *Ralstonia*, *Burkholderia* sp., *Variovorax* sp., *Magnetospirillum* sp. and Peptococcaceae bacterium. DGGE bands 1 and 3 from nitrate-amended enrichments are closely related to the reference species *Ralstonia*, *Burkholderia* sp., *Delftia acidovorans*, aniline-degrading bacterium HY99, *Acidovorax* and a *Variovorax* sp. DGGE band 1 fall into the same cluster with *Variovorax* sp.

These findings may be environmentally significant since such organisms are able to utilize a range of aromatic compounds often found in contaminated sites, including aniline, diphenylamine, catechol, PCBs, benzoate, toluene and vanillin. Among those reference species closely related to phylotyes detected in the nitrate-aniline community, *Delftia acidovorans*, *Azospirillum* and *Sphingomonas* sp. were reported to be mainly aerobic bacteria. For example, *Delftia acidovorans* can degrade aniline only under aerobic conditions (originally described by Loidle et al. 1990). Aniline-degrading bacterium HY99 has been proved be most similar to *Delftia acidovorans* by physiological and biochemical tests. It was shown to be able to degrade aniline under aerobic conditions via catechol *meta*-cleavage pathway and under nitrate-reducing conditions via 4-aminobenzoate pathway. Perhaps the most significant finding from the community analysis is the dominance of a phylotype closely related to *Magnetospirillum* in the aniline-nitrate enrichments (band 9, Figure 4.10). *Magnetospirillum* are known to reduce nitrate and contain genes to degrade toluene, phenol, benzoate and other aromatic compounds (Shinoda et al. 2005). Further, abundant nitrate reduction was observed in the microcosms and nitrate-amended enrichments. This suggests that an overall active community of denitrifyers was present.

The successful enrichments were set up from lightly contaminated freshwater canal microcosm triplicate #2 (#134-2) on day 1254 (Table 4.1, Set 1). This triplicate #2 was amended with approximately 100, 235, and 525 µM aniline on day 0, 100 and 173 respectively. From day 195 to day 343, there was less than or equal to 1 µM aniline in this microcosm for approximately 5 months. There were four re-amendments to this microcosm prior to transferring—i.e. 1630, 1415, 730 and 970 µM of aniline was

established on days 343, 551, 775 and 939, respectively. There was no sample taken on the transfer day (day 1254), but 6µM aniline remained in triplicate #2 on day 1284, which was 30 days after transferring.

The unsuccessful enrichments from the lightly contaminated freshwater canal included enrichment sets 2 to 5 (Table 4.1). Enrichment sets 2 to 4 were inoculated with microcosm triplicate #1 (Table 4.1, microcosm #133) on day 755. There were two aniline re-amendments to this microcosm prior to the transfer day (day 755) with 2570 and 685 µM of aniline established on days 343 and 551, respectively. There was no sampling point between day 551 and day 775. By day 775, the aniline concentration was lower than the detection limit, but there is no information regarding the aniline concentration between days 551 and 755. Enrichment set 5 was inoculated with microcosm triplicate #3 (Table 4.1, microcosm #135) on day 335. As described above, there was also less than 1 µM aniline in this microcosm between days 195 and day 343. Thus, these transfers occurred after long periods with little substate available.

The successful second transfer enrichments were set up from the successful enrichment triplicate #1 on day 76 (Table 4.1, second transfer set 1). This enrichment triplicate #1 (#134-1) was amended with approximately 500, 1350, 1000 and 973 µM aniline on day 0, 27, 57 and 75, respectively, before day 76 when the transfer occurred.

Eriksson et al. (2003) reported that most successful enrichment cultures that degrade PAHs under anoxic conditions were obtained from contaminated sediments and not from contaminated soils because of the low abundance of anaerobic PAH degraders in aerobic soils. There were unsuccessful enrichment transfers from groundwater aquifer and

freshwater canal sediment microcosms in our study. Here we hypothesize that low abundance of aniline degraders connected with long periods of incubation where aniline had been depleted, may have caused these unsuccessful transfers.

Table 4. 1. Nitrate-amended enrichment first, second and third transfer set up and activity summary. Transfers were inoculated with 10% (vol/vol) of slurry from active microcosms or from active enrichments.

Set[a] (bottle #)	Transfer	Aniline (µM)	Transfer from[a]	NH_4^+ (mM)	NO_3^- (mM)	reducing agents (mM)	Medium Ref.	Day	Activity
1 (#134-1 to #134-3)	1st	500	#134	10	32.6	na [b]	This study	1254[g]	+ + +[f]
2 (#133-1 to #133-3)	1st	500 to 1500	#133	10	32.6	0.9 [c]	Travkin et al., 2002	755	− − −
3 (#133-4 to #133-6)	1st	500 to 1500	#133	na[c]	32.6	0.9 [e]	This study	755	− − −
4 (#133-7 to #133-9)	1st	500 to 1500	#133	na[c]	32.6	na [b]	Kahng et al., 2000	755	− + −
5 (#135-1 to #135-3)	1st	500	#135	10	32.6	na [b]	Kahng et al., 2000	335	− − −
1 (#134-1A to #134-1F)	2nd	500	#134-1	10	32.6	0.9 [e]	This study	76 [h]	+ + +
2 (#134-3A to #134-3C)	2nd	500	#134-3	10	32.6	0.9 [e]	This study	492	− − −
1 (#134-1A-1 to #134-1A-3)	3rd	500	#134-1A	10	32.6	0.9 [e]	This study	233[i]	− − −

[a] *Bottle numbers #133, 134, 135 refer to triplicates #1, 2 and 3 for the nitrate-amended aniline microcosms from the lightly contaminated freshwater canal location (Figures 4.1 and 4.2). #133-1, 133-2, etc. refer to transfer from each original microcosm.*

[b] *Not amended with reducing agents.*

[c] *Amended with 0.45 mM Na_2S and 0.45 mM cysteine.*

[d] *Not amended with ammonia-N source.*

[e] *Amended with 0.9 mM Na_2S but without cysteine.*

[f] *+++: all triplicates show loss of aniline. −−−: none of them show activity.*

[g] *The enrichments were set up from lightly contaminated freshwater canal microcosm on day 1254.*

[h] *The second transfer enrichments were set up from the enrichment on day 76.*

[i] *The third transfer enrichments were set up from the second transfer enrichments on day 233.*

Table 4. 2. The radioactivity of the first [^{14}C] aniline stock solution and the change over time of the concentration of the second [^{14}C] aniline stock solution.

Stock solution	DPM	CPM	^{14}C-Aniline (μCi)	Date Measured
10 μL 1st stock solution	7,319,748	6,625,202	~1.1	Jul. 8, 2011[a]
100 μL 2nd stock solution	126,646	NA	~ 0.02	Jul. 12, 2011
100 μL 2nd stock solution	NA	109,097	~ 0.02	Sep. 8, 2011
100 μL 2nd stock solution	77,114	75,184	~ 0.02	Jan. 12, 2012
100 μL 2nd stock solution	74,766	68,141	~ 0.02	Mar. 21, 2012

[a] *First stock solution was prepared on Jul. 8, 2011 and second stock solution was prepared on Jul. 12, 2011.*

Table 4. 3. Radioactivity and concentrations of [^{14}C] aniline in microcosms and

enrichment study.

Treatment	Stock solution	Stock activities (μCi/ mL)	Stock conc. (μM)	Vol. added (μL)	Calculated final activities of treatment (μCi/ mL)	Calculated final conc. of treatment (μM)
Microcosm	2nd	~0.227	~4	100	~5.7 x 10^{-4}	~0.01
Enrichment	2nd	~0.227	~4	100	~5.7 x 10^{-4}	~0.01

Table 4. 4. [14]C labeled aniline experimental protocol. Concentrations given are the final

concentrations of the chemicals in the microcosm or second transfer

enrichment.

No.	Treatment	^{14}C-aniline (μCi)	Nitrate (mM)	^{12}C-aniline (μM)	Sediment Slurry (%)	Autoclaved
L1, L2, L3	Microcosm experiment live	0.02	30	50	20%	--
H1, H2	Microcosm experiment HPLC control	--	30	50	20%	--
134-1E, 134-1F	Enrichment experiment live	0.02	30	1300	0.2% (2nd Transfer Culture)	--
134-1B, 134-1D	Enrichment experiment HPLC control	--	30	1300	0.2% (2nd Transfer Culture)	--
134-1A, 134-1C	Enrichment experiment Killed control	0.02	30	1300	0.2% (2nd Transfer Culture)	Autoclaved

Table 4. 5. Universal bacterial primers used for PCR-DGGE analysis.

Primer	Sequence (5'→ 3')	Ref.
Bac338f-GC	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCG C-CCCCGCCCTCCTACGGGAGGCAGCAG	(Nakatsu, Torsvik, and Øvreås 2000)
Bac338f	CTCCTACGGGAGGCAGCAG	Nakatsu, Torsvik, and Øvreås 2000)
Univ519r	ATTACCGCGGCTGCTGG	Nakatsu, Torsvik, and Øvreås 2000)

Table 4. 6. Sequences of DGGE fragments obtained with primers 338fGC and 519r from

canal sediment nitrate-amended microcosms and nitrate-amended

enrichments.

Band name	Length (bp)	PCR products, sequence obtained with primers 338fGC and 519r
133-1	101	TCTTACGGGAGGCAGCAGTGAGGAATATTGCACAATGGGGGGAACCCTGAT GCAGCAACGCCGCGTGGAGGATGACGCATTTCGGTGTGTAAACTCCTTTT
133-2	138	AAAAGTGCTTTACAACCCGAAGGCCTTCATCGCACACGCGGCGTTGCTGGTT CAGGCTTTCGCCCATTGACCAATATTCCCTACTGCTGCCTCCCGTAGGAGGG CGGGGGCGGCGGGACGGGCGCGGGGCGCGGCGGG
133-3	158	TTGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACGGGAGG CAGCAGTAGGGAATATTGCACAATGGACGAAAGTCTGATGCAGCAACGCCG CGTGTGCGATGAAGGCCTTCGGGTTGTAAAGCACTTTTATGAGGGAAGAGC AAGG
133-4	141	TACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCA GCCATTCGGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGNTTTTGTCCG GAAGGAAAAGGCTCTCTTTAATACAGGGGGGTCATGAC
133-5	136	CCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACGGGAGGCAG CAGTAGGGAATATTGGTTAATGGGCGAAAGCCTGAACCAGCAACGCCGCGT GTGCGATGACGGCCTTCGGGTTGTAAAGCACTT
133-6	137	CCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACGGGAGGCAG CAGTAGGGAATATTGGTTAATGGGCGAAAGCCTGAACCAGCAACGCCGCGT GTGCGATGACGGCCTTCGGGTTGTAAAGCACTTT
134-1	154	TCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGAT CCAGCCATTCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGT ACGGAACGAAAAGGCTCTCTCTAATACAGGGGGCTCATGACGGTACCGTAA

Table 4. 6. Sequences of DGGE fragments obtained with primers 338fGC and 519r from canal sediment nitrate-amended microcosms and nitrate-amended enrichments (Continued).

Band name	Length (bp)	PCR products, sequence obtained with primers 338fGC and 519r
134-2	110	TACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAAGCCTGATCCAGCAATGCCGNGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTGTCCGGAAAGAAA
134-3	140	TCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCGCGTGAGGGAAGAAGGCCTTCGGGTCGTAAACCTCTGTCGGGGGGGGAAGAAACTCCGGGTGGTTAATACCCATCCGGA
135-1	133	TCATACGACTTACAAAGCCTTTTTTCTTCCATATAAAAGAAGTTTACAATCCAGAGGACCTTCATCCTCCACGCGGCATGGCTGGTTCAGACTTTCCTCCATTGACCAATATTCCTTACTGCTGCCTCCCGTA
135-2	118	TCCCGCCGCCCCCGCCCTCCTACGGGAGGCAGCAGTGGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCCTTCGGGTTGTAAAACTCTGTC
135-3	118	TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTTGCCCGGGAAGATAATGA
1	131	TCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCCATTCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGAAAAGGCTCTCTCTAATACA
3	117	GGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATGCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGAAAAGGTCTTTTCTAATAAAGAAGG

Table 4. 6. Sequences of DGGE fragments obtained with primers 338fGC and 519r from canal sediment nitrate-amended microcosms and nitrate-amended enrichments (Continued).

Band name	Length (bp)	PCR products, sequence obtained with primers 338fGC and 519r
5	135	TCCTACGGGAGGCAGCAGTAGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCCTCGGGTTGTAAAGCTCTTTTGTCCGGAAAGAAAAGCACCTGGTTAATACCCGGG
6	119	CTCCTACGGGAGGCAGCAGTGGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCCTTCGGGTTGTAAAACTCTGTCTTCAGGGAAGAAACAAA
7	98	CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTT
8	119	CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCGACGGGGACGATGATGA
9, 10,	120	CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCGACGGGGACGATGATGAC
12	115	CTCCTACGGGAGGCAGCAGGGAGGAATCTTGGGCAATGGGCGAAAGCCTGACCCAGCGACGCCGCGTGAGGGAAGAAGGCCTTCGGGTCGTAAACCTCTTTTGTGAGGGAAGAAG

Table 4. 7. Reference sequences, similarity, accession number and isolation source for matches to sequences obtained with primers 338fGC and 519r from PCR-DGGE of aniline-amended freshwater canal sediment microcosm bacterial community analysis.

DGGE band name	Close relative	Similarity (%)	Accession Number	Reference	Isolation source
133-1	Uncultured *Thiomicrospira* sp.	100	HQ162738	Unpublished	Lianjiang River sediment from electronic waste recycling site
133-2	*Levilinea saccharolytica*	98	NR_040972	(Yamada et al. 2005)	Mesophilic UASB granular sludge
133-3	Uncultured *Caldilineaceae*	97	FM176686	Unpublished	Rivulet
133-3	uncultured bacterium	99	GQ263921	(Field et al. 2010)	Simulated low level radioactive waste site
133-4	uncultured *Variovorax* sp.	96	JN125692	Unpublished	Biofilm sample in membrane biofilmreactor that bioreduced oxidized contaminants in drinking water
133-4	Uncultured *Comamonadaceae*	95	EU641895	(Mueller-Spitz, Goetz, and McLellan 2009)	Lake Michigan

Table 4. 7. Reference sequences, similarity, accession number and isolation source for matches to sequences obtained with primers 338fGC and 519r from PCR-DGGE of aniline-amended freshwater canal sediment microcosm bacterial community analysis (continued).

DGGE band name	Close relative	Similarity (%)	Accession Number	Reference	Isolation source
133-5, 133-6	uncultured bacterium	100	HM588745	(Militon et al. 2010)	Tert-butyl alcohol (TBA) contaminated sediment
133-5, 133-6	*Levilinea saccharolytica*	96	AB109439	(Yamada et al. 2005)	mesophilic UASB granular sludge *Chloroflexi*
134-1	Uncultured *Variovorax*	100	AM935564	(Militon et al. 2010)	Pilot-scale bioremediation process of a hydrocarbon-contaminated soil
134-2	*Burkholderia ubonensis*	98	EU024179	(Tayeb et al. 2008)	Type strain of *Burkholderia ubonensis*
134-3	uncultured *Desulfuromonadaceae* bacterium	94	JF747675	unpublished	Manantial del Toro hypersaline groundwater

Table 4. 7. Reference sequences, similarity, accession number and isolation source for matches to sequences obtained with primers 338fGC and 519r from PCR-DGGE of aniline-amended freshwater canal sediment microcosm bacterial community analysis (continued).

DGGE band name	Close relative	Similarity (%)	Accession Number	Reference	Isolation source
135-1	uncultured bacterium	96	JF341594	(Santo Domingo et al. 2011)	Concrete sewer biofilm
135-1	uncultured *Terrimonas* sp.	96	HQ018181	Unpublished	Rhizosphere soil not treated with nitrogen fertilizer
135-2	Peptococcaceae bacterium enrichment culture 13Fcon03	100	HQ625663	(Pilloni et al. 2011)	Toluene-degrading sediment SIP enrichments amended with ferric iron
135-3	*Sphingomonas* sp. T19	99	HQ647266	Unpublished	Rhizosphere
135-3	*Sphingomonas* sp. G20(2010)	99	HM591480	(Loaces, Ferrando, and Scavino 2011)	Grains
1	Uncultured *Variovorax* sp. clone MBfR_NS-136	100	JN125692	Unpublished	Biofilm sample in membrane biofilm reactor that bioreduced oxidized contaminants in drinking water

Table 4. 7. Reference sequences, similarity, accession number and isolation source for matches to sequences obtained with primers 338fGC and 519r from PCR-DGGE of aniline-amended freshwater canal sediment microcosm bacterial community analysis (continued).

DGGE band name	Close relative	Similarity (%)	Accession	Reference	Isolation source
1	*Acidovorax caeni*	98	AB639119	(Heylen, Lebbe, and De Vos 2008)	Activated sludge
3	*Acidovorax* sp. enrichment culture clone Van62	100	HQ222278	Unpublished	Anaerobic ^{13}C vanillin enrichment
5	uncultured *Thermomonas* sp.	99	GU257533	(Xia et al. 2010)	Activated sludge in a membrane bioreactor
6	Peptococcaceae bacterium enrichment culture NaFe56	100	JF820825	Unpublished	Iron-reducer enrichment culture
7	*Sphingomonas* sp. IGS55	100	JN680225	Unpublished	
8,9,10	*Magnetospirillum* sp. enrichment culture clone Van25	100	HQ222269	Unpublished	Anaerobic ^{13}C vanillin enrichment
12	uncultured *Chloroflexi*	97	AM935723	(Militon et al. 2010)	Hydrocarbon-contaminated soil

Table 4. 8. ^{12}C-aniline loss in two HPLC control nitrate-amended sediment microcosms

from the lightly contaminated freshwater canal location.

#	^{12}C-aniline Day 0 (μM) [a]	^{12}C-aniline Day 21 (μM) [a]	Average loss (%)
H1 and H2	57.1±3.8	25.2±6.8	44.1%

[a] *Average ± one standard deviation*

Table 4. 9. ^{14}C activity in captured gas from ^{14}C-aniline-nitrate-amended sediment

microcosms from the lightly contaminated freshwater canal location.

Day	#	DPM	CPM	Method
0	L1, L2, L3	NA[a]	NA	Add 100 mL of the "second" stock solution
22	L3	3,628	NA	Purge with Air 30 min. No HCl added.
22	L2	2,758	NA	Add 1 mL 6N HCl. Purge with Air 30 min.
58	L1	NA	499	Purge with Air 30 min. No HCl added.

[a] *Not measured.*

Table 4. 10. ^{14}C activity reading in second transfer enrichments from nitrate-amended aniline degrading microcosms.

Day	#	DPM	CPM	Method
--	Blank	326	135	Purge Air 30 min.
344	Killed control 134-1A	392	171	Purge Air 30 min.
344	Killed control 134-1C	334	131	Purge Air 30 min.
344	Live enrichment 134-1E	1,969	1,758	Purge Air 30 min.
344	Live enrichment 134-1F	5,178	4,729	Purge Air 30 min.
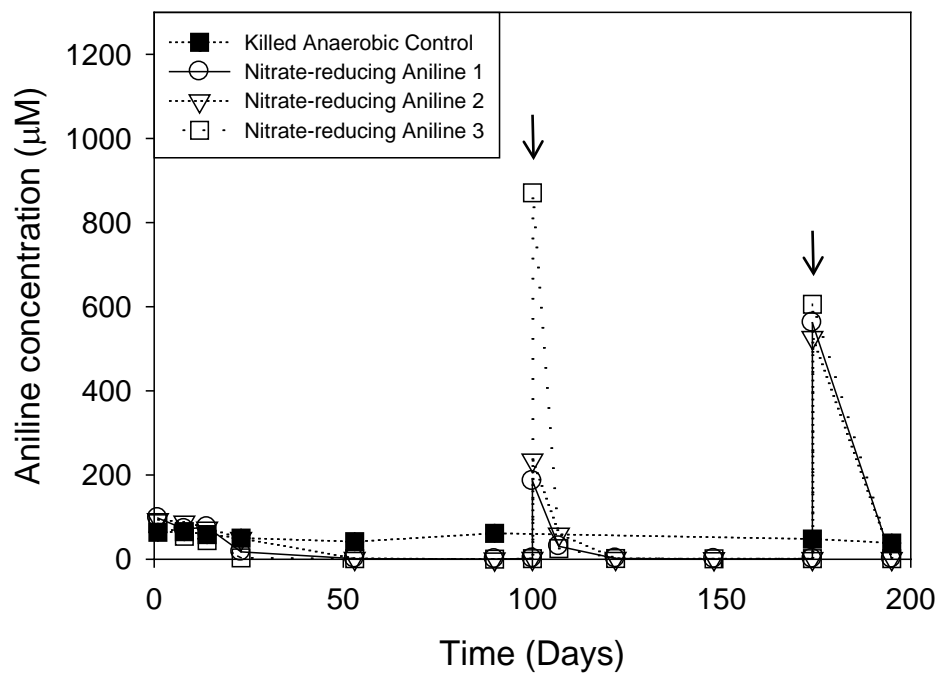
Figure 4. 1. Aniline in nitrate-amended and killed anaerobic sediment microcosms from

the lightly contaminated freshwater canal location, day 0 to 200. For killed

control, symbols are averages of triplicates and error bars represent one

standard deviation. The arrows indicate the re-amendment of substrate.
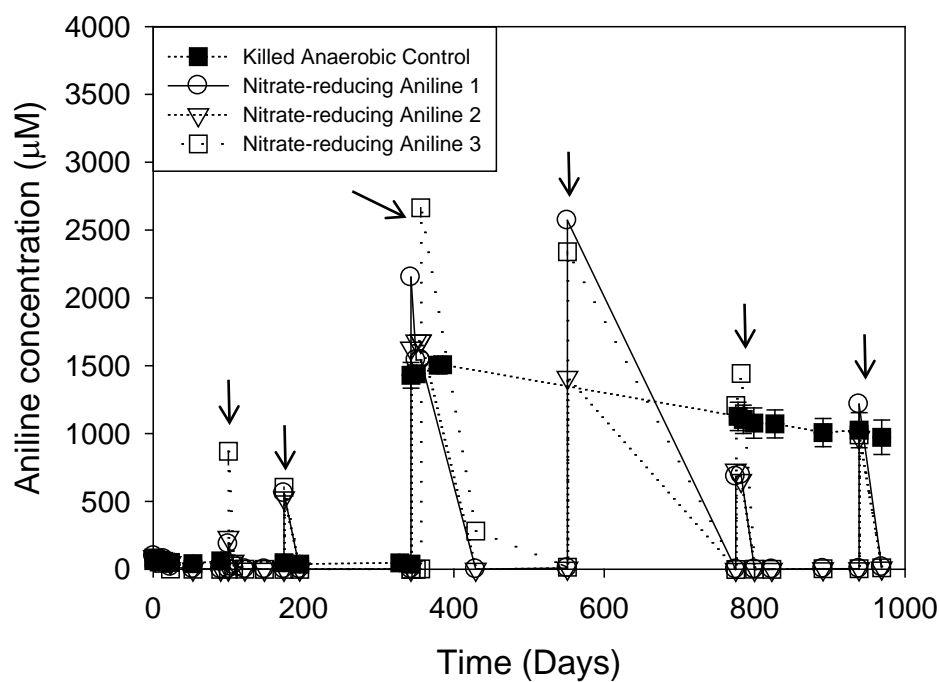
Figure 4. 2. Aniline in nitrate-amended and killed anaerobic sediment microcosms from

the lightly contaminated freshwater canal location, day 0 to 1000. For killed

control, symbols are averages of triplicates and error bars represent one

standard deviation. The arrows indicate the re-amendment of substrate.
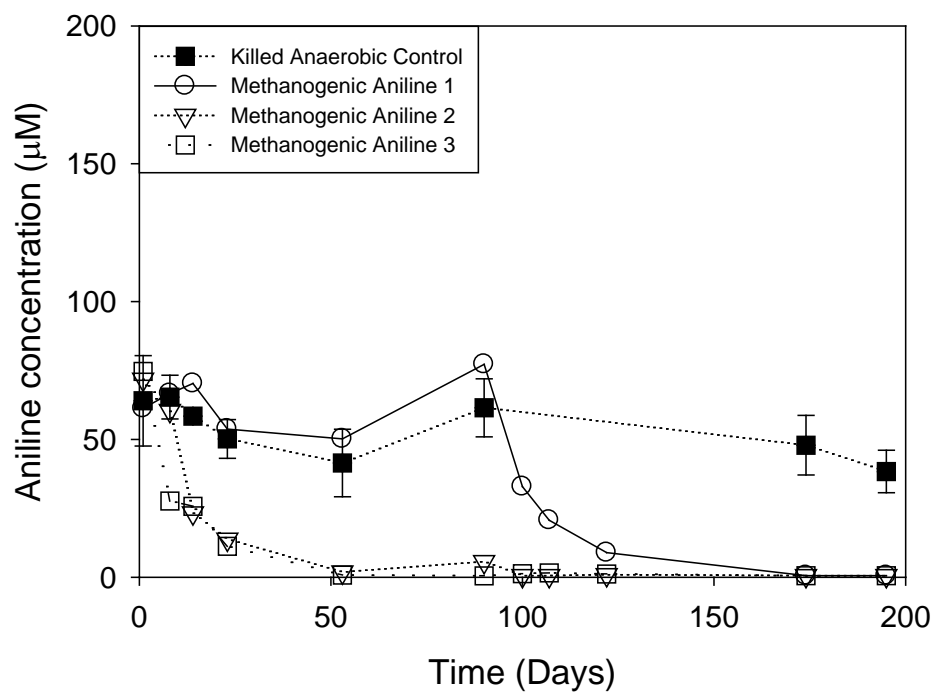
Figure 4. 3. Aniline in methanogenic and killed anaerobic control microcosms from the

lightly contaminated freshwater canal location, day 0 to 200. For killed

control, symbols are averages of triplicates and error bars represent one

standard deviation.
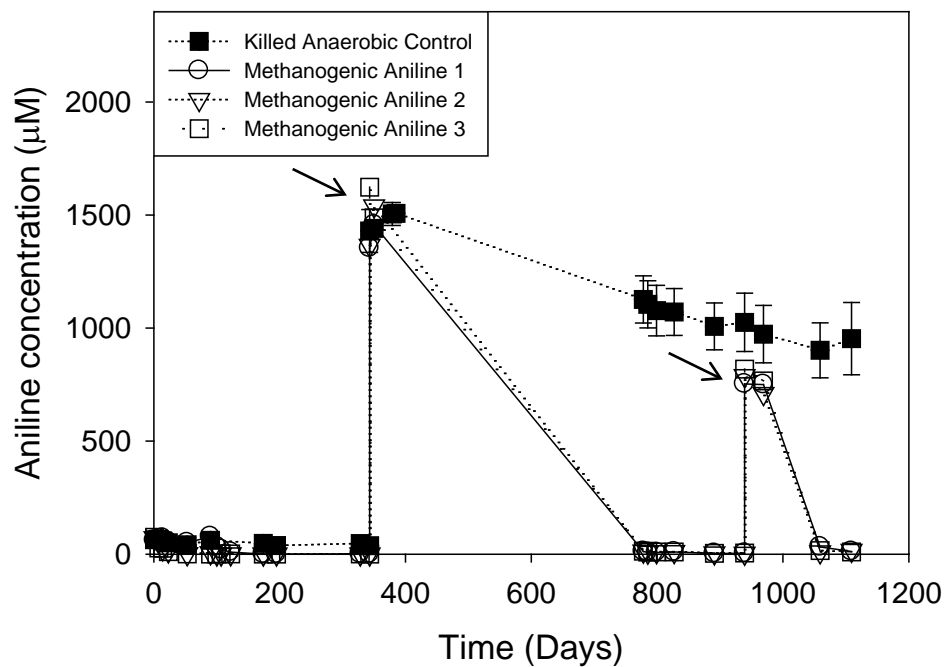
Figure 4. 4. Aniline in methanogenic and killed anaerobic control microcosms from the

lightly contaminated freshwater canal location, day 0 to 1200. For killed

control, symbols are averages of triplicates and error bars represent one

standard deviation. The arrows indicate the re-amendment of substrate.
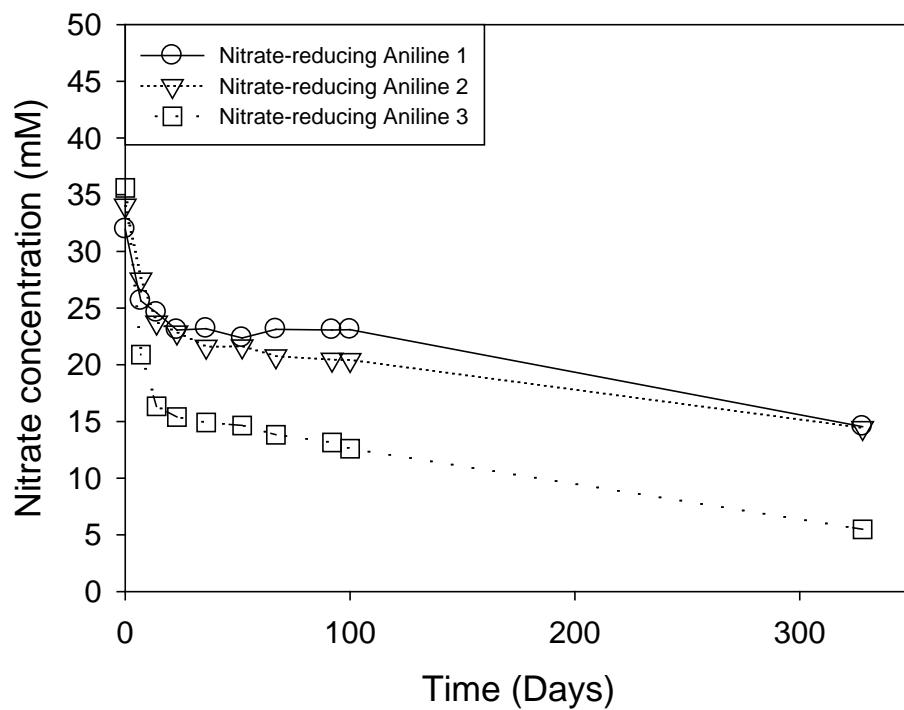
Figure 4. 5. Nitrate concentration in aniline spiked, nitrate-amended sediment

microcosms from the lightly contaminated freshwater canal location, day 0 to

350.
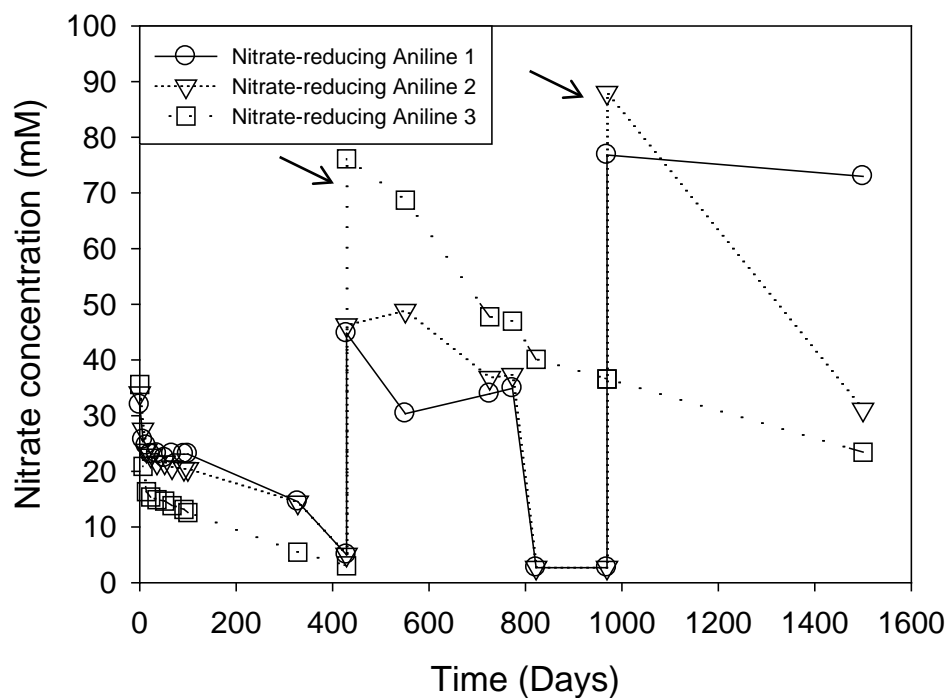
Figure 4. 6. Nitrate concentration in aniline spiked, nitrate-amended sediment

microcosms from the lightly contaminated freshwater canal location, day 0 to

1500. The arrows indicate the re-amendment of substrate.
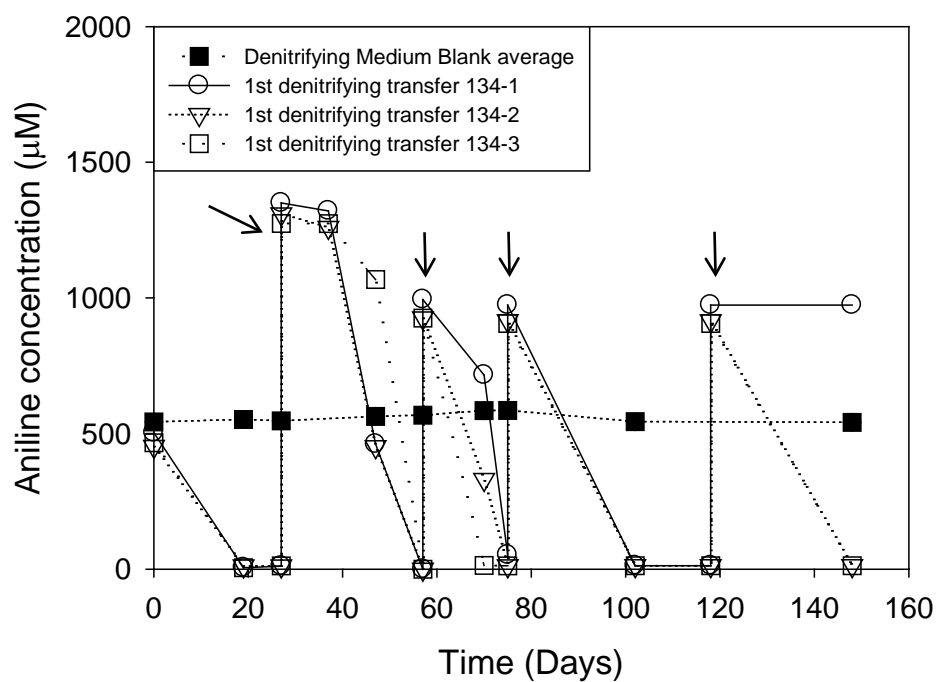
Figure 4. 7. Aniline in first transfer nitrate-amended enrichments from nitrate-amended

microcosms from the lightly contaminated freshwater canal location (bottles

#134-1 to 3). The arrows indicate the re-amendment of substrate.
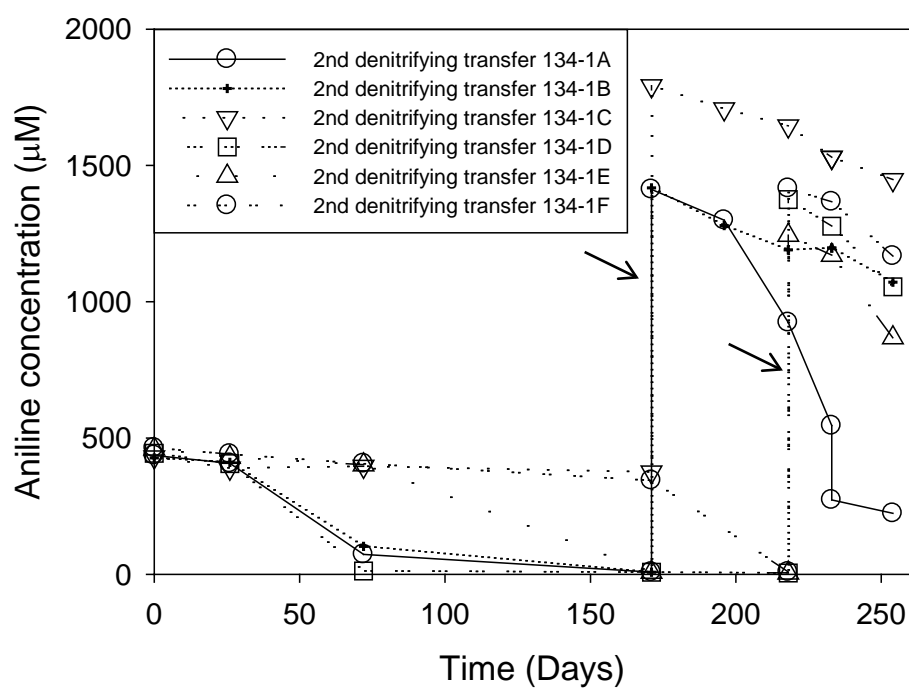
Figure 4. 8. Aniline in the second nitrate-amended transfer enrichments from nitrate-

amended microcosms from the lightly contaminated freshwater canal

location (bottles #134-1A to 1F). The arrows indicate the re-amendment of

substrate.
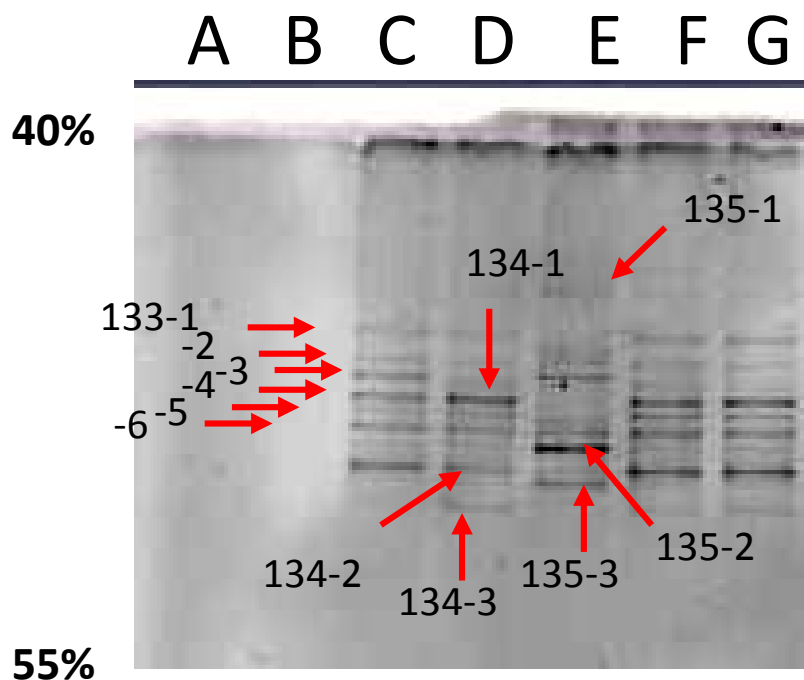
Figure 4. 9. DGGE image for the aniline-nitrate-amended sediment microcosms and first transfer enrichments from the lightly contaminated freshwater canal location. DGGE analysis is of PCR products for conserved 16S rRNA gene. Blank was labeled as A; canal native sediment sample was labeled as B; other samples were as follows: C, D, E – Canal sediment microcosms day 1342 (triplicate microcosm #133, 134, 135); F, G – Aniline-nitrate-amended enrichment day 0 (10% slurry transferred from microcosm #134, lane D).
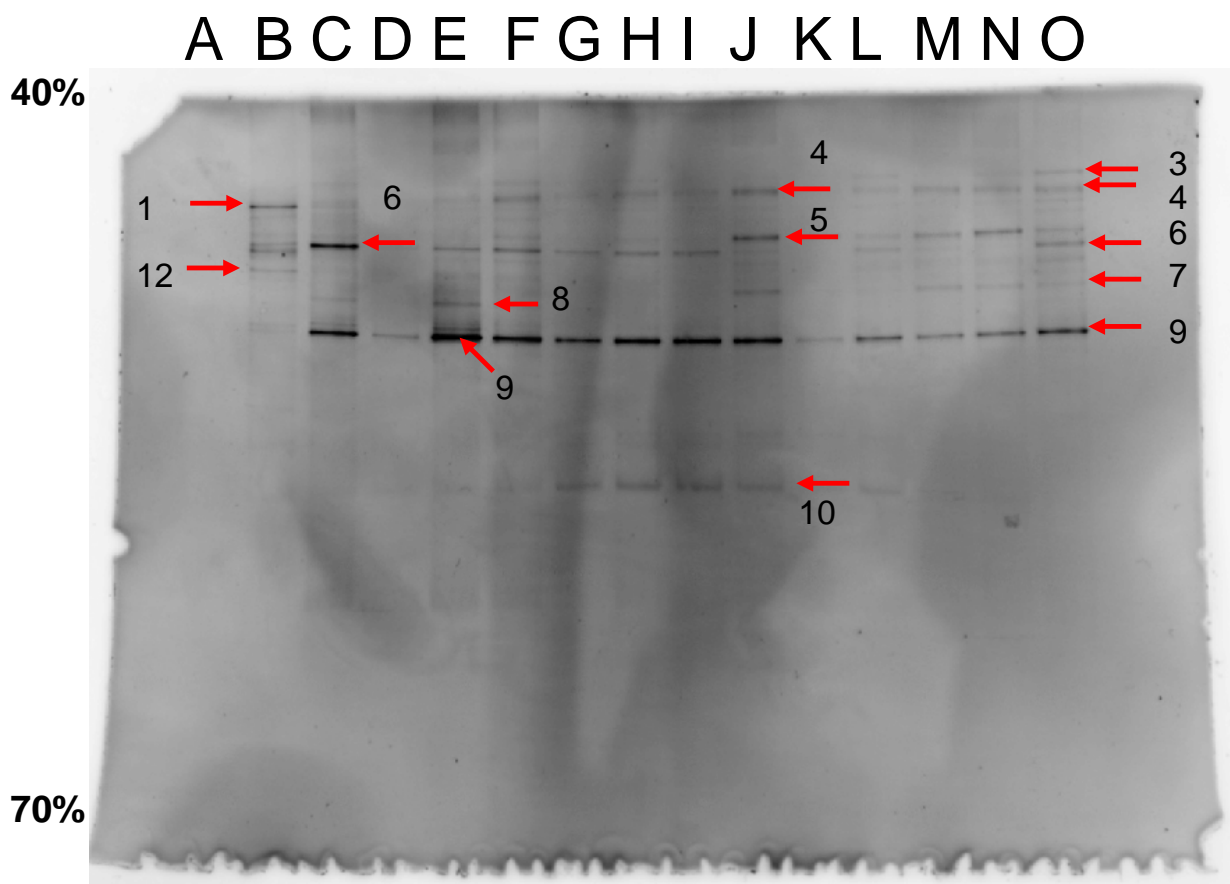
Figure 4. 10. DGGE image for aniline-nitrate-amended transfer enrichments from the

lightly contaminated freshwater canal location. DGGE analysis is of PCR

products (expected <200 bp) for conserved 16S rRNA gene. Blank was

labeled as A; other samples were as follows: B, C, D, E, F – Aniline-nitrate-

amended enrichment tripilicate #134-1 day 0, 37, 47, 57, 70 (10% slurry

transferred from canal sediment aniline-nitrate-amended microcosm #134,

inoculated w/nitrate and aniline); G, H, I – Second transfer enrichment day 0

(10% slurry transferred from #134-1, inoculated w/nitrate and aniline); J, K,

L – Second transfer enrichment day 218; M, N, O – Second transfer

enrichment day 254.
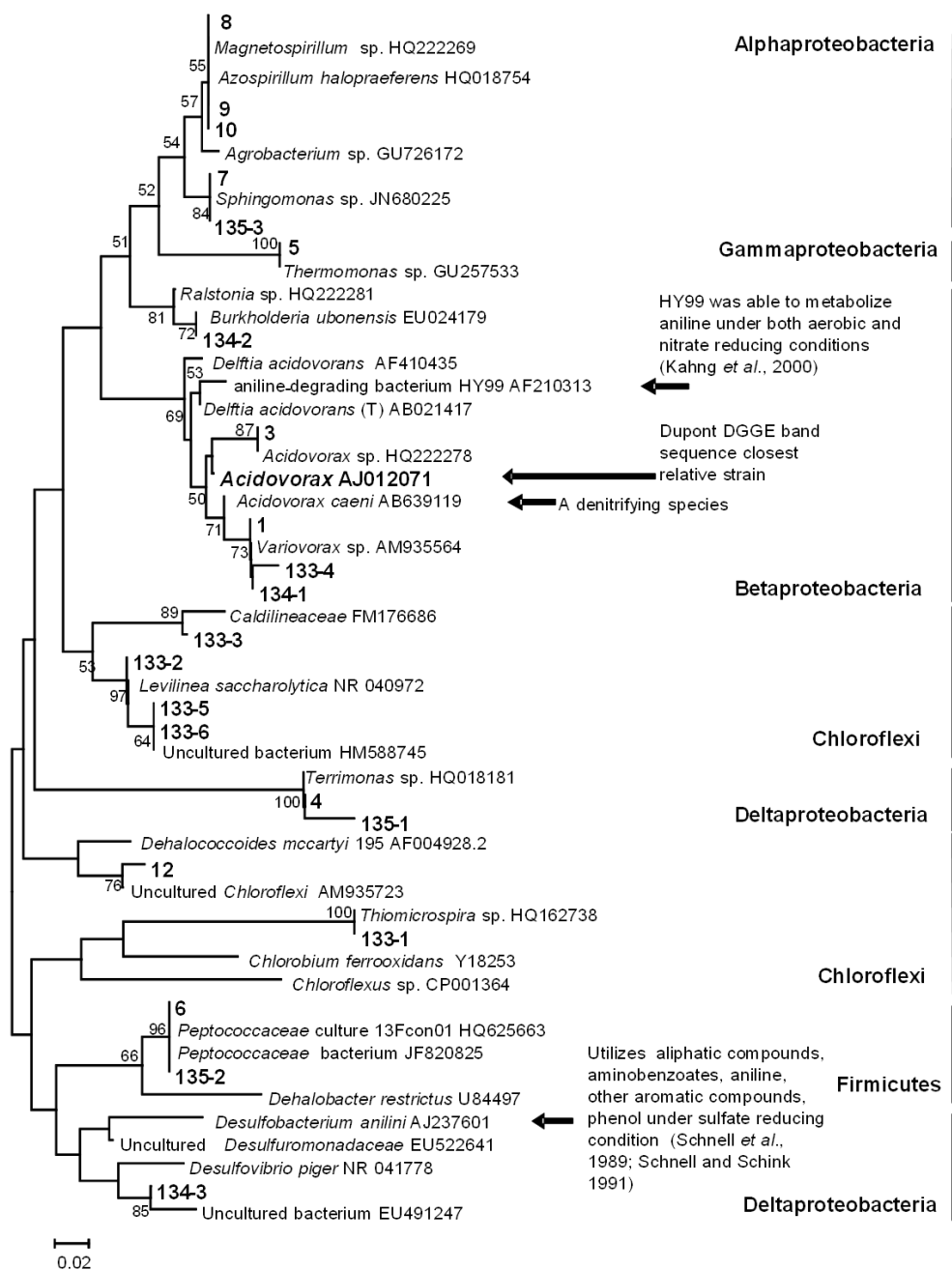
Figure 4. 11. Phylogenetic relationships between 16S rRNA genes obtained with primers

338fGC and 519r from DGGE of freshwater canal sediment, nitrate-amended

microcosms and nitrate-amended enrichments (Figure 4.9 and 4.10).
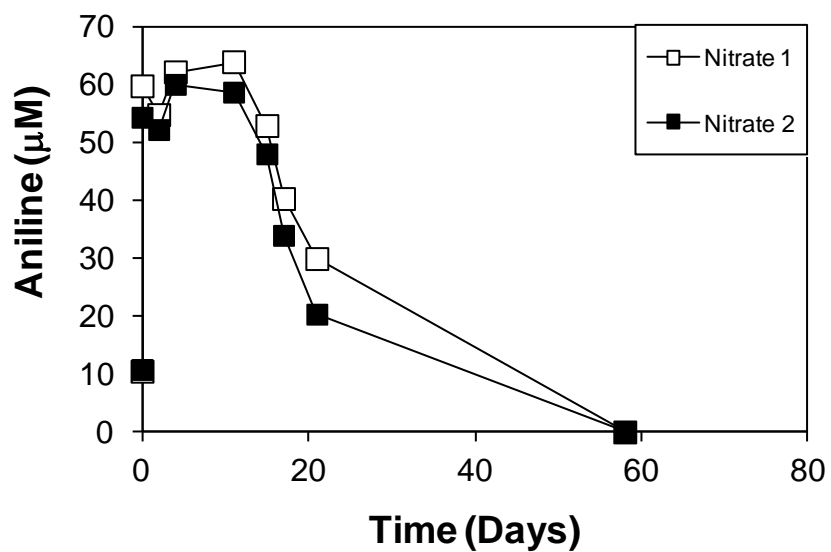
Figure 4. 12. Aniline in nitrate-amended sediment microcosms from the lightly

contaminated freshwater canal location, day 0 to 60. Sediment and canal

water samples were obtained in April 2011.
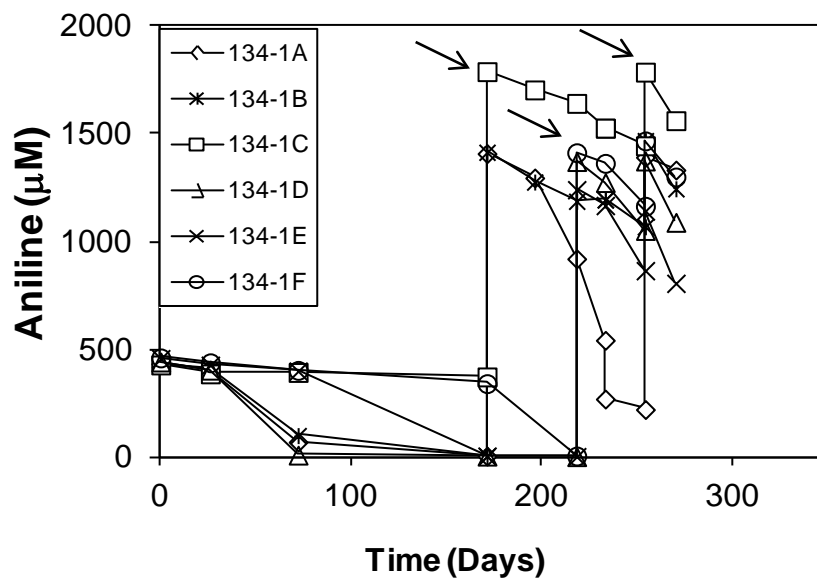
Figure 4. 13. Aniline in second transfer enrichments from nitrate-amended microcosms from the lightly contaminated freshwater canal location in Jan. 2007. The arrows indicate the re-amendment of substrate.
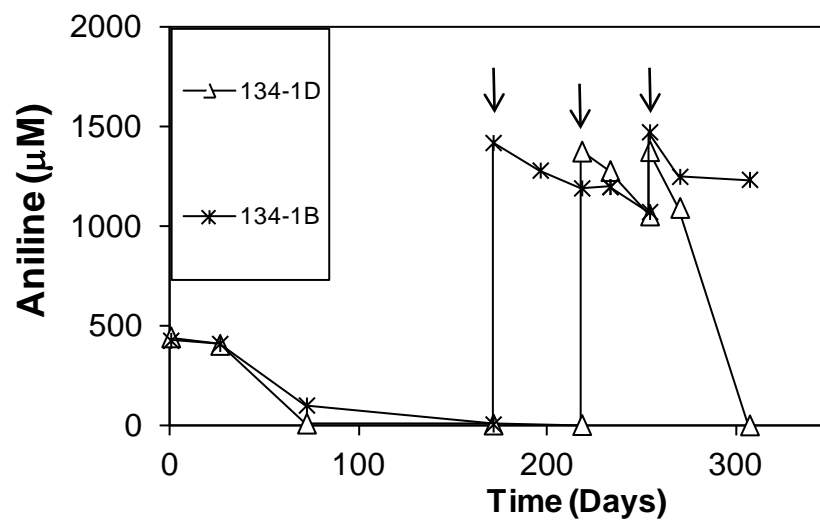
Figure 4. 14. Aniline in the live HPLC controls of second transfer enrichments from

nitrate-amended microcosms from the lightly contaminated freshwater canal

location in Jan. 2007. The arrows indicate the re-amendment of substrate.

Chapter 5   *Para*-chloroaniline Biotransformation under Methanogenic Conditions in Sediments from an Industrial Site

5.1 Abstract

Para-chloroaniline (PCA) which has been used as intermediate in the synthesis of certain drugs, dyes, pesticides and other substances is known as a pollutant of groundwater and sediments at chemical manufacturing sites. This study assessed the biological reductive dechlorination of PCA in sediments from an industrial site. Sediments were obtained from lightly and highly contaminated locations of a freshwater canal adjacent to an industrial site. In addition to PCA, the contaminated sediments contained co-contaminants such as aniline, dyes, PAHs, benzene and chlorinated benzenes. Microcosms were established under methanogenic conditions and were initially amended with 500 µM PCA. Methane production was detected indicating highly reduced conditions. After a period of inactivity, the PCA concentration was increased to approximately 1500 µM and lactate was added as a hydrogen source/electron donor. Microcosms from both lightly and highly contaminated canal sediments showed loss of

PCA and over hundreds of days of incubation and stoichiometric amounts of aniline (confirmed by gas chromatography-mass spectrometry) accumulated as a product of reductive dechlorination of PCA. The dechlorination was slow, with incubation periods over hundreds of days to accomplish conversion of PCA to aniline. Although first generation 10% transfers of the microcosm microbial communities exhibited activity, this activity slowed over time. Further, although PCA loss occurred in the transfer enrichments, no aniline was detected. Microbial community analysis using denaturing gradient gel electrophoresis detected *Dehalobacter* spp. directly from the microcosm sediment slurries, along with bacterial phylotypes associated with fermentative or syntrophic lifestyles. *Dehalobacter*-specific PCR confirmed the presence of these dehalogenators, which may be involved in PCA dechlorination at the site.

5.2 Introduction

4-chloroaniline (*para*-chloroaniline, PCA) and other choroanilines are used as precursors in the manufacture of synthetic dyes, pesticides, pharmaceutical compounds and other products (Bartha 1971; Freitag et al. 1984). PCA is a known animal carcinogen and is genotoxic (Pinheiro, Touraud, and Thomas 2004). Under aerobic conditions chloroaniline degradation has been studied extensively (Aoki et al. 1983; Bartha 1971; Bartha and Pramer 1971; Kahng et al., 2000; Nishino et al., 2000; You and Bartha 1982;

Zeyer and Kearney 1982; Latorre et al., 1984; Lyons et al., 1984). Monochloroanilines can be rapidly metabolized via catechol as an intermediate under aerobic conditions by many different bacterial species (Bachofer et al., 1975; Zeyer and Kearney 1982; Latorre et al., 1984; Spain et al., 2000; Liu et al. 2002). Several strains have been isolated which were able to degrade monochloroaniline by the *ortho*-cleavage pathway to the corresponding chlorocatechol (Radianingtyas, Robinson, and Bull 2003) (See Table 2.5 for a summary of these studies).

A mono-chlorinated compound such as PCA may be problematic in reduced groundwater aquifers and sediments. This is because lightly chlorinated compounds are often not dechlorinated by dehalorespiring bacteria as readily as more heavily chlorinated compounds (Holliger, Regeard, and Diekert 2003). This may be because of energetic considerations but is also related to presence of substrate-specific dehalogenases. For example, *Dehalococcoides mccartyi* strains (Löffler et al. 2013) have substanitial differences in substrate specificities for chlorinated ethenes. With respect to monochloroethene (vinyl chloride, VC) strain 195 (Maymo-Gatell et al. 1997) co-metabolizes VC to produce non-chlorinated ethene; strain BAV1 (He et al. 2003) dehalorespires VC to produce ethene; and strain FL2 (He et al. 2005) does not dechlorinate VC at all. For years it was thought that lightly chlorinated benzenes were not dechlorinated under anoxic conditions, but recently dichlorobenzene dechlorinating *Dehalobacter* spp. have been identified from sediment taken from the same industrial site described in our study (Nelson et al. 2011). For polychlorinated biphenyls, no bacteria have yet been identified that can accomplish complete dechlorination (Sowers and May 2013).

Several studies have reported negative results for reductive dechlorination of PCA or other monochloroanilines in reduced methanogenic enrichments or microcosms which dechlorinated higher chlorinated anilines or other halogenated aromatics such as chlorophenols or chlorobenzoates (Kuhn and Suflita 1989; Struijs and Rogers 1989; Haggblom and Young 1995; Sharak Genthner 1999; Okutman Tas and Pavlostathis 2005; Okutman Tas et al. 2006). Kazumi et al. examined biotransformation of aromatic compounds under Fe(III)-reducing conditions in Hudson River sediments but no loss of 2-, 3-, or 4-chloroanilines was observed (Kazumi et al. 1995a).

Aniline was identified as metabolite during dechlorination of dichloroanilines and PCA under aerobic conditions by *Acinetobacter baylyi* strain GFJ2 which was isolated from soil in areas where herbicides had been applied (Hongsawat and Vangnai 2011). Additionally, aniline was identified as a metabolite during dechlorination of dichloroanilines, ortho-chloroaniline and PCA under anoxic conditions in estuarine sediment (Susarla, Yonezawa, and Masunaga 1997). To date, however, there are no identified bacterial strains that dechlorinate monochloroanilines.

In this study we investigated the transformation of PCA under methanogenic conditions in freshwater canal sediments from an industrial site. We also characterized the bacterial communities in the microcosms and transfer enrichments and used a polymerase chain reaction method to detect known dehalorespiring genera in the systems.

5.3 Materials and Methods

5.3.1 Chemicals and reagents

4-chloroaniline (PCA) (sublimed, 99+ %), aniline (99.5+%, A.C.S. Reagent), 4-aminobenzoic acid (≥99%), sodium L-lactate (≥99.0%), azobenzene (99%) and pentafluorobenzaldehyde (98%) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (certified A.C.S.) was obtained from Fisher Scientific (Pittsburgh, PA). Methane (99.0%) was purchased from Matheson Tri-Gas (Montgomeryville, PA).

5.3.2 Sediment microcosm set up

Lightly and highly contaminated sediment and surface water were obtained from a freshwater canal adjacent to an industrial site in New Jersey in January of 2007. An additional sampling from the lightly contaminated location was performed in April of 2011. Canal sediments obtained in January of 2007 were believed to have become contaminated under the influence of a contaminated groundwater aquifer beneath a dyes manufacturing area adjacent to the canal. At the more highly contaminated location, the canal sediments also contained aniline, benzene, monochlorobenzene, dichlorobenzene, PAHs, dyes and other contaminants, in addition to PCA. Three 5 ft × 3. in. in diameter sediment cores in acetate sleeves were provided from both locations in the canal during the 2007 sampling event. The cores were cut into sections, labeled, capped and placed on ice for shipment overnight to Rutgers University. Canal water was provided in a 10 L

polyethylene carboy.   A grab sample of approximately 2 L of sediment from the depth

interval 0 to 0.5 ft and 10 L of canal water from the bottom of the water column were

obtained in April, 2011 by ponar recovery. Materials were shipped overnight on ice to

Rutgers University. All materials were stored at 4 °C for less than one month until use.

See Chapter 1, Section 1.2 for an extensive description of the site and the materials

obtained.

The sediment cores were composited under anoxic conditions using sterile instruments in

a disposable glove bag (Cole Parmer Instrument Company, Veron Hills, IL) under a

sterile nitrogen gas purge. After compositing, three 15 mL samples were removed per

location and placed in 15 mL polypropylene tubes at -80 °C for later microbial molecular

analysis. The remainder of the material was packed into sterile glass jars, and along with

canal water, was used to set up sediment slurry microcosms.

Canal water was purged with sterile, anoxic nitrogen prior to use. Jars of composited

sediment, site water, and autoclaved serum bottles and other materials were placed inside

the disposable glove bag in a chemical fume hood and were purged with sterile nitrogen

for 2 hours, prior to microcosm preparation.

Methanogenic microcosms were set up in triplicate in volumes of 100 mL sediment

slurry (20% composited canal sediment and 80% anoxic canal water) in sterile 160 mL

serum bottles following previously published methods (Monserrate and Haggblom 1997).

At time zero, 100 µM of PCA and 0.5 mg/L resazurin (as a redox indicator) were added

to each serum bottle based upon the final volume of 100 mL. Killed controls were

prepared identically to the methanogenic live treatment and then were autoclaved at 121

°C for 30 min on each of three consecutive days. Microcosms were incubated at 28 °C in the dark with no agitation.

On day 345 the PCA concentration was increased to approximately 1500 µM and 5 mM lactic acid was add to serve as an electron donor/hydrogen source. Re-amendment was performed on day 939.

5.3.3 Enrichment set up

Enrichment cultures were established by transferring microcosm slurry via sterile, anoxic syringes to fresh minimal medium in 160 mL serum bottles (containing 100 mL of medium) (Monserrate and Haggblom 1997). Transfers were inoculated with 10% (vol/vol) of slurry from microcosms and incubated at room temperature without agitation. No alternate electron acceptor was added so that methanogenic conditions were promoted. Methanogenic minimal medium was prepared as described previously (Fennell et al. 2004). The medium contained: 17.4 mM KCl, 1.5 mM KH_2PO_4, 20 mM NaCl, 9.3 mM NH_4Cl, 0.7 mM $CaCl_2 \cdot 2H_2O$, 0.9 mM $MgCl_2 \cdot 6H_2O$, 29.8 mM $NaHCO_3$, and 0.004 mM resazurin sodium salt. Trace salts and vitamin stock solution were also added to achieve final concentrations in the medium as follows: 0.4 µM d-biotin, 0.2 µM folic acid, 2.4 µM pyridoxine hydrochloride, 0.7 µM thiamine hydrochloride, 0.7 µM riboflavin, 2.0 µM nicotinic acid, 0.5 µM DL-calcium pantothenate, 0.04 µM vitamin B12, 1.8 µM *p*-aminobenzoic acid, 1.2 µM lipoic acid,1.3 µM 1,4-naphthaquinone, 4.1 µM nicotinamide, 0.1 µM hemin, 21.4 µM $MnCl_2 \cdot 6H_2O$, 8.1 µM H_3BO_3, 3.7 µM $ZnCl_2$, 2.1 µM $CoCl_2 \cdot 6H_2O$, 1.9 µM $NiCl_2 \cdot 6H_2O$, 2.2 µM $CuCl_2 \cdot 2H_2O$, 0.5 µM $NaMoO_4 \cdot 2H_2O$, 7.5 µM $FeCl_2 \cdot 4H2O$, 0.02 µM Na_2SeO_3 and 0.03 µM Na_2WO_4. Na_2S

stock solution was added to achieve 2.08 mM before adding the inoculum to ensure

reduced conditions.

5.3.4 Analytical methods

Microcosms were sampled for PCA and its potential dechlorination product, aniline,

initially weekly and thereafter periodically.

For analysis of PCA and its potential dechlorination product, aniline, one mL of well-

mixed slurry was withdrawn from each serum bottle using a sterile 1 mL plastic syringe

equipped with an 18 gauge stainless steel needle that had been pre-flushed with sterile

N_2/CO_2. Samples were placed in 1.5 mL Eppendorf tubes and either frozen at -20 °C or

extracted immediately. Extractions were performed using a slight modification of

previously described methods (Struijs and Rogers 1989) by adding 1 mL of acetonitrile,

mixing, and centrifuging at 10,000 rpm for 3 min. The supernatant was removed using a

plastic syringe, filtered through a 0.45 μm filter, and placed in a glass sample vial sealed

with a Teflon™-backed butyl rubber septum. Analysis was by an Agilent 1100 high

performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara,

CA) equipped with a diode array detector operating at 244 nm. Isocratic separations were

made on a Luna 5μ C18 (2) 120 column (250 ×2 mm) (Phenomenex®, Torrance, CA).

The column was held at 40°C. A water: acetonitrile (ACN) mixture (45:55 vol:vol) was

supplied at a flow rate of 0.33 mL min^{-1} as the mobile phase. PCA eluted at 3.7 min and

aniline eluted at 2.8 min. PCA and aniline were routinely identified by comparison of the

retention times to a known standards. The aniline metabolite from PCA dechlorination

was first confirmed by gas chromatography-mass spectrometry (section 5.3.5).

Concentrations in samples were quantified using a five point calibration curve. The detection limit was estimated to be approximately 0.15 μM.

Possible metabolites of anaerobic transformation of aniline were prepared as standards so that pathways could be investigated. Diphenylamine and azobenzene standards prepared in acetonitrile were detected by the same analytical method as aniline, and these potential metabolites of aniline transformation eluted at 9.6 and 6.1 min, respectively. 4-Aminobenzoate, benzoic acid and phenol standards were detected using a solvent system of acetonitrile (ACN):0.1% acetic acid buffer (25:75) supplied at a flow rate of 0.35 mL min^{-1}. Analysis was by a Agilent 1100 high performance liquid chromatography (HPLC) system equipped with a diode array detector operating at 220, 230, 254 and 280 nm. The wavelength of the fluorescence detector (FLD) was operated at: excitation wavelength (Ex) = 244, emission wavelength (Em) = 419 nm. Isocratic separations were made on a HyperClone 5μ ODS (C18) 250 × 2.00 mm 5μ (Phenomenex®, Torrance, CA) column.

Lactic, acetic, propionic and butyric acids were analyzed on a Beckman Coulter® System Gold^{TM} HPLC (Beckman-Coulter, Inc., Fullerton, CA) equipped with a Bio-Rad® Aminex HPX-87H organic acid analysis column (Bio-Rad Laboratories, Hercules, CA). UV detection was operated at a wavelength of 210 nm. The column was held at 60°C. Filtered 5.0 mM $H_2SO_4$was supplied at a flow rate of 0.6 mL min^{-1} as the eluent.

Extracted samples from certain time points collected from active microcosms were analyzed using these methods to determine if the potential metabolites could be detected.

The PCA concentrations and metabolite concentrations for each microcosm are presented

individually when replicates behaved substantially differently from each other. When

replicates behaved similarly, the average of the triplicates with error bars representing

plus or minus one standard deviation are presented.

Headspace methane content was analyzed with a gas chromatography system (Agilent

6890N G1530N network GC system city) equipped with a flame ionization detector. The

injection block was maintained at 250°C, the detector heater was maintained at 250°C

and the oven was 150°C. The column (Agilent 113-4332, GS-GasPro, capillary: 30 m x

0.32 mm I.D., Santa Clara, CA) was used with helium carrier gas at a flow rate of 1.3 mL

min^{-1}. The hydrogen flow to the flame jet was 40 mL min^{-1} and air flow to the detector

was 45 mL min^{-1}. Under these conditions, methane was eluted at 1.6 min.

5.3.5 Derivatization and gas chromatography-mass spectrometry for identification of aniline as a metabolite of PCA

A one-step derivatization and extraction of aniline and PCA were performed using

methods described previously (Chiang and Huang 2008). Derivatization transformed

aniline and PCA into products of similar chemical structure to improve GC separation.

One mL of slurry was withdrawn from each serum bottle using a sterile anoxic syringe,

placed in an Eppendorf tube and centrifuged at 10,000 rpm for 3 min. The supernatant

was removed and filtered through a 0.45 μm syringe filter into a 2 mL crimp-top vial

(VWR International, LLC, Bridgeport, NJ). Water blank samples were made from DI

water spiked with aniline and PCA. The spiked water blank and the microcosm

supernatant samples containing PCA and what was thought to be aniline were diluted to

achieve a concentration of about 0.5 μM. The pH of the spiked water blank and the

aqueous samples were adjusted to 4.2. Pentafluorobenzaldehyde dissolved in methanol (30 g/L) was used as the derivatization reagent and 0.5 mL acetone was used as the disperser solvent. These were mixed with 10 μL chlorobenzene. Chlorobenzene was used as the extraction solvent (Chiang and Huang 2008). Analytes were determined by a Hewlett Packard 6890 Gas Chromatograph (GC) coupled to a Hewlett Packard 5973 Mass Selective Detector (MSD) operating in Selective Ion Monitoring (SIM) mode and later in scan mode using a 30 m x 0.25 mm i.d. DB-5 (5% diphenyl-dimethylpolysiloxane) capillary column (J&W Scientific, Folsom, CA) with a film thickness of 0.25 µm as described previously (Du and Rodenburg 2007). Helium was used as a carrier gas at a constant flow rate of 1.0 mL min^{-1}. The initial GC oven temperature, 70°C, was held for 1 min and followed by a temperature ramp of 15°C/min to 295°C. The final temperature of 295°C was held for 1 min. The quadrupole and the source temperature were held at 150°C and 230°C, respectively. The derivatized product of aniline with an M/Z of 271 was expected based on findings reported by Chiang and Huang (2008).

5.3.6 Stoichiometry of PCA degradation

To examine stoichiometry of PCA dechlorination and aniline degradation, predicted values based on stoichiometric equations were applied to compare to those measured in degrading enrichments. The stoichiometric equations assume that aniline is completely mineralized to CO_2 (Struijs and Rogers 1989) (ignoring the production of cells) as shown in Equation 5.1:

Equation 5.1

$$C_6H_6NCl + \frac{1}{2}H_2 \rightarrow C_6H_7N + Cl^- + H^+ \text{ (Step 1)}$$

$$C_6H_7N + 7.5H_2O \rightarrow 2.5HCO_3^- + 3.5CH_4 + NH_3 + 2.5H^+ \text{ (Step 2)}$$
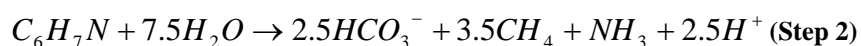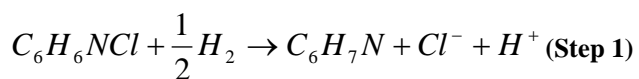
5.3.7 DNA extraction and PCR amplication of 16S rRNA genes

Total genomic DNA extraction was performed using the PowerSoil^{TM} DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's recommendation from 1 mL slurry samples from sediment microcosms and enrichment cultures, or from 0.25 to 1 g background sediment samples which had been stored at -80°C (See Section 2.1). Final DNA was eluted in 50 μL of PowerSoil^{TM} Solution C6.

The polymerase chain reaction (PCR) products for characterization of the bacterial community were generated with Bac338f-GC and Univ519r primers (See Table 4.5), resulting in an expected 200 bp DNA fragment of amplified bacterial 16S rRNA gene. Each 50 μL PCR reaction mixture contained the following: 1x PCR buffer (all reagents were obtained from USB Corp., Cleveland, OH, USA.), 2.5 mM of MgCl$_2$, 20 nmol of deoxynucleoside triphosphate, 10 pmol of each PCR primer (all primers were obtained from Integrated DNA Technology, Coralville, IA, USA), 1.5 U of Taq polymerase, and 4 μL of template DNA. 10 pmol μL^{-1} primers were made from 100 pmol μL^{-1} original stock by dilution in PCR quality water. The thermocycling program was as follows: initial denaturation at 94°C for 5 minutes, then 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds (30 cycles), and a final elongation at 72 °C for 7 minutes. Final hold was set at 4 °C for an indefinite time. The expected PCR amplicon

sizes were verified by running on a 1.5 % agarose gel. A blank (negative control) using DI water as template was run with each set of samples.

The polymerase chain reaction (PCR) products for phylotypes from specific dehalogenating bacterial genera were generated using DHB477f and DHB647r primers for *Dehalobactor* spp. or DHC774f and DHC1212r primers for *Dehalococcoides mccartyi* spp. (Table 5.1), resulting in an expected 438 bp or 170 bp DNA fragment of amplified bacterial 16S rRNA gene, respectively. Each 50 µL PCR reaction mixture contained the following: 1x PCR buffer (all reagents were obtained from USB Corp., Cleveland, OH, USA; all primers were obtained from Integrated DNA Technology, Coralville, IA, USA.), 2.5 mM of $MgCl_2$, 20 nmol of deoxynucleoside triphosphate, 10 pmol of each primer, 1.5 U of Taq polymerase and 4 µL of template DNA. 10 pmol $µL^{-1}$ primers were made from 100 pmol $µL^{-1}$ original stock by dilution in PCR quality water. The *Dehalobacter* and *Dehalococcoides*-specific PCR thermocycling program was as follows: initial denaturation at 94°C for 5 minutes, then 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds (30 cycles), and a final elongation at 72 °C for 7 minutes. Final hold was set at 4 °C for an indefinite time. The expected PCR amplicon sizes were verified by running the products on a 1.5 % agarose gel. A blank (negative control) using DI water as template was run with each set of samples. The positive control was genomic DNA extracted from a tetrachloroethene-dechlorinating enrichment from landfill material which had been confirmed to contain a *Dehalobacter* phylotype (Loudon 2011). [10 mL enrichment was centrifuged and DNA was extracted from the pellet using a PowerSoil[TM] DNA Isolation Kit (MoBio laboratories, Carlsbad, CA, USA) as described above.]

5.3.8 DGGE and sequence analysis of bands excised from DGGE gels

The PCR products generated from universal bacterial PCR primers were subjected to

Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer et al., 1993) using 8%

polyacrylamide gels with a 40 to 70% urea-formamide gradient to resolve DNA

fragments of different bacterial phylotypes. Amplified DNA products were

electrophorized for 300 min at 150 V and 60°C using a DCode mutation detection system

(Bio-Rad Laboratories, Hercules, CA, USA). DNA was visualized after 0.1% ethidium

bromide staining by UV transillumination on a Molecular Imager Gel Doc XR system

(Bio-Rad Laboratories, Hercules, CA, USA). Each prominent band detected in the DGGE

gel was excised using a sterile razor blade and placed in 20 μL of sterilized DI water

overnight at 4°C. The DNA eluted from the gel to the DI water was then used as template

DNA and re-amplified using the Bac338f and Univ519r primer set (Table 4.4).

The PCR products from the genera-specific reactions were subjected to denaturing

gradient gel electrophoresis (DGGE) according to the protocol of Muyzer *et al*. (1993).

The protocol was adapted by using an 8% polyacrylamide gel with a 50 to 65% urea-

formamide gradient for 16 h at 60 V and 60°C with a DCode mutation detection system

(Bio-Rad Laboratories, Hercules, CA, USA). DNA was visualized after 0.1% ethidium

bromide staining by UV transillumination on a Molecular Imager Gel Doc XR system

(Bio-Rad Laboratories, Hercules, CA, USA). Each prominent band detected in the DGGE

gel was excised using a sterile razor blade and placed in 20 μL of sterilized DI water

overnight at 4°C. The DNA eluted from the gel to the DI water was then used as template

DNA and reamplified using *Dehalobacter*-specific primers DHB477f and DHB647r (Table 5.1).

Following PCR, the resulting amplicons were verified by a 1.5 % agarose gel and purified with an UltraClean PCR Clean-up kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Purified PCR products were then sequenced (Genewiz Inc., South Plainfield, NJ).

5.3.9 Construction of phylogenetic trees

Sequencing results for the 16S rRNA gene fragments obtained from DGGE were analyzed and compared to the sequences in GenBank® database using BLAST® BlastN (National Center for Biotechnology Information database) (http://www.ncbi.nlm.nih.gov). Phylogenetic trees were constructed from unique sequences and the closely related sequences searched from the GenBank® database. Bootstrap values were determined from 100 iterations of the maximum likelihood calculation.

5.4 Results

5.4.1 Background concentrations

After set up of microcosms (day 0) and before spiking with PCA stock solution (day 1), samples were taken from three random microcosms and analyzed for background PCA concentrations. The concentration of PCA in freshwater canal materials was near the detection limit of HPLC method (0.15 µM). Because the headspace of microcosms was purged with nitrogen during set up, the initial background methane concentration in the headspace was assumed to be low.

5.4.2 Microcosm results

PCA concentrations in methanogenic microcosms prepared with material from the lightly contaminated freshwater canal location are shown in Figure 5.1 and 5.2. Microcosms were initially amended with 100 µM PCA and then increased to 1500 µM PCA at day 345. By day 20, microcosms showed an average of 41% loss of PCA in methanogenic live microcosms and 29% loss in methanogenic killed control microcosms. Thus, no substantial loss of PCA was noted when live microcosms were amended with 100 µM PCA without the addition of any electron donor/hydrogen source when compared to similar losses in killed controls from day 20 to day 345 (Figure 5.1).

There was an average of 1100 µM accumulated methane in methanogenic live microcosms on day 357 (See Table 3.10). There was 10 µM, 1000 µM and 50 µM of

methane detected in the headspaces of anaerobic killed controls triplicates # 1, 2 and 3 on day 357, respectively. The substantial methane production in killed control #2 indicated that autoclaving at 121°C for 30 min on each of three consecutive days was not enough to kill all microorganisms from the lightly contaminated freshwater canal site, or that killed control #2 was inadvertently re-inoculated with live microbes at some time. Note that on day 383, the killed controls were autoclaved again over three consecutive days.

After increasing the PCA concentration and adding lactate, PCA loss was observed in the methanogenic microcosms. Microcosms were amended with 1500 µM PCA on day 345. By day 427, no loss of PCA was noted and no aniline was detected in methanogenic live microcosms. By day 720, all the triplicates showed an average of 42% loss of PCA and concurrent accumulation of 370 µM of a metabolite determined to be aniline (Figures 5.2 and 5.3). There was no sampling time point between day 427 and 804 and thus the time progression of the loss of PCA is not available for that time period.

Aniline was identified using GC-MS as dechlorination product from PCA. Figure 5.3 shows the results of GC-MS analysis of the derivatized metabolite (see Section 5.3.5) and the aniline standard showing an ion with an expected an M/Z of 271 as previously described (Chiang and Huang 2008).

From day 427 to day 804, the average concentration of PCA in these active microcosms decreased from 1360 µM to 380 µM. The average aniline concentration in the active microcosms detected on day 804 was 750 µM. As the average PCA concentration dropped to 300 µM, the dechlorination slowed, and all the triplicates were re-amended with PCA on day 939. The average concentration of PCA decreased from 1300 µM to

270 µM again from day 939 to day 1281. Aniline accumulated to 1230 µM by day 1099

and then its concentration began to decrease after day 1116. On day 1281, the

concentration of aniline dropped to 720 µM indicating that the dechlorination of PCA and

transformation of aniline may have both been proceeding simultaneously in

methanogenic live microcosms. A 38.5% loss of PCA was noted in the killed controls,

which were not amended with electron donor/hydrogen source from day 345 to day 1281

(Figure 5.1). No aniline metabolite was detected in the killed controls.

Dechlorination of PCA to aniline in methanogenic microcosms from the highly

contaminated freshwater canal were also observed in two of the live triplicates. Loss of

PCA with respect to the killed anaerobic controls was difficult to discern in triplicate

microcosms prepared under methanogenic conditions (Figure B.50). However, aniline

was detected in triplicates #1 and #2 of the methanogenic microcosms. There was 285

and 507 µM aniline detected in triplicate #1 on days 890 and 1281, respectively, and

there was 404 µM, 542 µM and 187 µM aniline detected in triplicate #2 on days 890,

1116 and 1281, respectively (data not shown on graphs). No aniline was detected in

killed controls.

No benzoic acid, phenol, 4-aminobenzoate, diphenylamine or azobenzene was detected in

the live microcosms (or the concentrations were lower than 5 µM). Further no lactic acid,

acetic acid, propionic acid or butyric acid was detected in the live microcosms (or the

concentrations were lower than 0.5 mM). Thus, the fate of aniline was not established.

Aniline loss was observed in separate microcosms prepared under methanogenic

conditions amended with aniline only, using the lightly contaminated canal sediments, as

reported in Chapter 4 (Figure 4.3 and 4.4). Together, these results confirm the potential for complete loss of PCA via dechlorination to aniline and subsequent biodegradation.

Note that no loss of PCA was observed under methanogenic conditions in microcosms prepared using lightly contaminated (Figure B.6) or highly contaminated sediments from the adjacent ground water aquifer (Figure B.26).

5.4.3 Enrichments from PCA amended microcosms

Active microcosms were re-amended with PCA at least twice (along with lactate), and then enriched via 10% transfers into minmal media and additional amendment with PCA and lactate. Figures 5.4 to 5.7 show the results of PCA analysis for the enrichments. Note that of four attempts to transfer and maintain activity, two were apparently successful (PCA loss exhibited) and two showed no PCA loss. Inexplicably, at no time in the operation of the active enrichments was aniline detected as a metabolite. Further, activity slowed in the two active sets of enrichments (Figure 5.5 and 5.6) as sequential re-amendments with PCA and lactate were made. Attempts to identify other intermediates expected to be produced from aniline degradation were unsuccessful. No further analyses of these enrichments were performed.

5.4.4 Phylogenetic analysis of bacterial community in methanogenic microcosms

Bacterial community structure for microcosms dechlorinating PCA under methanogenic conditions was obtained using PCR-DGGE. Figure 5.8 shows the DGGE image, and Table 5.2 shows the identification of the detected phylotypes for the PCA-dechlorinating methanogenic microcosms after prolonged incubation (day 1046). Bands 7, 8 and 12 are

phylotypes with high similarity to *Clostridium* spp. (100% similarity to *Clostridium crotonatovorans*). Clostridia produce hydrogen during fermentation, which can be further used by methanogens to produce methane under anaerobic conditions [See for example (Wang *et al*. 2003)] and, could also support hydrogen-using dehalogenators.

DGGE band 10 had 100% similarity with uncultured *Pelospora* sp. isolated from a fatty acid-oxidizing consortium (Chauhan and Ogram, 2006). Bacteria from the genus *Pelospora* belong to the family Syntrophomonadaceae, which are also well-known for producing hydrogen by oxidizing short-chain carbon compounds during syntrophic association with hydrogen users. For example, *Pelospora glutarica* strain WoGl3T grows using glutarate, methylsuccinate and succinate (Matthies *et al*. 2000). It is possible that both these phylotypes could be involved in the fermentation of lactate. The phylotype represented by DGGE band 9 is closely related to an uncultured *Dehalobacter* sp., which was isolated from a 1,2-dichloroethane contaminated aquifer (Marzorati et al. 2010). This was the only dehalogenating phylotype detected by DGGE.

The PCR-DGGE analyses for the dehalorespiring bacterial genera *Dehalococcoides*, did not result in amplification of a product—no bands were observed during PCR-DGGE analysis for the lightly contaminated freshwater canal sediment, methanogenic PCA microcosms (data not shown). However, amplification with *Dehalobacter*-specific primers produced a DGGE band with a sequence that matched to *Dehalobacter* spp. (Figure 5.9). The direct detection suggests that *Dehalobacter* was present as one of the dominant bacteria in the microcosms and confirmed the direct detection by DGGE using universal 16S rRNA gene primers (Figure 5.8). A phylogenetic tree relating the

Dehalobacter phylotype detected via 16S rRNA gene-based DGGE to closely related

species is shown in Figure 5.10. Sequences (127 bp, Table 5.3) were compared by

BLAST® to sequences existing in the GenBank® database (Table 5.4). The

Dehalobacter control DNA was provided by Ms. Jennifer Loudon of Rutgers University,

Department of Environmental Sciences. [Ms. Loudon enriched and transferred

dehaloginating bacteria from a NJ leachate recirculating bioreactor landfill by feeding the

enrichment with PCE and butyric acid (Loudon, 2011).]

The phylogenetic analysis of the 16S rRNA gene sequences obtained with *Dehalobacter*-

specific primers DHB477f and DHB647r confirmed the detection of *Dehalobacter*. By

comparing with reference species, DGGE bands 151, 152 and 153 matched closely with

Dehalobacter spp. The sequence of bands 151 and 153 had 100% similarity with

Dehalobacter sp. MS. The sequence of band 152 and the *Dehalobacter* control had 99%

and 100% similarity with the type strain *Dehalobacter restrictus* strain PER-K26,

respectively. *Dehalobacter restrictus* PER-K26 was isolated from tetrachloroethene-

dechlorinating, Rhine River sediment mixed with ground anaerobic granular sludge

(Holliger et al. 1998) and was reported to be able to couple oxidation of dihydrogen and

reductive dechlorination of PCE to growth (Smits et al. 2004). *Dehalobacter* sp. strain

MS is reported to grow during reductive dechlorination of chloroform to dichloromethane

(Justicia-Leon et al. 2012). *Dehalobacter* is a strictly anaerobic bacterium that

reductively dechlorinates chloroethenes, chloroethanes, chloromethanes and

chlorocyclohexanes, and is implicated in the dehalogenation of PCBs and polychlorinated

dibenzo-*p*-dioxins (PCDDs). An important recent finding was the enrichment of

Dehalobacter spp. from the same industrial site, and from sediments near the same

location as we report in this dissertation. The *Dehalobacter* detected previously dechlorinate dichloro- and mono-chlorobenzene (Nelson et al. 2011), which are also important contaminants at the site.

In our experiments, two different PCR primer sets confirmed detection of *Dehalobacter* sp. from the microcosms where PCA was dechlorinated to aniline. To conclusively link *Dehalobacter* to this activity would require additional experimentation, and successful transfer of the activity.

5.5 Discussion

Microcosms were established under methanogenic conditions and initially amended with 500 µM PCA and then later increased to 1500 µM PCA. Microcosms from lightly and highly contaminated canal sediments showed no loss of PCA when amended with 500 µM PCA without an additional electron donor/hydrogen source. After increasing the PCA concentration and adding lactate to a final concentration of 5 mM to serve as an electron donor/hydrogen source, PCA loss was observed. Stoichiometric amounts of aniline accumulated as a product of reductive dechlorination of PCA, as confirmed by HPLC and GC-MS analysis. Aniline loss was also observed in these and separate microcosms prepared under methanogenic conditions using the lightly and highly

contaminated freshwater canal sediments (See Chapter 3). This suggests that anaerobic dechlorination of PCA and subsequent degradation of aniline could occur under methanogenic conditions in the freshwater canal sediments. A major question and concern about the observed activity is the extremely long lag times and slow rates that were observed in the microcosms. Further, first generation transfers of the activity were successful only half the time. Additionally aniline was not detected in the active PCA degrading enrichments and the rates of PCA loss in those enrichments that did show activity slowed as the number of PCA re-amendment events increased.

Dehalobacter was detected by PCR-DGGE in the PCA-dechlorinating microcosms and confirmed by sequencing. *Dehalococcoides* spp. was not detected using *Dehalococcoides*-specific PCR primers. *Dehalobacter* spp. was detected and enriched from chlorobenzene-dechlorinating microcosms from the same industrial site (Nelson et al. 2011). Finding an association of *Dehalobacter* with PCA dechlorination in the current study confirms that *Dehalobacter* spp. may be important mediators of pollutant biotransformation at this industial site.

One previous study has reported aniline as metabolite during dechlorination of dichloroanilines, ortho-chloroaniline and PCA under anoxic conditions in estuarine sediment (Susarla, Yonezawa, and Masunaga 1997). This is despite many reports over the years of dechlorination of higher chlorinated anilines in a variety of environmental media (Struijs and Rogers 1989; Häggblom and Young 1995; Kuhn et al. 1990; Sharak Genthner 1999; Tas and Pavlostathis 2005; Tas et al. 2006). The lack of documented PCA-dechlorinators, few reports of PCA dechlorination (one other study beside this one)

and the slow rates of dechlorination observed here, suggests that this activity may be rare in the environment or that the proper environmental conditions have not been maintained in laboratory systems such as these microcosms that allow cultivation of the organisms.

As mentioned in Chapter 3, additional microcosms were observed to produce aniline from PCA—for example in Fe(III)-amended microcosms prepared with material from the lightly contaminated freshwater canal location (Figures B.30 and 3.22). These systems were not analyzed further, but provide supporting evidence that this activity could be intrinsic at this site.

The observation of this activity is novel and further study of the capability of *Dehalobacter* from this industrial site is warranted.

Table 5. 1. *Dehalobacter* and *Dehalococcoides-* specific 16S rRNA gene primers used

for PCR-DGGE analysis.

Primer	Sequence (5'→ 3')	Reference
DHB [a] 477f	GATTGACGGTACCTAACGAGG	(Grostern and Edwards 2006)
DHB647r	TACAGTTTCCAATGCTTTACGG	(Grostern and Edwards 2006)
DHC774f	GGGAGTATCGACCCTCTC	(Hendrickson *et al.* 2002)
DHC1212r	GGATTAGCTCCAGTTCACACTG	(Hendrickson *et al.* 2002)

[a] DHB, *Dehalobacter*; DHC, *Dehalococcoides*.

Table 5. 2. Reference sequences, similarity, accession number and isolation source for matches to bacterial 16S rRNA gene sequences obtained with primers 338fGC and 519r from PCR-DGGE analysis of methanogenic PCA-amended sediment microcosms.

DGGE band name	Close relative	Similarity (%)	Accession number	Reference	Isolation source
6	methanogenic bacterium enrichment culture clone	99	FJ802166.1	(van der Zaan et al. 2009)	Ebro River Delta sediment
7	*Clostridium* sp. C5S17	99	AB539906.1	Unpublished	rice paddy field soil
8	uncultured *Clostridium* sp.	100	FR774831.1	Unpublished	Soil
9	uncultured *Dehalobacter* sp.	97	FM204994.1	(Marzorati et al. 2010)	1,2-dichloroethane contaminated aquifer
10	uncultured *Pelospora* sp.	100	DQ173910.1	(Chauhan and Ogram 2006)	Wetland
12	*Clostridium crotonatovorans*	100	AY742899.1	Unpublished	Anaerobic granular sludge

Table 5. 3. Sequences obtain via *Dehalobacter*-specific PCR using primers DHB477f

and DHB647r from canal sediment methanogenic microcosms.

Band name	Length (bp)	PCR products, sequence obtained with primers DHB477f and DHB647r
151	127	GCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATCATT GGGCGTAAAGGGCGCGTAGGCGGGCTTATAAGTCTGATGTGAAAG TGCGGAGCTTAACTCCGTAAAGCATTGGAAACTGTAA
152	127	GCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATCATT GGGCGTAAAGGGCGCGTAGGCGGCTATATAAGTCTGATGTGAAAG TGCGGAGCTTAACTCCGTAAAGCATTGGAAACTGTAA
153	127	GCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATCATT GGGCGTAAAGGGCGCGTAGGCGGGCTTATAAGTCTGATGTGAAAG TGCGGAGCTTAACTCCGTAAAGCATTGGAAACTGTAA
Dehalobacter '+' control	127	GCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATCATT GGGCGTAAAGGGCGCGTAGGCGGCAATATAAGTCTGATGTGAAAG TGCGGAGCTTAACTCCGTAAAGCATTGGAAACTGTAA

Table 5. 4. Reference sequence, similarity, accession number and isolation source for

matches to sequences obtained with primers DHB477f and DHB647r from

PCR-DGGE of PCA-amended freshwater canal sediment microcosm

bacterial community analysis.

DGGE band name	Close relative	Similarity (%)	Accession number	Reference	Isolation source
151	*Dehalobacter* sp. MS	100	DQ663785.1	(Grostern and Edwards 2006)	NA
152	*Dehalobacter restrictus* strain PER-K26	99	NR_026053.1	(Smits *et al.* 2004)	Anaerobic bacterium; couples oxidation of dihydrogen and reductive dechlorination of PCE to growth; type strain of *Dehalobacter restrictus*
153	*Dehalobacter* sp. MS	100	DQ663785.1	(Grostern and Edwards 2006)	NA
Dehalobacter '+' control	*Dehalobacter restrictus* strain PER-K26	100	NR_026053.1	(Smits *et al.* 2004)	Anaerobic bacterium; couples oxidation of dihydrogen and reductive dechlorination of PCE to growth; type strain of *Dehalobacter restrictus*
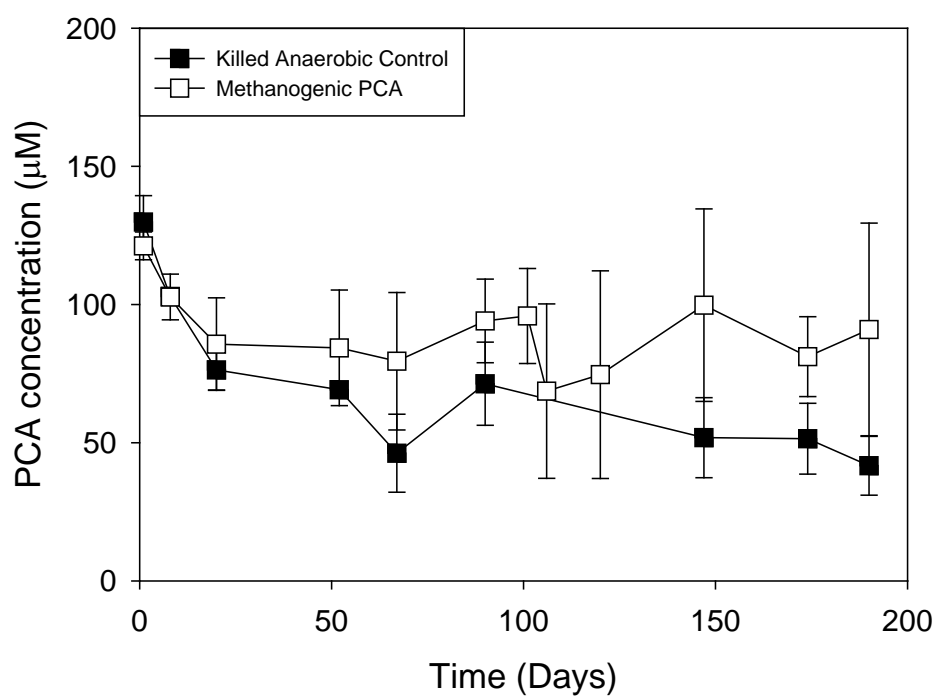
Figure 5. 1. PCA in methanogenic and killed anaerobic microcosms from the lightly

contaminated canal location, day 0 to day 190. Symbols are averages of

triplicate microcosms and error bars are one standard deviation.
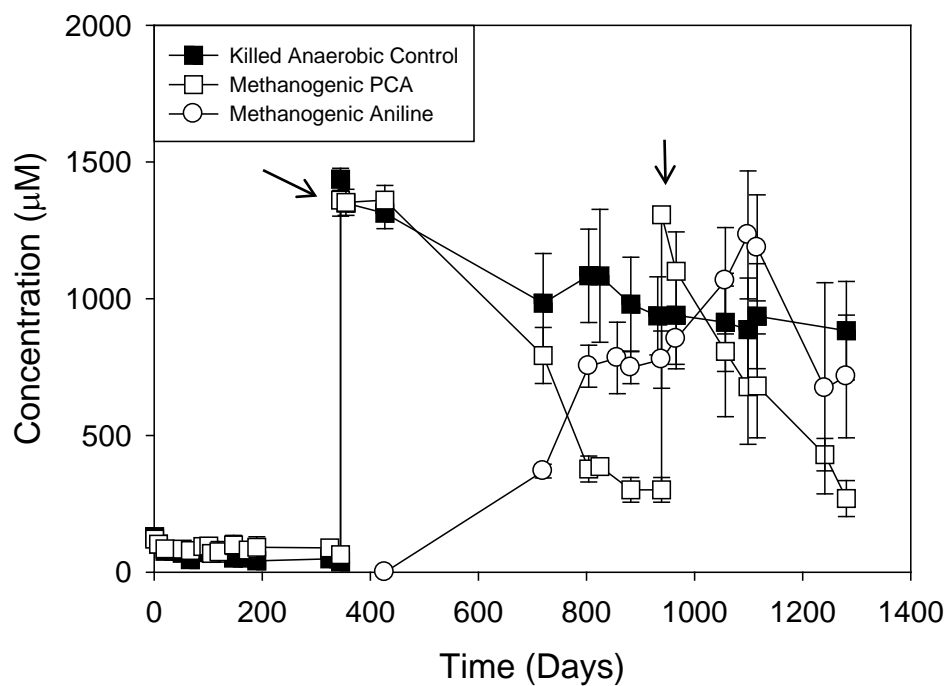
Figure 5. 2. PCA and aniline in methanogenic and killed anaerobic microcosms from the

lightly contaminated freshwater canal location, day 0 to day 1281. Symbols

are averages of triplicate microcosms and error bars are one standard

deviation. The arrow indicates the re-amendment of PCA.
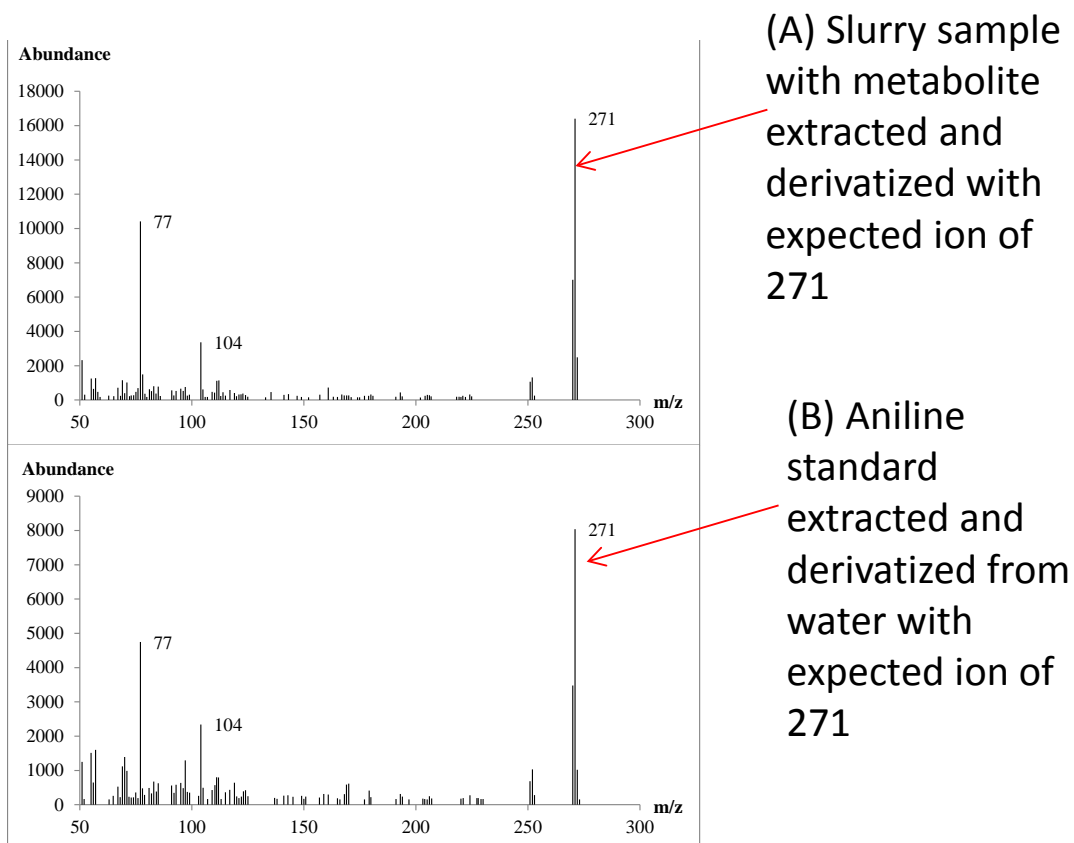
Figure 5. 3. Comparison of GC-MS output for derivatized aniline standard to derivatized

metabolite produced in methanogenic microcosms from the lightly

contaminated freshwater canal location.
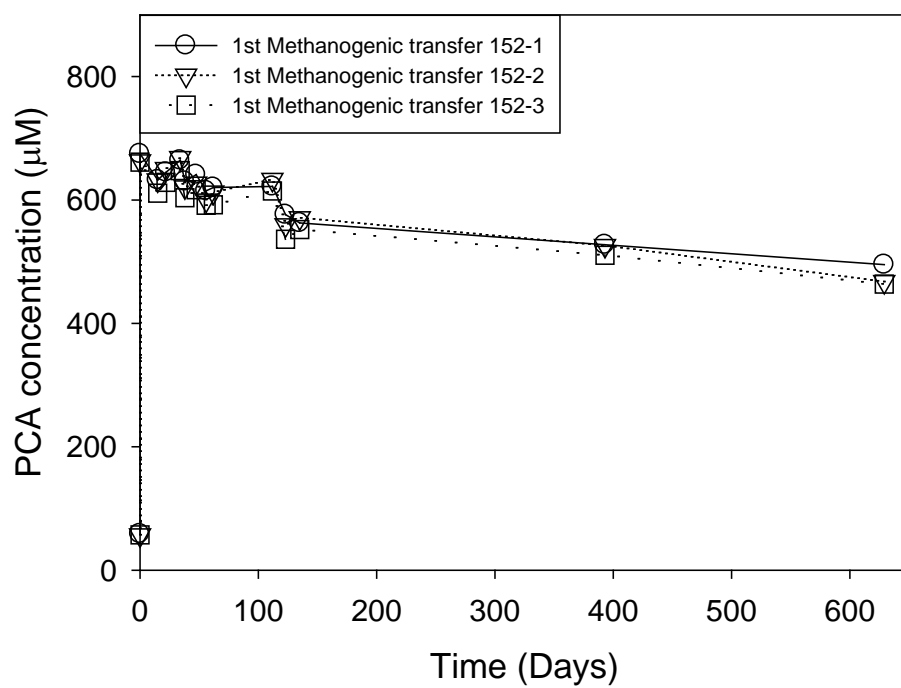
Figure 5. 4. PCA in methanogenic enrichment established by transferring from triplicate

microcosm #2 on day 1007 from the lightly contaminated freshwater canal
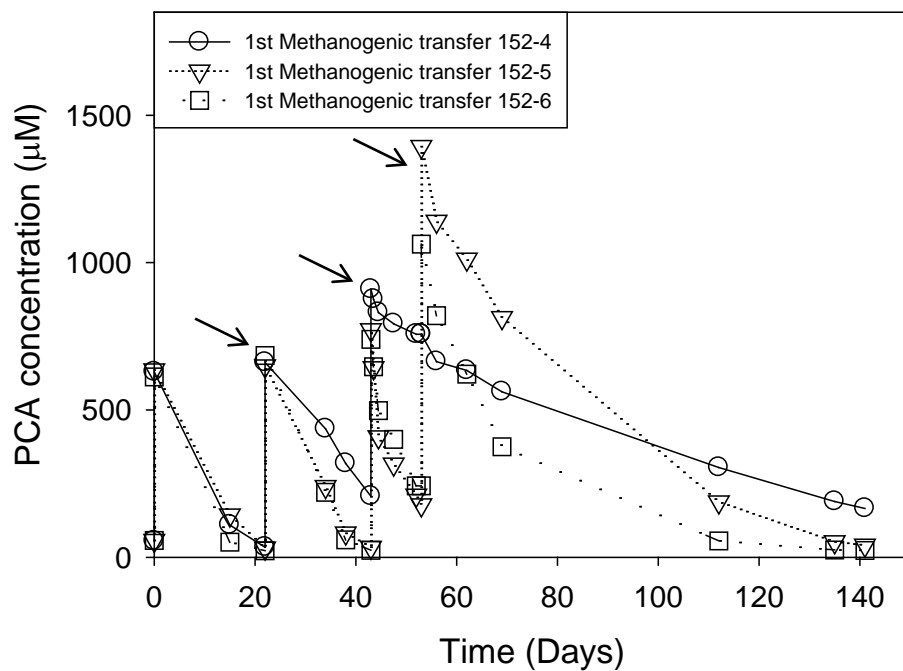
location.

Figure 5. 5. PCA in methanogenic enrichment established by transferring from triplicate

microcosm #2 on day 1007 from the lightly contaminated freshwater canal

location. The arrows indicate the re-amendment of PCA.
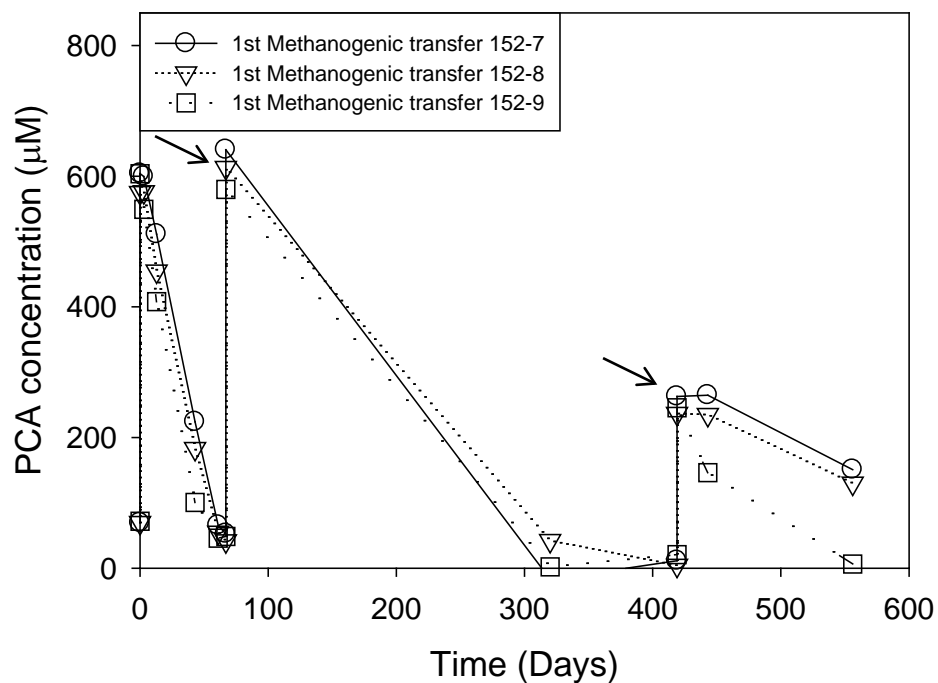
Figure 5. 6. PCA in methanogenic enrichment established by transferring from triplicate

microcosm #2 on day 1080 from the lightly contaminated freshwater canal

location. The arrows indicate the re-amendment of PCA.
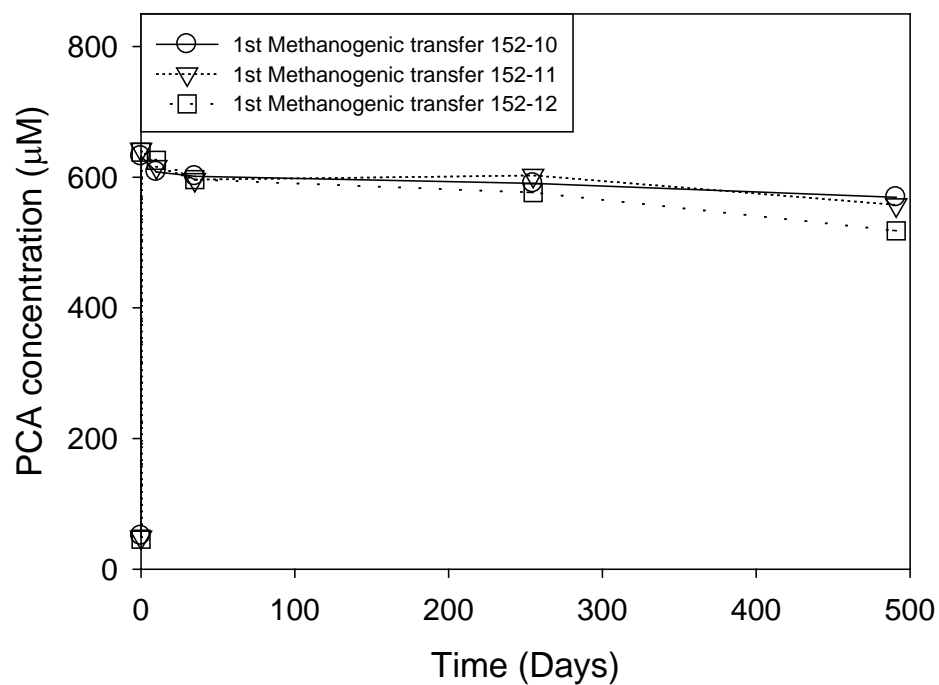
Figure 5. 7. PCA in methanogenic enrichment established by transferring from triplicate

microcosm #2 on day 1145 from the lightly contaminated freshwater canal
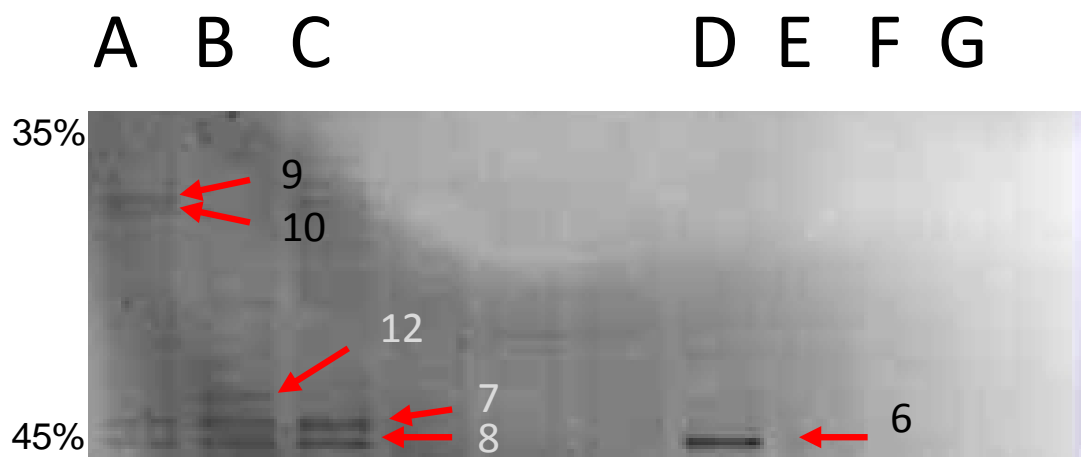
location.

Figure 5. 8. DGGE of PCA-methanogenic sediment microcosm samples from lightly and

highly contaminated freshwater canal background location. DGGE analysis is

of PCR products for conserved bacterial 16S rRNA gene. Blank was labeled

as G; other samples were: A,B,C – Lightly contaminated freshwater canal

sediment (methanogenic) day 1046; D,E,F – Highly contaminated freshwater

canal sediment (methanogenic) day 932.
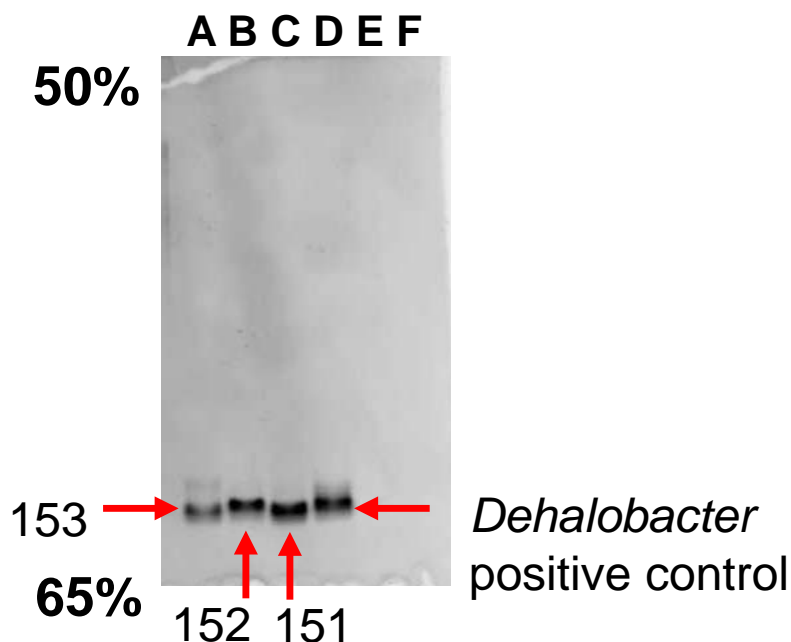
Figure 5. 9. DGGE of PCA-methanogenic microcosm samples from the lightly

contaminated freshwater canal location. DGGE analysis is of *Dehalobacter*-

specific primer amplified PCR products for conserved 16S rRNA gene.

Blank was labeled as F; canal native sediment sample was labeled as E; other

samples were as follows: A, B, C – Lightly contaminated freshwater canal

location microcosms established under methanogenic conditions with PCA

and lactic acid amendment-Day 1046; D– *Dehalobacter* control from landfill

microcosm enrichment (Loudon, 2011). 151, 152 and 153 refer to PCA-

methanogenic triplicate microcosms #1, 2 and 3, respectively.
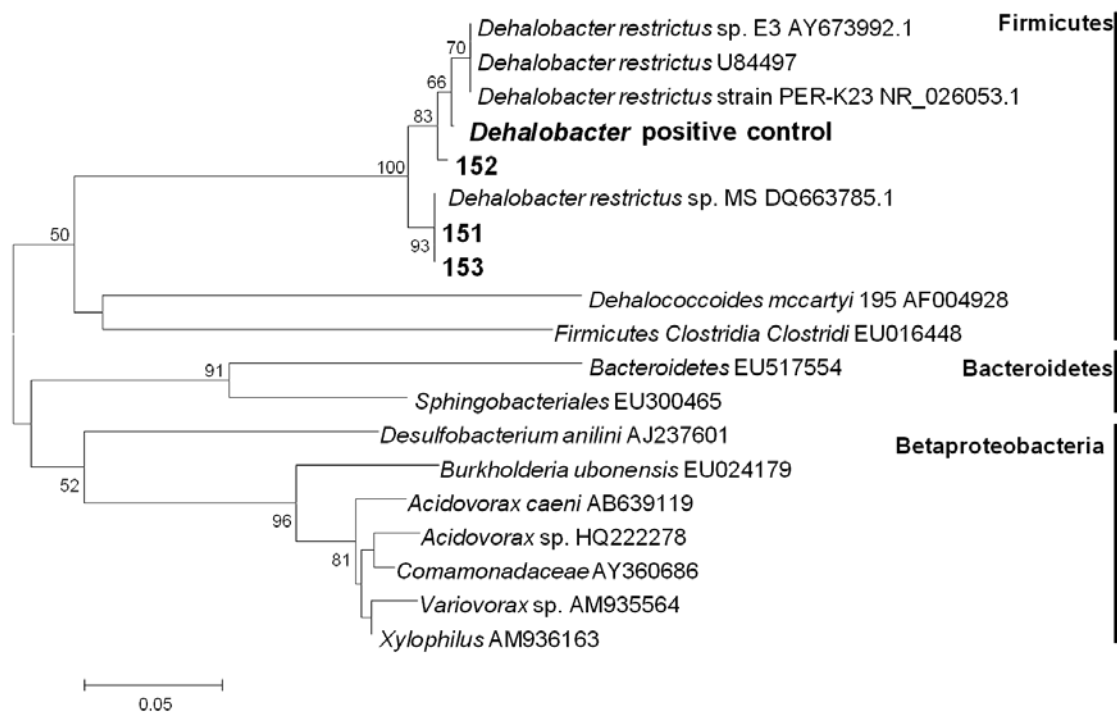
Figure 5. 10. Sequencing analysis confirming detection of *Dehalobacter* and showing

phylogenetic relationships.

Chapter 6 Summary and Environmental Implications

6.1 Summary

This study describes research on microbial transformations of aniline and *para*-chloroaniline (PCA) under anoxic conditions in freshwater canal sediments and groundwater aquifer solids recovered from a chemical industrial site. Additionally, a mixed culture containing *Dehalococcoides mccartyi* strain 195 was tested for ability to dechlorinate chlorinated anilines.

In Chapters 3 through 5, microcosm studies using four types of environmental matrix—lightly and heavily contaminated freshwater canal sediments and lightly and heavily contaminated aquifer sediments—from the industrial site are described. Figure 6.1a and 6.1b summarize the most effective treatments for aniline and PCA when added at both 100 µM and 1500 µM. The results for the lightly contaminated groundwater aquifer location are summarized in Figure 6.1a because of its distinctly different geographic

location. All three other locations are presented in Figure 6.1b. The treatments are

considered as most effective if loss of substrate was observed for all three triplicate

microcosms set up using materials from the location. These treatments are labeled on

each location on Figure 6.1a and 6.1b. For example, Figure 6.1a shows that lightly

contaminated groundwater aquifer sediment microcosms showed loss of 100 µM aniline

under aerobic, nitrate-amended, Fe (III)-amended and sulfate–amended conditions from

all three triplicates (refer to Chapter 3, Table 3.3 and 3.4). Those treatments with one or

more of the triplicates not showing loss of substrate were not considered significantly

different from the killed control and are not labeled on maps. This consideration was

based on a nonparametric one sample sign test. This test makes no assumption about the

shape of the population distribution. The null hypothesis is that the proportion of the

active microcosms among the triplicate microcosms was assumed to be 0.5. The

alternative hypothesis is that the proportion of the active microcosms among the triplicate

microcosms was greater than 0.5. The p value of the binomial random variable with a

probability mass function is estimated based on Equation 6.1.

$$P(X = x) = \binom{n}{x} p^x (1-p)^{n-x}, x = 0, 1, ..., n.$$

(Equation 6.1)

where:

p is the proportion of the active microcosms among the triplicate microcosms under the

null hypotheses; n is the sample size and x is the observed active microcosm numbers

among the triplicate microcosms. The problem of the test is that the sample size is too

small (n=3), so there is an increased risk of a Type II error—an error that occurs when

one accepts a null hypothesis that is actually false.

Results presented in Chapter 3 showed that aniline and PCA in sediment and aquifers were amenable to biological treatment under a variety of conditions. Groundwater aquifer and freshwater canal sediment microcosms showed loss of aniline under nitrate-, Fe (III)- and sulfate–amended conditions, and PCA loss under nitrate-amended conditions. PCA loss was also observed in sediments from the lightly and heavily contaminated canal site under methanogenic conditions after prolonged incubation (from day 427 to 720) and an increase in the PCA concentration (from 100 µM to 1500 µM), accompanied by addition of the electron donor/hydrogen source, lactate. PCA loss was not observed under methanogenic conditions in microcosms prepared using aquifer sediments from either location.

In Chapter 4, aniline loss under nitrate-amended conditions in microcosms prepared using sediments from the lightly contaminated location is described in more detail. The loss was substantial compared to loss in killed controls, and repeated re-amendments were consumed. Further, this activity was observed in 10% first and second transfers to fresh minimal medium. Attempts to track the fate of the aniline via a mineralization test resulted in low recovery (5 to 10 %) of $^{14}CO_2$ from added ^{14}C-aniline under nitrate-amended conditions for both freshwater canal sediment microcosms and enriched cultures. Additionally, no volatile fatty acids, benzoate, phenol, 4-aminobenzoate, diphenylamine, azobenzene or any other major theoretically possible metabolites were detected under nitrate-amended conditions from either freshwater canal sediment or enrichments. Although degradation was not conclusively determined, phylotypes detected via bacterial community analysis of the nitrate-aniline microcosms and enrichments do

cluster with known denitrifying genera *Variovorax* and *Acidovorax*. Further, the

dominance of a phylotype closely related to *Magnetospirillum* was observed in the

aniline-nitrate enrichments (Figure 4.10). *Magnetospirillum* are known to reduce nitrate

and contain genes to degrade toluene, phenol, benzoate and other aromatic compounds

(Shinoda et al. 2005). While it seems likely that these organisms were maintained in the

microcosms and transfers by degradation of added aniline; it cannot be ruled out that

other contaminants may have been present in the original sediment recovered from the

field site and these may have supported the growth of bacteria.

In Chapter 5, the biological reductive dechlorination of PCA in both lightly and heavily

contaminated freshwater canal sediments under methanogenic conditions was reported.

PCA disappearance was concurrent with production of stoichiometric amounts of aniline.

This transformation occurred in all three replicates, but was exceedingly slow, occurring

over months and only after increasing the PCA concentration from 500 μM to 1500 μM

with addition of lactate. Dechlorination of PCA to aniline was also observed in two

replicates of the methanogenic heavily contaminated canal sediment microcosms. Aniline

was detected in triplicate #1 and #2 of the methanogenic microcosms. A concentration of

285 and 507 μM of aniline was detected in triplicate #1 on days 890 and 1281,

respectively. Aniline at 404 μM, 542 μM and 187 μM was detected in triplicate #2 on

days 890, 1116 and 1281, respectively. Loss of PCA also occurred in two anaerobic

killed controls prepared under methanogenic conditions (Figure B.50). However, no

aniline was detected in the killed controls.

Dehalobacter spp. was detected in methanogenic PCA-dechlorinating microcosms

through PCR-DGGE analysis with either universal bacterial or *Dehalobacter* specific 16S rRNA gene primers. This suggests that these organisms were major bacterial community members under these conditions. Recently, the involvement of *Dehalobacter* spp. in the dechlorination of chlorobenzenes at the same site was reported (Nelson et al. 2011). Transfer enrichments of PCA-dechlorinating microcosms did show PCA loss, however, aniline production was not detected. Further investigation should be performed to conclusively link PCA dechlorination to *Dehalobacter*. Other bacterial phylotypes detected in the PCA-dechlorinating microcosms were *Clostridium* and *Pelospora*. These phylotypes are indicative of a fermentative environment that should have been brought about by the addition of lactate. Since aniline loss was also observed in separate microcosms prepared under methanogenic conditions as discussed in Chapter 3, results from this study support the possibility for complete dechlorination of monochloroaniline to aniline and subsequent degradation of aniline under anoxic conditions. In the absence of conclusive knowledge of the fate of aniline in these systems, and the slow rates of transformation of PCA, however, further information is needed to confirm biodegradation of aniline and thus the complete transformation of PCA.

Although it could not be concluded which bacteria or degradation pathways were directly responsible for the observed aniline and PCA loss under the various redox conditions, this study provided information about the presence of denitrifying bacteria, sulfate-reducing bacteria, dehalogenating bacteria and fermenting bacteria in nitrate- or sulfate-amended microcosms, and dechlorinating, methanogenic microcosms, respectively. The results also confirmed that the consumption of nitrate or sulfate was associated with aniline or PCA loss. Further, methane production was highest in microcosms operated

under methanogenic conditions, except for those microcosms operated under Fe(III)-amended and sulfate-amended conditions after hundreds of days of operation. Methane productions in these systems are suggestive of methanogenic niche formation as the other terminal electron acceptors were depleted.

In Figure 5.8, more diversity was observed in lightly contaminated freshwater canal sediment than in highly contaminated freshwater canal sediment, but this figure is not clear and this comparison was made after the sediment microcosms were incubated for more than a thousand days. Thus the community profile at this timepoint may not represent the background microbial communities. This research shows that aniline and PCA biotransformation capacity was present in different sediments at the site, and that biodegradation potential associated with different microbial communities in sediments varies with the geochemical properties of the sediments. Addition of aniline, PCA and the electron acceptors may alter the composition of microbial communities in sediments and groundwater.

Several issues of concern were noted in this study that should be addressed in further investigations of the site. For example, only half of the attempts to transfer the aniline-nitrate-amended microcosms to produce enrichments were successful. Further, this activity was not robust and although some second transfers were successful, the activity was eventually lost and no isolation of pure cultures was achieved. Eriksson et al. (2003) reported that most successful enrichment cultures that degrade PAHs under anoxic conditions were obtained from contaminated sediments and not from contaminated soils because of the low abundance of anaerobic PAH degraders in aerobic soils. There were

unsuccessful enrichment transfers from groundwater aquifer and freshwater canal sediment in our study. Here we hypothesis that the low abundance of aniline degraders caused the unsuccessful transfers.

No loss of the PCA at the lower concentration level (<200 μM) under methanogenic conditions suggests that the bacterial enzymes might work best at a higher concentration of PCA (1500 μM) or that the correct enzymes may not be induced at the lower PCA concentrations. Enrichments on PCA under methanogenic conditions were successful, but the activity slowed over time, the dechlorination product, aniline was not detected, and additional transfers were not successful.

Different anoxic enrichment medium were formulated for transferring from aniline or PCA amended microcosms in an attempt to achieve successful transfers. These manipulations did not appear to increase the success rates. Low populations in the initial microcosms may have caused difficulties in enrichment transfers. Another reason might be that a broad diversity of microbial community members in the initial microcosms provided support to remove end-products of initial reactions more efficiently, and this need was not provided in the 10% transfers. Periodical replacement of culture medium with fresh medium was designed for end-product removal; however, most of the first enrichment transfers were not activated by the first aniline or PCA amendment. The addition of a reducing agent in enrichments was used to ensure that the observed activity was not related to oxygen leakage and establishment of microaerophilic conditions. However, in this study, addition of Na_2S as a reducing agent did not enhance of transfer activities for nitrate-amended medium.

Because the intermediates of aniline degradation were not detected and tests of mineralization resulted in recovery of low amounts of CO_2, the main evidence for biological activity being responsible for aniline loss depends heavily on observation of loss in live microcosms versus killed controls. In the case of aniline loss under nitrate-amended and methanogenic conditions in the lightly contaminated canal sediments (Chapter 4), this was clearly observed. Although some loss occurred in killed controls, compared to the robust loss of multiple re-amendments of aniline in the live microcosms (See Figure 4.2, 4.3 and 4.4), it could be reasoned that the loss in killed controls is dominated by different mechanisms than that in live microcosms (i.e., abiotic versus biotic). A second example of this observed phenomenon that supports biological activity is shown in Figure 3.15 for the heavily contaminated groundwater aquifer location. Aniline concentration in methanogenic live microcosms was relatively stable for a greater than 100 day lag period, then relatively rapid loss of aniline beginning at different times in different replicates was observed. In contrast the aniline concentration in the corresponding anaerobic killed controls was stable over almost 400 days. The loss of aniline therefore appears to be linked to biological activity.

However, for some microcosms, the autoclaved, killed controls showed similar activity to the live microcosms. As shown in Chapter 3, for example, in Figure B.2, PCA in aerobic and killed aerobic aquifer microcosms from the lightly contaminated groundwater aquifer location was lost at approximately the same rates. In Figure B.3, PCA in Fe(III)-amended and killed Fe(III)-amended aquifer microcosms from the lightly contaminated groundwater aquifer location had a very similar and rapid rates of loss between day 0 and 20. A similar phenomenon was observed in Figure B.31, for PCA in sulfate-amended

and killed anaerobic sediment microcosms from the lightly contaminated freshwater canal location, between day 0 to 350. Initial rapid rates of loss of PCA were similar in live and killed microcosms in this case. Potential mechanisms for loss in abiotic systems are discussed in the next section, 6.2 Environmental Implications.

Finally, because there are no known dechlorinators of PCA or other chlorinated anilines that have been identified, the activity of *Dehalococcoides mccartyi* strain 195 was also tested on these compounds (Appendix I). A fully grown mixed culture containing *Dehalococcoides mccartyi* strain 195 grown on tetrachloroethene was used to produce 0.5% and 1% transfer cultures that received PCNB or PeCA (Figure A.4-A.7). These cultures showed dechlorination activity with production of tetrachloroaniline (TeCA), trichloroaniline (TCA) and dichloroaniline (DCA) detected in both PCNB and PeCA treatments (Figure A.8). Monochloroanilines were not detected after 80 days of incubation with the mixed culture (Figure A.2-A.7). Further, the mixed culture containing strain 195 did not dechlorinate PCA when it was added as a sole halogenated substrate (Figure A.1).

6.2 Environmental Implications

Microcosm studies help determine the biodegradation/biotransformation activity and

rates, biotransformation products formed, and can help in initial characterization of the bacterial community at a site (Madsen 1991; Fennell and Gossett 2003).

The extensive microcosm study for this manufacturing site was focused primarily on detecting biological activity for aniline and PCA removal under anoxic conditions. Potential mechanisms for loss of aniline and PCA from the environment are show in Figures 6.2 and 6.3. In the case of aniline, there are several confirmed methods of its removal from the environment as shown in Figure 6.2. Aniline can be carboxylated and activated through the benzoyl-CoA pathway under anaerobic conditions (Heider and Fuchs 1997). Under nitrate-amended and sulfate-amended conditions, aniline was metabolized via the *p*-aminobenzoate pathway (Schnell et al. 1989; Schnell and Schink 1991; Kahng et al. 2000), through which the *p*-aminobenzoate was anaerobically oxidized to benzoyl-CoA plus CO_2 by a series of enzymatic reactions. As described in Chapters 3 and 5, samples from certain time points collected from active anoxic microcosms were extracted and analyzed. The concentrations of potential anaerobic metabolites (Figure 6.2), including benzoic acid, phenol, 4-aminobenzoate, diphenylamine and azobenzene, were no higher than 0.5 µM (the estimated detection limits for these compounds).

Previous studies indicated that *Pseudomonas* sp. in pure mineral medium with aromatic substrate degraded phenylacetate under denitrifying conditions via the benzoyl-CoA pathway. Benzoyl-CoA accumulated and accounted for 80% of the initial phenylacetate. Mohamed *et al*. (1993) indicated that benzoyl-CoA accumulation correlated with the absence of strong reductant in the in vitro system of phenylacetate oxidation (Mohamed

et al. 1993). Thus, it is possible that benzoyl-CoA could have accumulated in the microcosms. We did not directly attempt to measure benzoyl-CoA in our systems, and so, this possibility cannot be excluded.

In the absence of detectable intermediates, or potential soluble polymerization products (azobenzene or diphenylamine) a mineralization test with ^{14}C-labeled aniline was performed to determine if [14]CO_2 was a major end product in the microcosms. Only 5 to 10% of the ^{14}C-label was detected in the gaseous phase of the live systems. Thus, complete mineralzation of aniline was also not evident. Therefore, more work is needed to conclusively link the loss of aniline we observed to biodegradation. On-going work for this project is using stable isotope probing (SIP) and detection of ^{13}C from labeled aniline in the DNA of bacteria in active microcosms from this study. Some success has been shown [Applying innovative environmental molecular diagnostics for informing bioremediation at contaminated sites: identification of *in situ* aniline biodegraders by stable isotope probing. 2013. Weimin Sun, Lora Mcguinness, Lee Kerkhof, Max Haggblom and Donna Fennell. Poster presented at the 114th General Meeting of the American Society for Microbiology, Denver, CO., USA] and a manuscript is in preparation.

Still, the possibility of substantial loss of both aniline and PCA from the sediments by abiotic processes cannot be excluded, especially in light of the losses noted in killed microcosms (For example, see Figures B.2, B.8, B.16, B.33 B.36, B.37, B.39 to B.42, B.46, B.47, B.49 and B.50). The moderate water solubility and vapor pressure for aniline and PCA suggests low volatility from aqueous solutions (Thomas 1990). Evaporation from soil was reported to be in the range of 0.11–3.65% of applied PCA, depending on

soil type and sorption capacity (Kilzer et al. 1979). Thus volatilization is unlikely to be a major pathway of loss from the environment or from the microcosms. The exception may be the aerobic microcosms where incubations were performed in flasks with foam plugs wherein the flask headspace was open to the environment. However, even in this case, loss of aniline from aerobic incubations was more rapid than the loss from killed controls (see Figure 3.1 and B.8).

Sorption could be a significant mode of loss from the microcosms. The sorption pathway of aniline to pond sediment was studied by sequential extraction of sediment treated with ^{14}C-labeled aniline (Weber, Colón, and Baughman 2001). In that study, sorption of aniline to sediments occurred with an initial rapid loss of aniline from the aqueous phase followed by a much slower rate of disappearance. Sorption was evident over hundreds of hours at concentrations of 5 µM to 1000 µM, although irreversible binding sites appeared to become saturated at higher concentrations. Investigation of the early stages of incubation of some of the microcosms in this study exhibit data that do appear to support rapid sorption as an important loss mechanism in killed controls (see Figures 3.1, 3.2 and 3.17). For PCA-amended microcosms, abiotic loss also appeared to occur.

As mentioned in Section 6.1, in Figure 5.12, PCA in aerobic and killed aerobic aquifer microcosms from the lightly contaminated groundwater aquifer location was lost at approximately the same rates. In Figure 5.13, PCA in Fe(III)-amended and killed Fe(III)-amended aquifer microcosms from the lightly contaminated groundwater aquifer location had rapid rates of loss between day 0 and 20, very similar to the two stage losses attributed to sorption noted by Weber et al. (Weber, Colón, and Baughman 2001). A similar phenomenon was observed in Figure 5.53 for PCA in sulfate-amended and killed

anaerobic sediment microcosms from the lightly contaminated freshwater canal location, during day 0 to 350. Initial rapid rates of loss of PCA were similar in live and killed microcosms in this case.

Despite the apparent role of sorption on direct loss of aniline and PCA in our systems, the more rapid loss noted in live verses killed controls that occurred in individual replicates after long lag periods seem more indicative of biologically mediated reactions (see Figures 3.18, 3.20, 3.22, 3.23,B.33 and B.40).

Interestingly, substantial PCA loss was observed in the killed anaerobic control microcosms from the highly contaminated canal location (Figures B.46 and B.47). This loss persisted despite repeating the autoclaving on day 383. Note that the highly contaminated location killed control microcosms for the Fe(III)-amended set did not exhibit loss (Figure B.44). While biodegradation of aniline and PCA were the process we sought to verify here, it is also possible that biotransformation reactions could have included polymerization mediated by microbial enzymatic processes (Figure 6.2 and 6.3).

Aniline and PCA can be covalently bound to soil humic material by abiotic catalysts such as birnessite (δ-MnO_2) or by enzymes including laccases and peroxidases (Park et al., 1999; Kong et al. 2013). Bollag (1992) proposed that the binding of xenobiotics to humus not only reduced the toxicity of the parent compound but also prevented the contamination of aquatic envrionments. The binding has been proposed as a remediation approach by adding extracellular enzymes or abiotic catalysts or by changing physicochemical conditions (Bollag 1992). However, the stability of coupling this process with natural humic material was studied and the re-release of free chloroanilines was identified as a potential issue at low pH (Kong et al., 2013). Thus, biologically-

mediated polymerization to sediment organic matter could be a reversible process, and is not superior to complete biomineralization.

To check our microcosms for possible polymerization it is suggested that a back extraction using harsh solvents or under low pH conditions could provide further information about the fate of aniline and PCA.

Reductive dechlorination could be a useful process to remediate PCA contaminated sediments where PCA could be transformed to aniline under methanogenic conditions. As described extensively in Chapter 5, dechlorination of PCA to aniline in methanogenic microcosms from this site was observed when the PCA concentration was increased from 100 µM to 1500 µM. This dissertation work has demonstrated biological reductive dechlorination of PCA in sediments concurrent with production of stoichiometric amounts of aniline. This is a novel finding that has been reported only once previously. Lack of activity on PCA at the lower concentrations suggested that the bacterial dehalogenase enzyme might only be induced at higher concentrations and that these microbes may not act upon low concentrations of PCA. Such "thresholds" for dechlorination were reported for Aroclor 1254, Aroclor 1248 and hexachlorobenzene for other microbial communities (Rhee et al. 1993; Magar et al. 2005; Yuan et al. 1999). Recently a dichlorobenzene dechlorinating *Dehalobacter* sp. has been identified from sediment taken from the same industrial site described in our study (Nelson et al. 2011). Those authors observed a 15 µM threshold with monochlorobenzene (under this concentration biotransformation did not occur) (Nelson 2012). Further work should be performed to determine if this apparent "threshold" could be responsible for persistence of some of the contamiants at the industrial site. *Dehalobacter* sp. was also present in the

PCA-amended methanogenic microcosms and given their connection with chlorobenzenes at the site, further investigation of the ability to dechlorinate PCA should be performed. Additionally, based on the finding from this study that *Dehalococcoides maccartyi* strain 195 dechlorinates PeCA to DCA, a co-culture of *Dehalococcoides and Dehalobacter* spp. should be investigated as a means to completely dechlorinate PeCA to aniline..

Finally, in addition to observing loss of aniline and PCA under a variety of redox conditions, this work also highlighted the activity of natural microbial communities to reduce a variety of electron acceptors. For the lightly contaminated groundwater aquifer location, we observed loss of nitrate (Figure 3.4), sulfate (Figure 3.9) and production of methane (Table 3.7) in aniline-amended microcosms. We observed methane production (Table 3.8), but did not observe loss of nitrate (Figure 3.13) or sulfate (Figure B.7) in PCA-amended microcosms from this location.

For the highly contaminated groundwater aquifer location, very little production of methane (Table 3.7) was observed in the aniline-amended microcosms, and loss of nitrate (Figure B.11), and sulfate (Figure B.15) was also not observed. Loss of nitrate (Figure 3.13) or sulfate (Figure B.7) was not observed in PCA-amended microcosms from this location. Further, there was no methane production (Table 3.8) in PCA-amended microcosms from this location.

In the lightly contaminated freshwater canal sediment location, loss of nitrate (Figure 4.5), sulfate (Figure 3.21) and production of methane (Table 3.9) was observed in aniline-amended microcosms. Loss of nitrate (Figure B.29), sulfate (Figure B.34) and production

of methane (Table 3.10) was also observed in PCA-amended microcosms. In the highly contaminated freshwater canal sediment location, production of methane (Table 3.9) was observed in aniline-amended microcosms, but loss of nitrate (Figure B.34) or sulfate (Figure B.38) was not observed. Finally, methane production (Table 3.10) was observed, but loss of nitrate (Figure B.43) or sulfate (Figure B.48) was not observed in PCA-amended microcosms from this location.

In particular, the heavily contaminated aquifer location exhibited very little biodegradative microbial activity and it cannot be ruled out that the heavy contamination has caused toxicity or inhibition of microorganisms at this location. Knowledge of the potential dominant redox processes that could be supported at different site locations could help guide overall remediation efforts in the future.

Finally, as noted previously there have been other observations of anaerobic degradation/loss of aniline. Only two of these studies presented evidence of intermediates or growth of the organisms (Schnell et al. 1989; Kahng et al. 2000). Other studies did note aniline loss in environmental microcosms, but presented no further evidence of biodegradation (detection of metabolites). To determine conclusively, for example, that aniline has biodegraded it is important that metabolites or endproducts (CO_2) be detected to rule out abiotic loss as the most important mechanism. In this study, loss of aniline (and PCA) in the microcosms appears to be more than abiotic and there appears to be a strong microbial component. Because aniline and PCA have the potential for complex abiotic reactions and sorption, in addition to biodegradation, the degradation and fate of these compounds in anoxic environments may be highly complex and dependent on many factors besides presence of active microorganisms.

Figure 6.1a. Effective treatments in the lightly contaminated groundwater aquifer (sample ID: GDS-11). Treatments are considered as most effective if loss (+) of substrate was observed from all three triplicates.
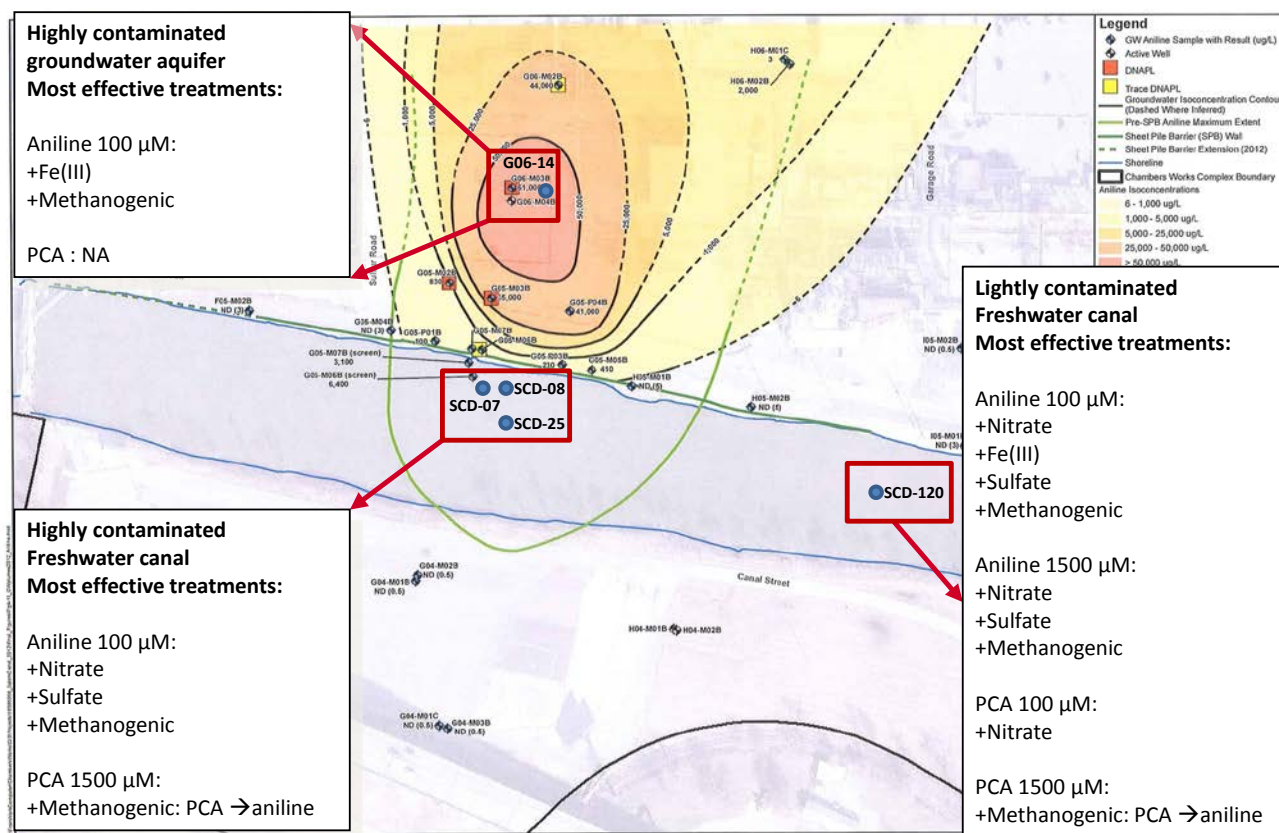
Figure 6.1b. Effective treatments in the highly contaminated groundwater aquifer (sample ID: G06-14), lightly contaminated

freshwater canal (sample ID: SCD-120), and highly contaminated freshwater canal (sample ID: SCD-7, 8 and 25).

Treatments are considered as most effective if loss (+) of substrate was observed from all three triplicates.
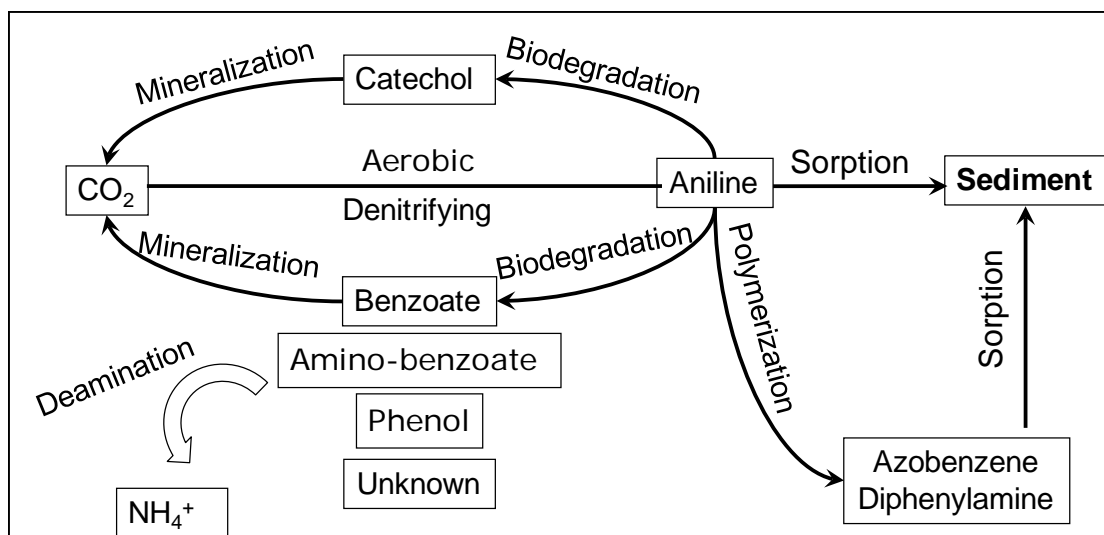
Figure 6. 2. Potential models of removal and potential metabolites for aniline in environmental systems.
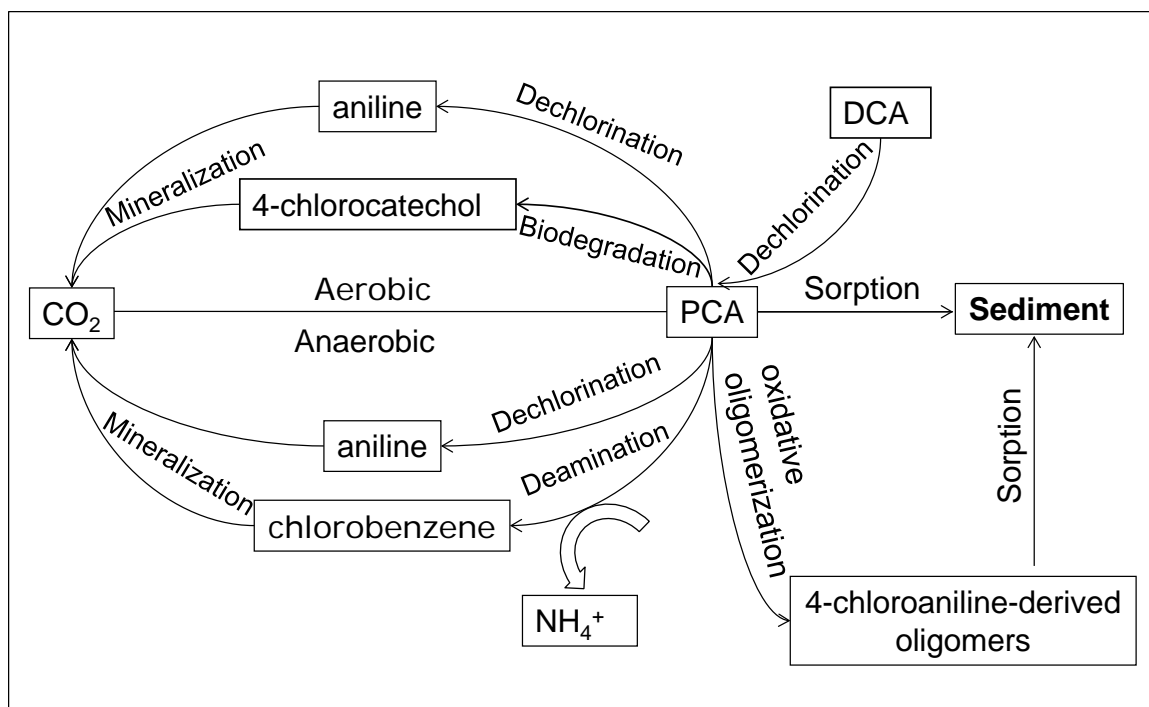
Figure 6. 3. Potential models of removal and potential metabolites for PCA in

environmental systems. PCA, *para*-chloroaniline; DCA, dichloroaniline.

Appendix I   Transformation of Pentachloronitrobenzene and Dechlorination of Chloroanilines by a Mixed Culture Containing *Dehalococcoides mccartyi* strain 195

A.1 Abstract

Dehalococcoides mccartyi strain 195 was originally enriched and isolated on tetrachloroethene (PCE). This dechlorinator has a wide substrate range that includes chlorophenols and chlorobenzenes. A previous study has shown that amendment of Anacostia River, Washington DC, sediment with pentachloronitrobenzene (PCNB) as a halogenated co-substrate enhanced dechlorination of polychlorinated biphenyl (PCBs) by native dechlorinators and when bioaugmented with strain 195. Here we report the transformation and reductive dechlorination of PCNB and pentachloroaniline (PeCA), respectively, by a mixed culture containing *Dehalococcoides mccartyi* strain 195. Mixed culture containing *D. mccartyi* strain 195 pre-grown on butyrate and PCE, with yeast extract added as a nutrient source were amended with 20 μM PCNB, PeCA or *para*-

chloroaniline (PCA). Triplicate 1% transfer cultures amended with 10 μM PCNB or 5 μM pentachloroaniline (PeCA) and 0.5% transfer cultures amended with 5 μM PCNB or 2.5 μM PeCA were also established along with transferred live controls amended with tetrachloroethene (PCE). Butyrate was added as electron donor and hydrogen source.

PCNB, PeCA, tetrachloroanilines (TeCA), trichloroanilines (TCA), dichloroanilines (DCA) and monochloroanilines (MCA) were determined by gas chromatography–mass spectrometry (GC-MS). The fully grown mixed culture, the 1% and the 0.5% transfer cultures that received PCNB or PeCA, all exhibited showed dechlorination activity. Dechlorination of PCE to vinyl chloride (VC) was observed in the live control treatment, indicating a successful transfer from the mixed culture to fresh medium in new serum bottles. PeCA was detected in the live cultures and killed controls a few hours after amendment with PCNB, suggesting rapid abiotic conversion of PCNB to PeCA. TeCA, TCA and DCA dechlorination products were detected in both PCNB and PeCA treatments, but no monochloroanilines were detected after 60 days of incubation. No monochloroaniline dechlorination products were detected after 335 days of incubation in the PCNB or PeCA treatment with the fully grown mixed culture. These results suggest that *D. mccartyi* strain 195 readily dechlorinates PeCA to dichloroanilines. Slow dechlornation rate in transfer cultures precluded measurement of growth on chloroanilines. Finally in PCA-amended cultures, no dechlorination or loss of PCA was observed over 335 days.

A.2 Introduction

Chlorinated organic compounds are widespread environmental contaminants and many of these are or are known or suspected to be carcinogenic (Öberg, 2002). The *Dehalococcoides* bacteria have varied dechlorination abilities and can dechlorinate an array of chlorinated organic compounds (Adrian *et al*., 2007). During reductive dechlorination, chlorinated compounds act as terminal electron acceptors in metabolism and anaerobic bacteria could obtain energy for growth from the energy released during the dechlorination reaction.*Dehalococcoides mccartyi* strain 195^T, the type species of the genus was formerly designated '*Dehalococcoides ethenogenes*' strain 195. This dechlorinator is well known for its abilities to reductively dehalogenate persistent environmental pollutants (Löffler *et al*. 2012). So far, the *Dehalococcoides* group of bacteria are the only known isolated organism capable of fully dechlorinating PCE and the other chloroethenes to ethene (Hendrickson *et al*., 2002, Duhamel *et al.,* 2004).

Dehalococcoides spp. have been utilized in field scale bioremediation of chlorinated solvents (Hendricksen et al. 2002; Lendvay et al. 2003). In addition to dechlorination of the chlorinated ethenes, Dehalococcoides dechlorinate polychlorinated biphenyls (PCBs) (Fennell et al. 2004; Liu and Fennell 2008; Bunge et al. 2003; Bedat et al 2008). A previous study has showed bioaugmentation with mix culture containing *Dehalococcoides mccartyi* strain 195 and biostimulation with a halogenated co-substrate, pentachloronitrobenzene (PCNB) enhanced dechlorination of polychlorinated biphenyl

(PCBs) (Krimins et al. 2009; Park *et al*. 2011). It is believed that by adding halogenated co-substrate, the increasing production of the degradative enzymes may maximize catalytic efficiency or stability (Vangnai and Petchkroh, 2007). In the PCBs microcosm experiment, PCR-DGGE was used to study *Dehalococcoides* sp. for their existence in differently-amended sediments. The results demonstrated an increase of the population of the native *Dehalococcoides* sp., but not *Dehalococcoides mccartyi* strain 195. It was assumed that co-substrate supplementation may increase growth rates and thus the biodegradation efficiency of different *Dehalococcoides* sub-populations that differ by dechlorination activity (Krumins et al. 2009). To our knowledge, there has been no report of attempts to test the dechlorination of PCNB or chloroanilines by *Dehalococcoides* strain 195. Here we report the activity of a mixed culture containing *Dehalococcoides mccartyi* strain 195 on PCNB and chloroanilines.

A.3 Materials and Methods

A.3.1 Mixed culture protocol

A mixed culture containing *Dehalococcoides mccartyi* strain 195 was pre-grown at 25°C on butyrate and PCE, with yeast extract and a vitamin solution added as nutrient sources as described previously (Fennell *et al*. 1997; Fennell *et al*. 2004; Fennell and Gossett

1998). The culture is fed PCE (110 µM) with butyrate (440 µM) as a hydrogen source to moderate competition between *D. mccartyi* strain 195 and methanogens (Fennell *et al*. 1997; Fennell *et al*. 2004).

A.3.2 Chemicals and reagents

Pentachloronitrobenzene (PCNB, 99%), internal standard 1,3,5-tribromobenzene (TBB, 98%), 4-chloroaniline (PCA, sublimed, 99+ %), PCE (>98% purity) and butyric acid (>99% purity) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Standards of expected PCNB dechlorination products were obtained as follows: pentachloroaniline (PeCA, 96%) was obtained from Alfa Aesar (Ward Hill, MA); 2,3,4,5-tetrachloroaniline (TeCA) (98%) and 2,3,5,6-TeCA (99%) were purchased from UltraScientific (North Kingstown, RI); 2,3,4-trichloroaniline (TCA) (97%) was obtained form Sigma-Aldrich Co. (St. Louis, MO); 2,4,5-TCA (97%), 2,4,6-TCA (98+%) and 3,4,5-TCA (97%) were obtained from Alfa Aesar (Ward Hill, MA); 2,4-dichloroaniline (DCA) (99%), 2,5-DCA (99%), 2,6-DCA (98%), 3,4-DCA (98%) and 3,5-DCA (98%) were purchased from Sigma-Aldrich Co. (St. Louis, MO) and 3-chloroaniline (CA) (99%) was purchased from Sigma-Aldrich Co. (St. Louis, MO), and 2-CA (99.7%, Riedel-de Haën) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Standards of potential PCA dechlorination products, 2,3,4,6-TeCA, 2,3,6- and 2,3,5-TCA were not available during this experiment. Prepurified nitrogen and a 70% N_2/30% CO_2 gas mixture purchased from Air Gas (Piscataway, NJ) were used as anaerobic purge gases.

A.3.3 Culture set up

For each treatment using the fully grown mixed culture, a set of three 60 mL glass serum bottles was sterilized. The bottles were capped with Teflon-coated butyl rubber stoppers, crimped with aluminum seals and autoclaved for 40 minutes. Bottles were made anoxic by purging with filter-sterilized nitrogen for 20 minutes. Anoxic, autoclaved bottles were filled with 50 mL of the pre-grown mixed culture. One set, amended with PCE, served as a transfer control to ensure that the bacterial culture was active and could dechlorinate its known growth substrate, PCE. Butyric acid (440 µM) was added to each transfer control.Three filled bottles from each set were autoclaved to serve as killed controls. The other six bottles (in each set) were amended with either 1 mL of a 20 mM PCA aqueous stock solution, 25 µL of a 50 mM PCNB stock solution made in acetone, or 60 µL of 20 mM PeCA stock solution made in acetone, respectively. The preparation of the stock solutions and treatments of the bottles are summarized in Tables A.1 and A.2. The PCA stock solution was delivered using sterile disposable syringes equipped with 25G needles, which were preassembled and flushed with sterile nitrogen prior to use. The PCNB and PeCA stock solutions were delivered using seperate glass syringes rinsed with acetone and dedicated for use with this culture. Needles and tops of bottles were swabbed with alcohol and flamed between each needle entry into the bottle.

For each treatment using 1% and 0.5% transfers of the mixed culture, a set of three 160 mL glass serum bottles was prepared. Autoclaved bottles were filled with 100 mL of fresh anaerobic medium. A transfer control fed PCE was set up to ensure that the bacterial culture was active. No killed controls were prepared. The 1% transfers were

amended with 1 mL of pre-grown mixed culture. The 0.5% transfers were amended with

0.5 mL of pre-grown mixed culture. The 1% transfer PCNB treatments were amended

with 24 µL of a 50 mM PCNB stock solution. The 0.5% transfer PCNB treatments were

amended with 12 µL of a 50 mM PCNB stock solution. The 1% transfer PeCA treatments

were amended with 48 µL of a 20 mM PCNB stock solution. The 0.5% transfer PeCA

treatments were amended with 24 µL of a 20 mM PCNB stock solution. The final

concentrations of PCNB and PeCA in the culture are shown in Table A.3. The same

PCNB and PeCA stock solution were used as described in the fully grown mixed-culture

microcosms experiment (Table A.1). Butyric acid was added to all treatments at 440 µM

and pre-fermented yeast extract (FYE) solution (50 g/L) as a trace nutrient source was

added to all treatments at 20 µL per 100 mL (Fennell *et al*. 1997; Fennell *et al*. 2004;

Fennell and Gossett 1998).

A.3.4 Sample extraction for chlorinated compounds

1 mL samples were taken from each serum bottle using sterile and anoxic techniques.

Samples were stored in 7 mL screw-cap vials and frozen at -20°C until analysis.

Liquid/liquid extraction was performed to analyze and quantify PCNB and

chloroanilines. 1 mL of hexane was added to each vial and vials were capped and

vigorously shaken by hand for 2 minutes, and then mixed on a shaker overnight.The

extraction vials were centrifuged at 1000 rpm for 15 minutes and the supernatant was

transferred with a glass pipette to a 2 mL clear glass auto-sampler vial. 0.1 mL of internal

standard with 0.787 µg 1,3,5-tribromobenzene (TBB), was added with a 0.5 mL glass-

Teflon®-stainless-steel gas-tight syringe to each auto-sampler vial. The 2 mL vials were

crimped and frozen at -20°C until analysis.The extracts were analyzed for the

halogenated parent and daughter compounds using gas chromatography–mass

spectrometry (GC-MS) as described below.

A.3.5 Analytical methods

PCNB and chloroanilines were analyzed on a Hewlett-Packard 6890 gas chromatograph

(GC6890) coupled with a Mass SelectiveDetector (HP 5973) (Agilent Technologies,

Atlanta, GA, USA). TBB was used as an internal standard. The mass of the TBB added to

each 2 mL glass vial was constant, but the fraction of the gas phase injected into GC was

unknown. This fraction can be corrected by the ratio of the peak area of TBB verses the

mass of TBB. The calibration curve was estimated by the relationship shown in Equation

A.1.

$$\frac{Area(CAs)}{Area(TBB)} = \frac{a \times Mass(CAs)}{Mass(TBB)} + b$$

(Equation A.1)

where:

Area (CAs) = the GC-MS peak area of PCNB, PeCA or chloroaniline compounds

detected during dechlorination; Area (TBB) = the GC-MS peak area of the internal

standard TBB; Mass (TBB) = the mass of internal standard TBB, which was 0.787 µg, as

described above; a, b are constants found from the standard curve. The unknown Mass

(CAs) can be obtained from the calibration curves.

Separation of analytes by gas chromatography was performed using a J & W Scientific

(Folsom, CA) column (i.d.=0.25 mm, L=30 m,) with DB-5 as the stationary phase (film

thickness 0.25 µm) and with the detector operated under monitoring (Scan) mode.

Separation of the parent compounds involved injecting 2.0 µL of the sample into the front

inlet of the gas chromatograph operating at 250°C and operated in splitless mode.The

constant flow of helium (carrier gas) was 1.0 mL min^{-1}. The oven program was as

follows: The initial temperature was 40°C, the temperature was raised at a rate of

5°C/min to 250°C.The analysis time for one sample was about 52 min. The retention

times and qualifying ions for PCNB, PeCA and possible chloroanilines formed by the

reductive transformation of PCNB or PeCA are summarized in Table A.4.

PCE and its daughter products vinyl chloride (VC) and ethene, and methane, were

determined using an Agilent Technology 6890N (Agilent Technologies, Inc. Santa Clara,

CA, USA) gas chromatograph (GC) with a flame ionization detector (FID). 250 µL

headspace samples were injected into the GC-FID equipped with a GS-GasPro capillary

column (30 m × 0.32 mm I.D.; Agilent Technologies, Inc. Santa Clara, CA, USA) with

helium as the carrier gas. The injector temperature was 50°C. The constant flow of

helium (carrier gas) was 1.0 mL min^{-1}. The oven temperature was programmed to hold at

50°C for 2 min, increase to 180°C at 15°C/min, and then hold at 180°C for 2 min.

Retention times of individual compounds were 12.08, 10.624, 9.809, 8.507, 5.393, 1.349,

and 0.862 min for PCE, TCE, *cis*-DCE, *trans*-DCE, VC, ethene, methane, respectively.

A.4 Results

A.4.1 Dechlorination in the Dehalococcoides mccartyi strain 195 mixed-culture

The transfer controls were active and dechlorinated PCE to ethene and traces of vinyl chloride within 7 days (data not shown). Methane was also formed from fermentation of butyric acid.

No dechlorination of PCA by the mixed culture was observed. The loss of PCA in the live cultures was not substantially different from the loss in the killed control samples after 335 days (Figure A.1).

PeCA, 2,3,4,5-TeCA, 2,3,5,6-TeCA, 3,4,5-TCA, 2,4,5-TCA, 2,4,6-TCA, 2,6-DCA, 2,5-DCA were detected after 60 days of incubation of the PCNB amended cultures (Figure A.2). Microcosms were amended with approximately 25 µM PCNB. An average of 4.86 µM (19.4 mol%) PCNB was recovered in PCNB live treatment on day one. An average of 6.71 µM (26.8 mol%) and 16.8 µM (67.3 mol%) PeCA was observed on day 1 and 5 in the PCNB live treatments, respectively. An average of 8.14 µM (32.6 mol%) PCNB was recovered in the PCNB killed controls on day one. An average of 5.67 µM (22.7 mol%) and 18.5 µM (73.9 mol%) PeCA was observed on days 1 and 5 in the PCNB killed controls, respectively. No PCNB was detected on day 19 in the PCNB live treatments and no PCNB was detected on day 10 in the PCNB killed controls. Thus,

transformation of PCNB to PeCA occurred in both live and killed cultures at similar rates.

2,3,4,5-TeCA was identified on day 14 in PCNB live treatments with an average concentration of 0.024 µM (0.10 mol%). Although 2,3,5,6-TeCA was not detected on day 14 or day 19, it was detected at other sampling times and remained in the culture until day 62 with concentrations ranging from 0.02 to 0.12 µM. TeCAs were further dechlorinated to 2,4,5-TCA, 2,4,6-TCA, 3,4,5-TCA, 2,5-DCA and 2,6-DCA by 19 days of incubation. PCNB was re-amended on day 48. Accumulation of 2,6-DCA was observed as the major final daughter product with its concentration increasing between days 62 to day 130 (data not shown). No monochloroanilines were detected in the culture after 130 days.

Similarly, 2,3,4,5-TeCA, 2,3,5,6-TeCA, 3,4,5-TCA, 2,4,5-TCA, 2,6-DCA, 2,5-DCA were also detected in the PeCA-amended cultures as the major dechlorination products, with 2,6-DCA as the major, final end product. (Figure A.3).

A.4.2 Dechlorination in 1% and 0.5% transfer cultures

PCE was dechlorinated within two weeks in transfer controls (data not shown), indicating successful transfers.

1% transfer PCNB live treatment cultures (Figure A.4) were amended with approximately 12 µM PCNB. An average of 1.76 µM (14.7 mol%) and 0.91 µM (7.6 mol%) PCNB was observed on day 0 and 1, respectively. No PCNB was detected by day 11. An average of 1.46 µM (12.2 mol%) and 8.82 µM (73.5 mol%) PeCA was observed on day 1 and 60, respectively. 2,3,4,5-TeCA and 2,3,5,6-TeCA were detected on day 95 with an average concentration of 0.13 µM (1.1 mol%) and 0.02 µM (0.2 mol%),

respectively. An average of 0.11 µM (0.9 mol%) 2,4,6-TCA was observed on day 11. 2,4,5-TCA, 3,4,5-TCA, 2,4-DCA, 2,5-DCA, 2,6-DCA, or monochloroanilines were not detected after 173 days.

The 0.5% transfer PCNB live treatment microcosms (Figure A.5) were amended with approximately 6 µM PCNB. An average of 0.70 µM (5.8 mol%) and 0.39 µM (3.2 mol%) PCNB was observed on day 0 and 1, respectively. No PCNB was detected by day 11. An average of 1.88 µM (15.7 mol%) and 3.74 µM (31.2 mol%) PeCA was observed on day 1 and 11, respectively. An average of 0.82 µM (6.9 mol%) PeCA was observed on day 105 and no PeCA was detected by day 173 (data not shown). 2,3,4,5-TeCA was detected on day 11 with an average concentration of 0.07 µM (0.6 mol%). An average of 0.08 µM (0.6 mol%) 2,3,5,6-TeCA was observed on day 77. An average of 0.11 µM (0.9 mol%) 2,4,6-TCA, 2,4,5-TCA and 3,4,5-TCA were observed on day 77. 2,6-DCA was detected on day 173 (data not shown). 2,4-DCA, 2,5-DCA or monochloroanilines were not detected after 173 days.

The 1% transfer PeCA amended cultures (Figure A.6) were amended with approximately 10 µM PeCA. An average of 0.14 µM (1.4 mol%) PeCA remained in culture on day 173 (data not shown). 2,3,4,5-TeCA was identified on day 11 with an average concentration of 0.50 µM (5.0 mol%). 2,3,5,6-TeCA was identified on day 60 with an average concentration of 0.25 µM (2.5 mol%). An average of 0.04 µM (0.4 mol%) 2,4,6-TCA, 0.48 µM (4.8% mol%) 3,4,5-TCA and 0.32 µM (3.2% mol%) 2,4,5-TCA were observed on day 60. 2,4-DCA, 2,5-DCA, 2,6-DCA, or monochloroanilines were not detected after 173 days.

The 0.5% transfer PeCA amended cultures (Figure A.7) were amended with approximately 5 µM PeCA. An average of 6.02 µM (60.2 mol%) PeCA remained in

culture on day 105. No PeCA was detected after 173 days of incubation. 2,3,4,5-TeCA was identified on day 60 with an average concentration of 0.25 µM (2.5 mol%). 2,3,5,6-TeCA was identified on day 77 with an average concentration of 0.15 µM (1.5 mol%). 2,4,5-TCA was observed on day 77. 2,4,6-TCA and 3,4,5-TCA were detected on day 95. 2,4-DCA and 2,6-DCA were observed on day 173. 2,5-DCA or monochloroanilines were not detected after 173 days (data not shown).

The pathways of PCNB and PeCA transformation in the *D. mccartyi* strain 195 mixed-culture is shown in Figure A.8. [Note that standards of 2,3,4,6-TeCA, 2,3,6- and 2,3,5-TCA were not available during this experiment.]

A.5 Discussion

PCNB was transformed to PeCA and PeCA was dechlorinated to 2,3,4,5-TeCA, 2,3,5,6-TeCA, 2,4,5-TCA, 2,4,6-TCA, 3,4,5-TCA, 2,5-DCA and 2,6-DCA by a mixed culture containing *Dehalococcoides mccartyi* strain 195. No monochlorinated aniline daughter products were detected. Further there was no dechlorination of PCA over 335 days by the culture (Figure A.1). Kuhn et al. also found that monochlorinated anilines were not transformed in methanogenic aquifer slurries from two sites adjacent to the Norman Municipal Landfill (Kuhn et al., 1989). It might be possible that *D. mccartyi* strain 195

can only dechlorinate more highly chlorinated anilines (such as PeCA) along with benzene and phenol derivatives to less chlorinated products, as has been reported previously (Adrian et al. 2007; Fennell et al. 2004).

We intended to document the growth of *Dehalococcoides mccartyi* strain 195 on the chlorinated anilines. To accomplish this, transfer cultures were established so that increases in *Dehalococcoides* 16S rRNA genes could be quantified as repeated dosages of PCNB or PeCA were added. Because only low concentrations of dechlorination products were observed in the 1% and 0.5% transfers (Figures A.4-A.7), it was not possible to quantify growth. It is possible that with further incubation time, or with incubation at 35 °C (the temperature optimum for strain 195, Maymo-Gatell et al. 1997) this experiment could have been improved and growth demonstrated. Recently, Zhen et al. 2013 attempted to show growth of strain 195 on PCBs and polychlorinated dibenzo-*p*-dioxins (PCDDs) using a similar approach and were unsuccessful. In those experiments, PCB and PCDD dechlorination eventually slowed and ceased in transfer cultures. Here, dechlorination of PeCA appears to increase over time (Figure A.7) suggesting that growth could occur, but that longer incubation times are necessary. Certainly PeCA and PCNB supported dechlorination activity by strain 195. PCB dechlorination was enhanced in PCNB-amended sediments bioaugmented with strain 195 (Krumins et al. 2009). This increased activity could have been caused by enhanced expression of dehalogenases over the short-term. Further study is needed to determine the usefulness of strain 195 in remediation PCNB or PeCA-contaminated environments.

Table A. 1. Stock solutions for experiments on reductive dechlorination of PCNB, PeCA

and PCA.

Stock [a] Solution	Solvent	Stock Concentration	Volume added (μL)	Mixed culture final concentration (μM)
PCA	DI water	20 mM (stored at 4°C)	1000	400
PeCA	Acetone	20 mM (stored at 4°C)	60	24
PCNB	Acetone	50 mM (stored at 4°C)	25	25
PCE	--	--	0.6	97.8
HBu	--	Neat HBu (density=0.96 g/mL)	2	436

[a] PCA-*para*chloroaniline

PeCA-pentachloroaniline

PCNB-pentachloronitrobenzene

PCE-tetrachloroethene

HBu- butyric acid

Table A. 2. Description of mixed culture treatments performed in triplicate.

Treatment[a, b]	4-CA (μM)	PeCA (μM)	PCNB (μM)	PCE (μM)	HBu (μM)	Autoclave
Transfer control	--	--	--	97.8	436	--
PCA Killed Control	400	--	--	--	--	Killed
PCA live	400	--	--	--	436	--
PeCA Killed Control	--	24	--	--	--	Killed
PeCA live	--	24	--	--	436	--
PCNB Killed Control	--	--	25	--	--	Killed
PCNB live	--	--	25	--	436	--

[a] Concentrations given are the final concentrations of the chemicals in the culture.

[b] PCA-*para*chloroaniline

PeCA-pentachloroaniline

PCNB-pentachloronitrobenzene

PCE-tetrachloroethene

HBu- butyric acid

Table A. 3. Description of 1% and 0.5% transfer mixed culture treatments prepared in

triplicate.

Treatment [a, b]	Medium volume (mL)	transfer culture volume (mL)	PeCA (μM)	PCNB (μM)	PCE (μM)	HBu (μM)	FYE
Transfer control	100	--	--	--	97.8	436	
1% transfer PCNB	100	1	--	12	--	436	20 μL/
0.5% transfer PCNB	100	0.5	--	6	--	436	100mL
1% transfer PeCA	100	1	10	--	--	436	culture
0.5% transfer PeCA	100	0.5	5	--	--	436	

[a] No autoclaved controls were prepared.

[b] PCA-*para*chloroaniline

PeCA-pentachloroaniline

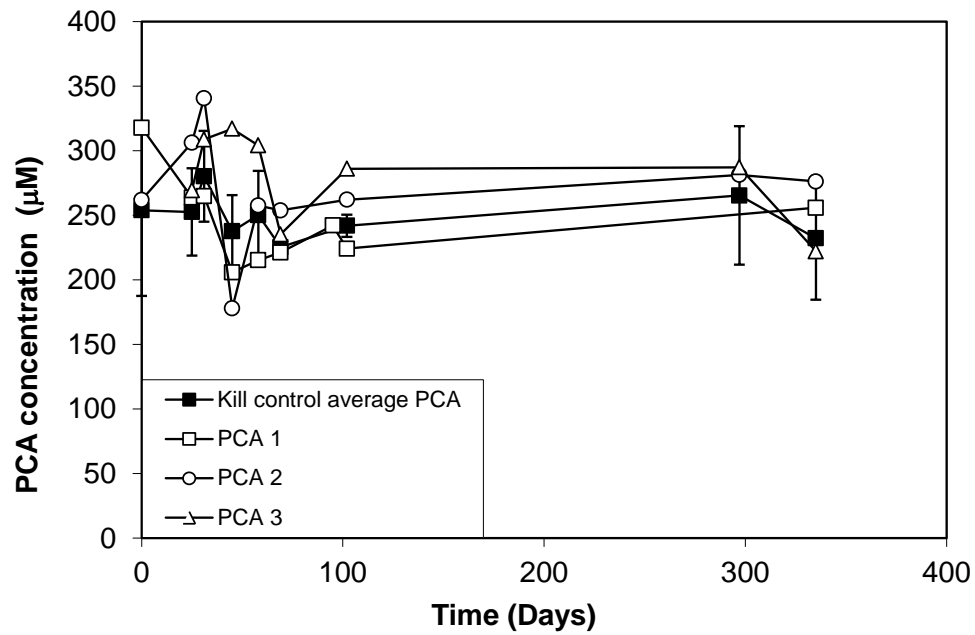PCNB-pentachloronitrobenzene

PCE-tetrachloroethene

HBu- butyric acid

Table A. 4. The retention times and quantification ions for PCNB, PeCA and possible

chloroanilines[a] formed by the reductive transformation of PCNB or PeCA.

Chemical	Retention time (min)	Qualifying ion (M/Z)	Secondary ions (M/Z)
PCNB	28.7	295	237
PeCA	30.2	265	267
TBB	20.6	316	235
2,3,4,5-TeCA	29.1	231	229, 232, 233
2,3,5,6-TeCA	25.7	231	229, 232, 233
3,4,5-TCA	26.4	197	195
2,4,5- TCA	23.8	197	195
2,4,6- TCA	20.5	197	195
2,3,4- TCA	24.5	197	195
2,3-DCA	19.1	161	163
2,4- DCA	18.43	161	163
2,5- DCA	18.5	161	163
2,6- DCA	20.38	161	163
3,4- DCA	21.1	161	163
3,5- DCA	15.9	161	163
PCA	14.9	127	129
3CA	14.9	127	129, 65
2CA	12.9	127	129

[a]Standards of 2,3,4,6-TeCA, 2,3,6- and 2,3,5-TCA were not obtained for this experiment.
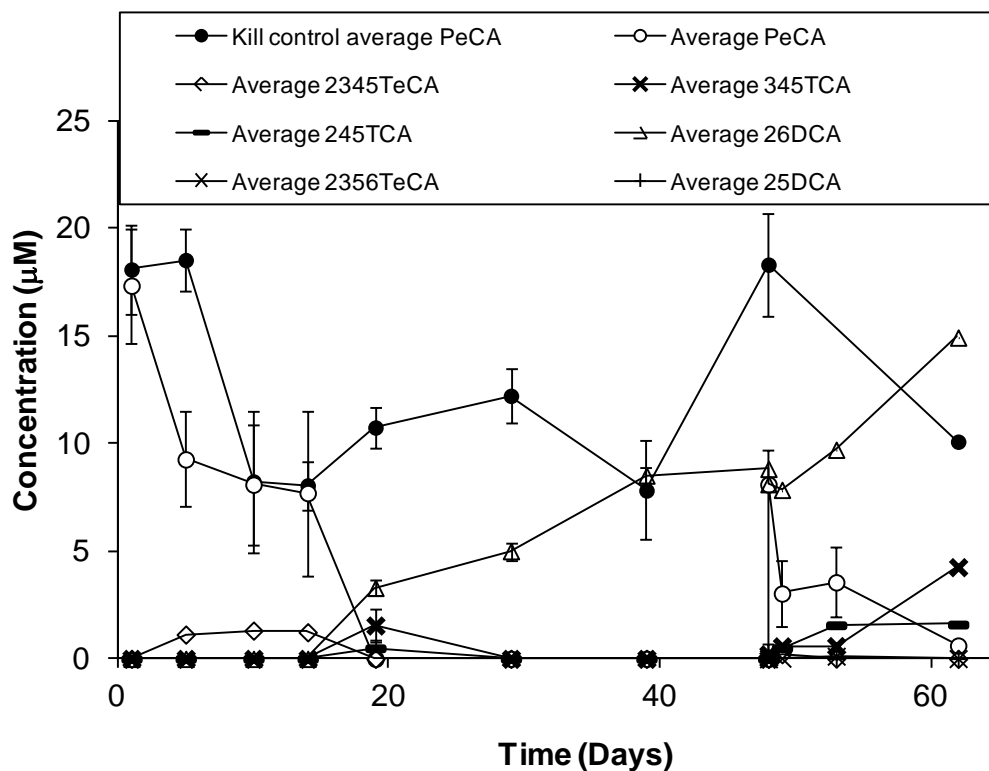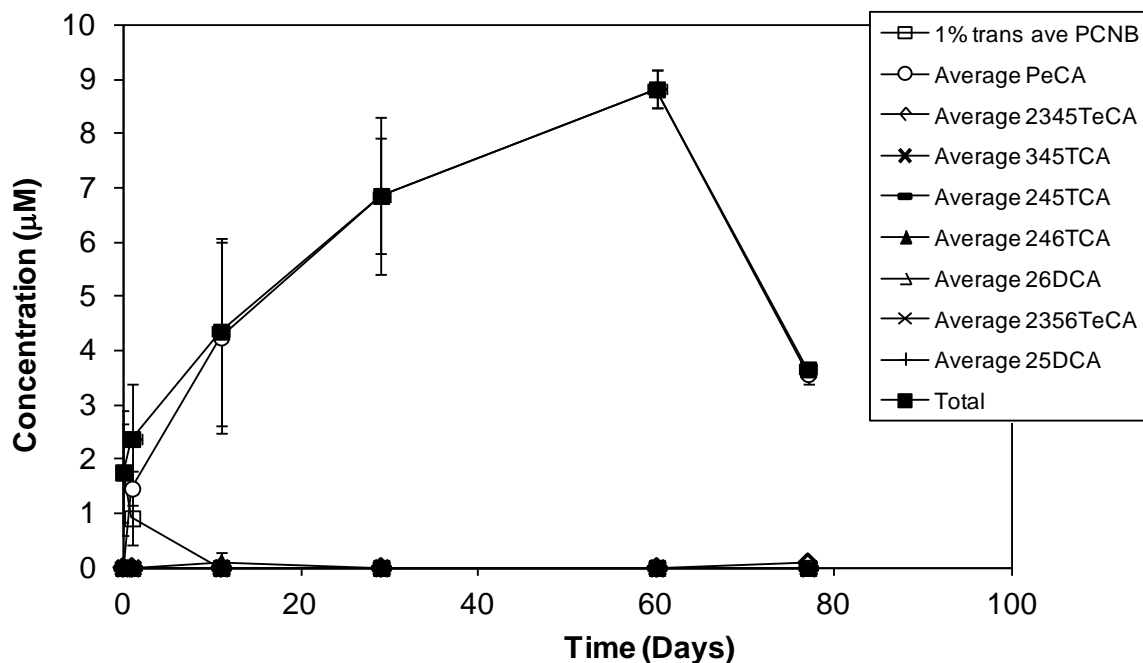
Figure A. 1. *Para*-chloroaniline (PCA) in the *Dehalococcoides mccartyi* strain 195 mixed-culture and killed controls.
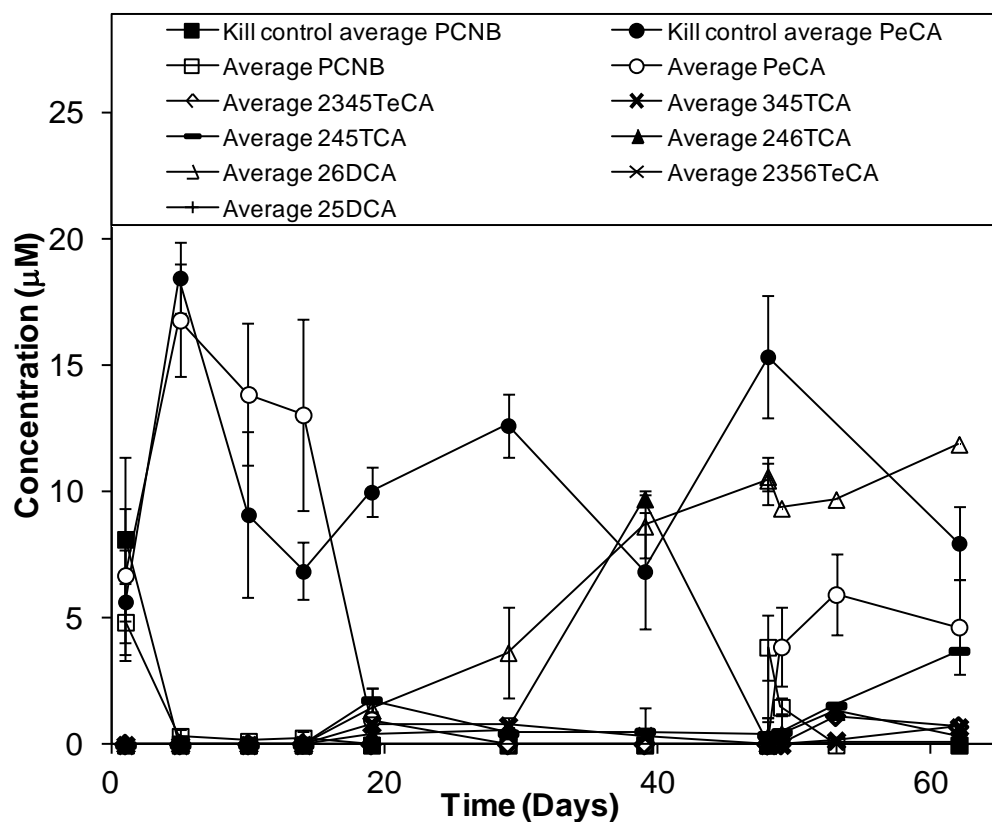
Figure A. 2. Pentachloronitrobenzene (PCNB) and transformation product concentrations

in *Dehalococcoides mccartyi* strain 195 mixed-culture and killed control cultures. [PeCA,

pentachloroaniline; TeCA, tetrachloroanilines; TCA, trichloroanilines; DCA,

dichloroanilines.]

Figure A. 3. Pentachloroaniline (PeCA) and dechlorination product concentrations in
Dehalococcoides mccartyi strain 195 mixed-culture and killed control
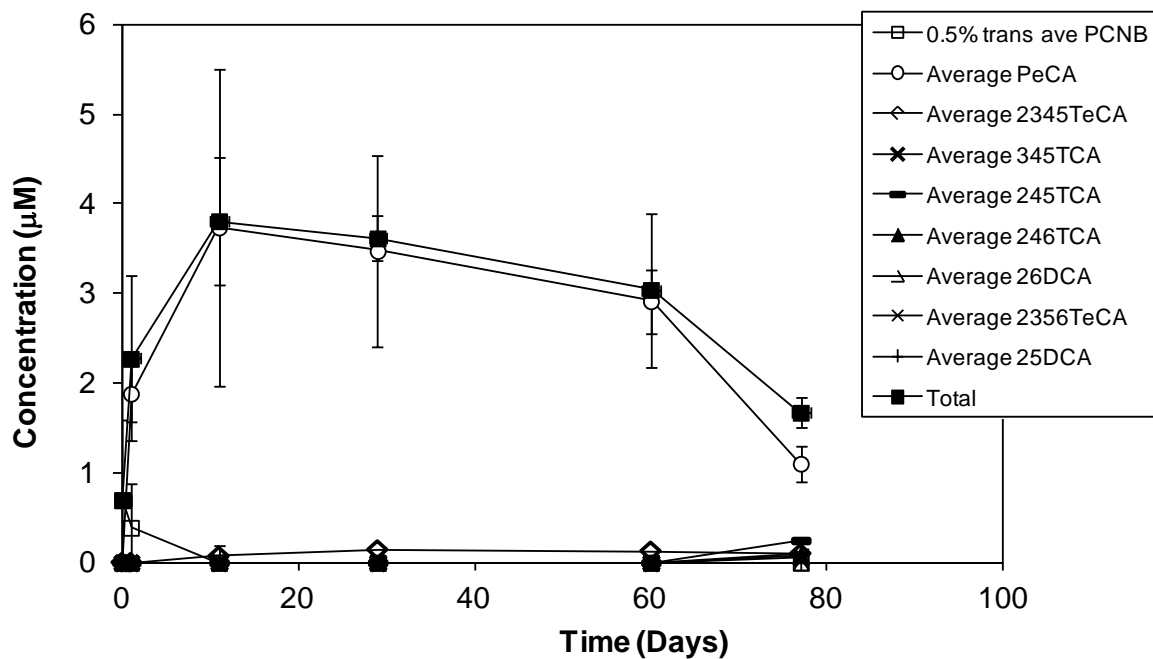cultures. [TeCA, tetrachloroanilines; TCA, trichloroanilines; DCA,
dichloroanilines.]

Figure A. 4. Pentachloronitrobenzene (PCNB) and transformation product concentrations

in 1% *Dehalococcoides mccartyi* strain 195 mixed-culture. [PeCA,

pentachloroaniline; TeCA, tetrachloroanilines; TCA, trichloroanilines; DCA,

dichloroanilines.]

Figure A. 5. Pentachloronitrobenzene (PCNB) and transformation product concentrations

in 0.5% *Dehalococcoides mccartyi* strain 195 mixed-culture. [PeCA,

pentachloroaniline; TeCA, tetrachloroanilines; TCA, trichloroanilines; DCA,

dichloroanilines.]
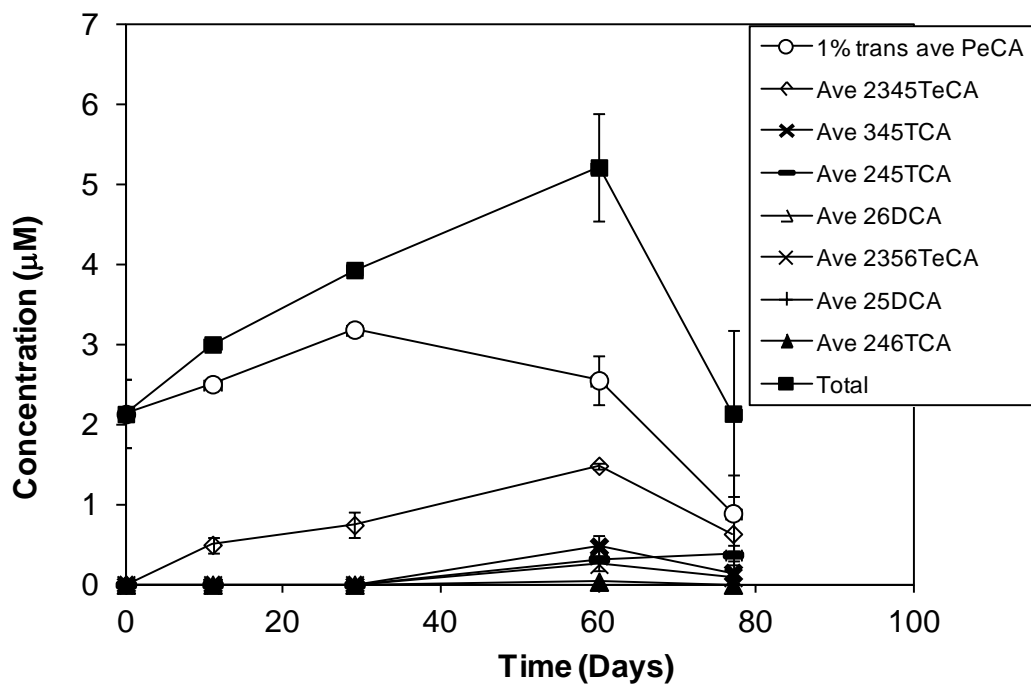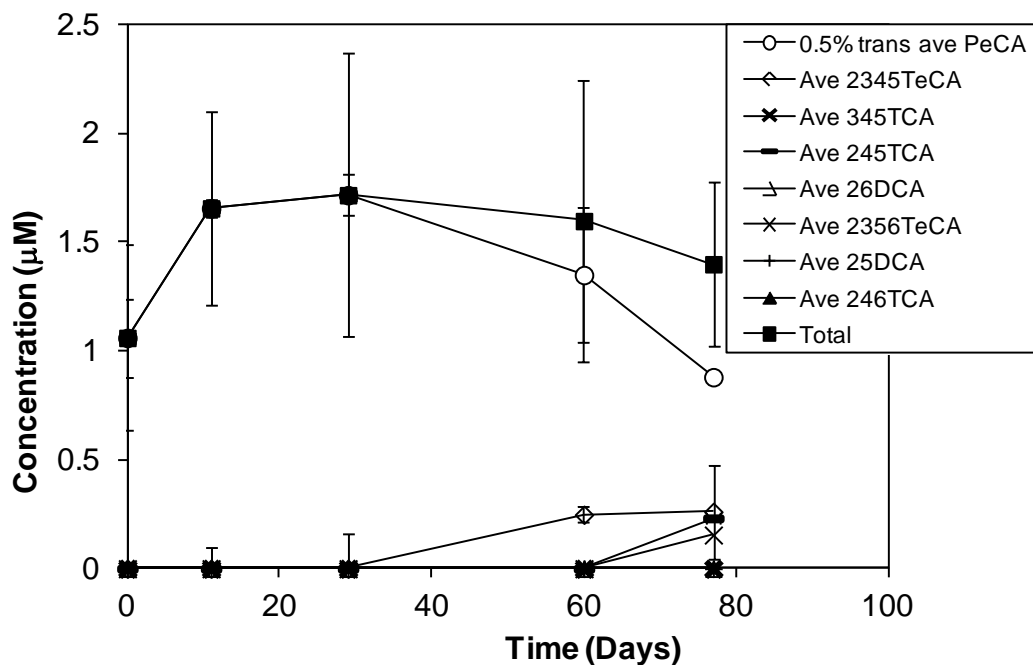
Figure A. 6. Pentachloroaniline (PeCA) and dechlorination product concentrations in 1%

Dehalococcoides mccartyi strain 195 mixed-culture. [TeCA,

tetrachloroanilines; TCA, trichloroanilines; DCA, dichloroanilines.]

Figure A. 7. Pentachloroaniline (PeCA) and dechlorination products in 0.5%

Dehalococcoides mccartyi strain 195 mixed-culture. [TeCA,

tetrachloroanilines; TCA, trichloroanilines; DCA, dichloroanilines.]

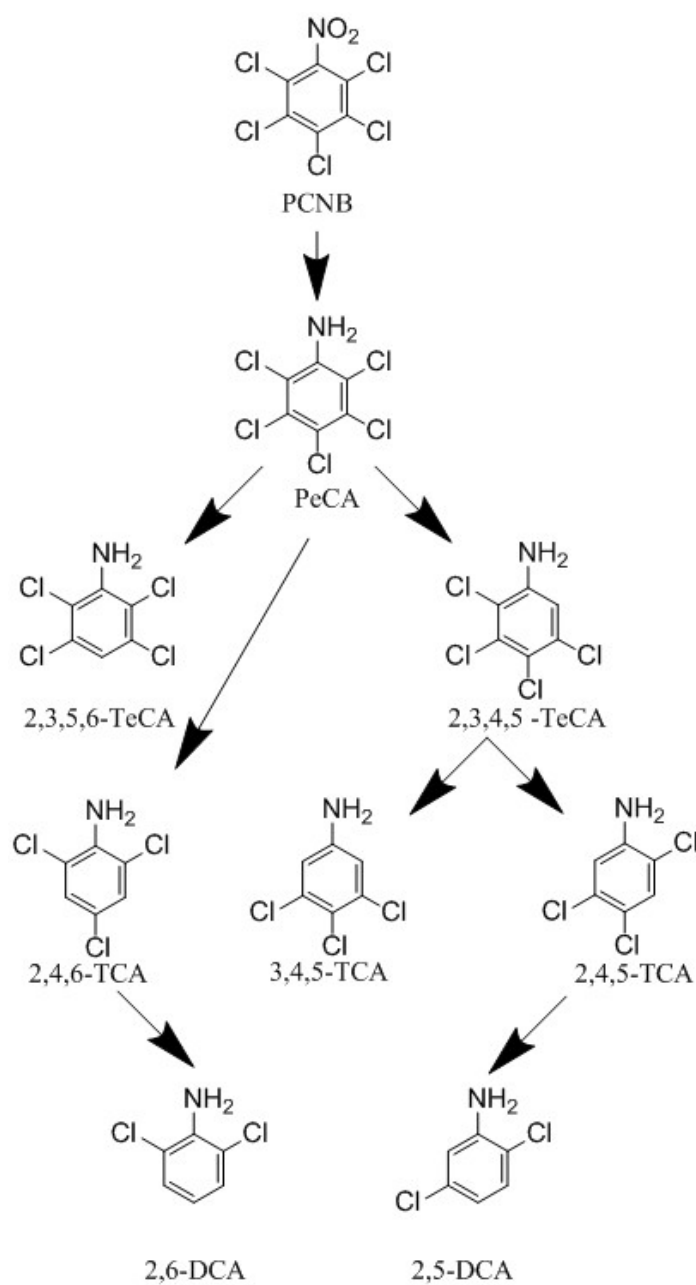**Figure A. 8.** Pathways of PCNB and PeCA dechlorination in *Dehalococcoides mccartyi* strain 195 mixed culture. Note that the first step also occurs in killed cultures. 2, 6-DCA was the major final dechlorination product in the fully growth culture.

Appendix II   Additional Figures to Accompany

Chapter 3
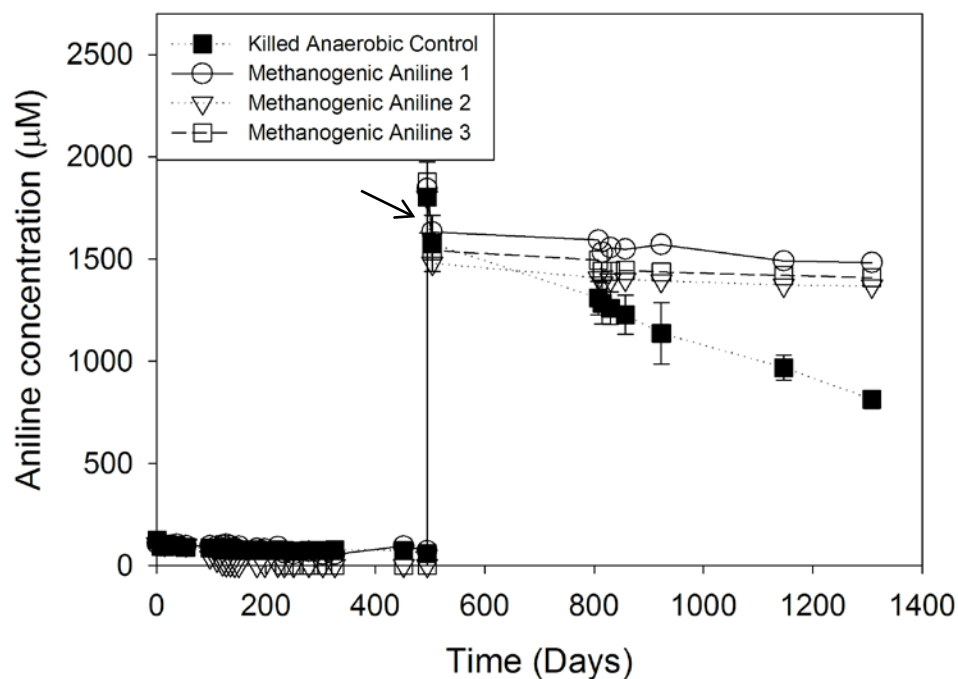
Figure B. 1. Aniline in methanogenic and killed anaerobic aquifer microcosms from the

lightly contaminated groundwater aquifer location, day 0 to 1300. For the

killed control, symbols are averages of triplicates and error bars represent one

standard deviation. The arrow indicates the re-amendment of substrate.
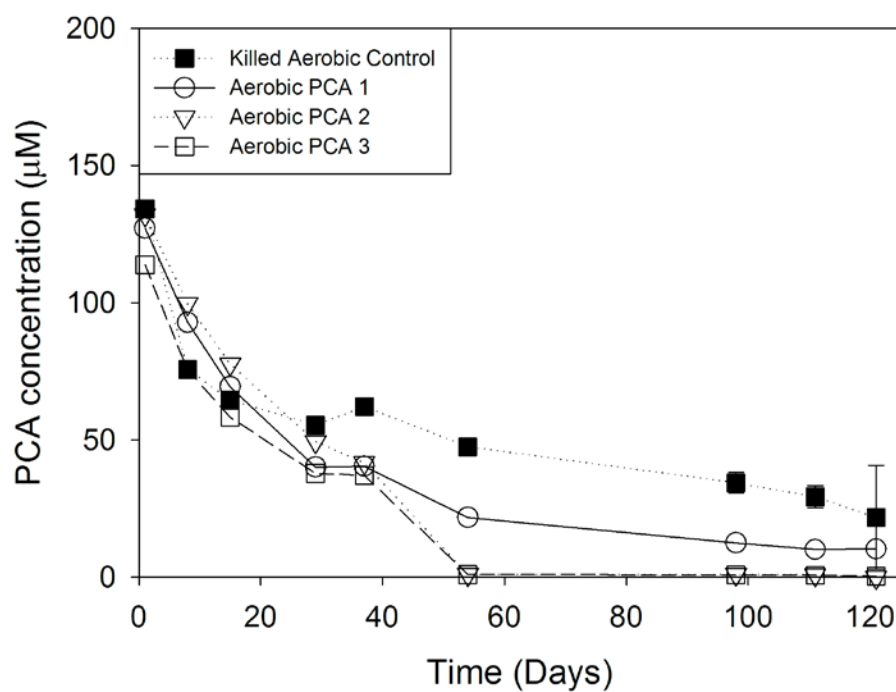
Figure B. 2. PCA in aerobic and killed aerobic aquifer microcosms from the lightly

contaminated groundwater aquifer location, day 0 to120. For the killed

control, symbols are averages of triplicates and error bars represent one

standard deviation.

Figure B. 3. PCA in Fe(III)-amended and killed Fe(III)-amended aquifer microcosms

from the lightly contaminated groundwater aquifer location, day 0 to 350.

Symbols are averages of triplicates and error bars represent one standard

deviation.

Figure B. 4. PCA in Fe(III)-amended and killed Fe(III)-amended aquifer microcosms

from the lightly contaminated groundwater aquifer location, day 0 to 1300.

Symbols are averages of triplicates and error bars represent one standard

deviation. The arrow indicates the re-amendment of substrate.
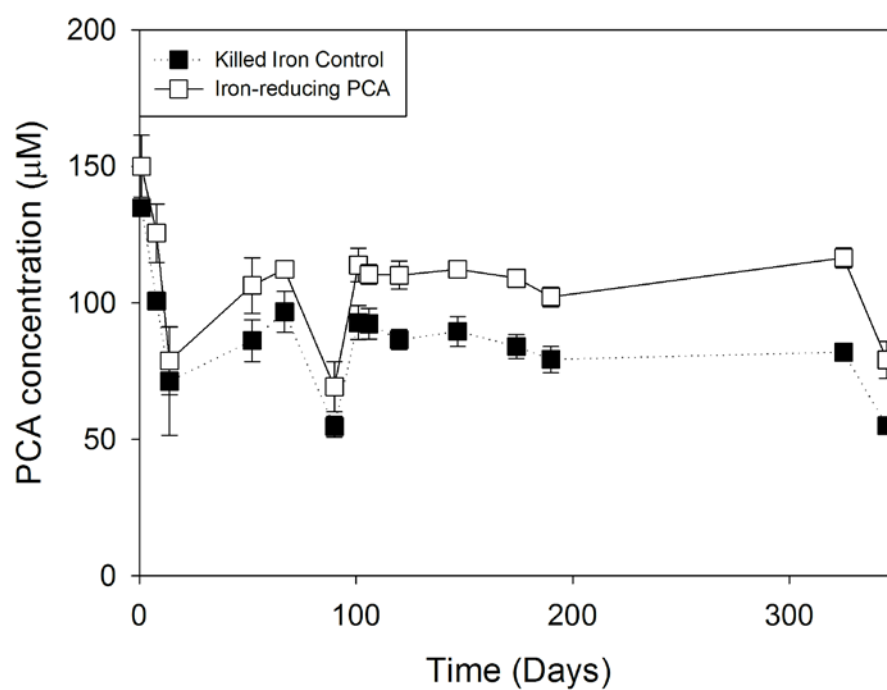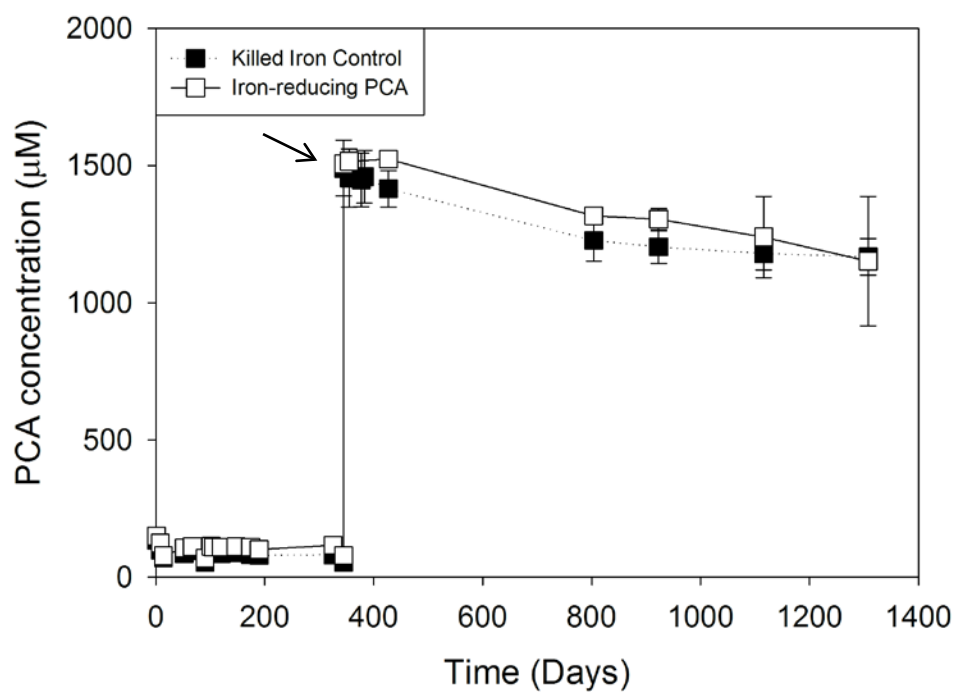
Figure B. 5. PCA in sulfate-amended, methanogenic, and killed anaerobic aquifer

microcosms from the lightly contaminated groundwater aquifer location, day

0 to 300. Symbols are averages of triplicates and error bars represent one

standard deviation.

285

Figure B. 6. PCA in sulfate-amended, methanogenic, and killed anaerobic aquifer microcosms from the lightly contaminated groundwater aquifer location, day 0 to 1300. Symbols are averages of triplicates and error bars represent one standard deviation. The arrow indicates the re-amendment of substrate.

Figure B. 7. Sulfate concentration in PCA-sulfate-amended aquifer microcosms from the

lightly contaminated groundwater aquifer location.

Figure B. 8. Aniline in aerobic and killed aerobic aquifer microcosms from the highly

contaminated groundwater aquifer location. For the killed controls, symbols

are averages of triplicates and error bars represent one standard deviation.

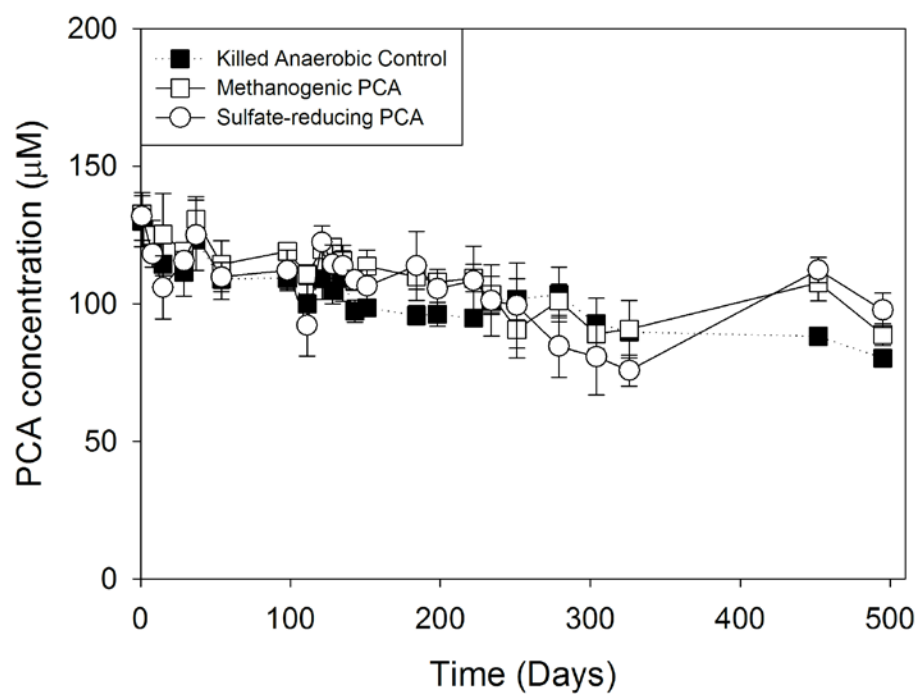The arrow indicates the re-amendment of substrate.

Figure B. 9. Aniline in nitrate-amended and killed anaerobic aquifer microcosms from

the highly contaminated groundwater aquifer location, day 0 to 500. Symbols

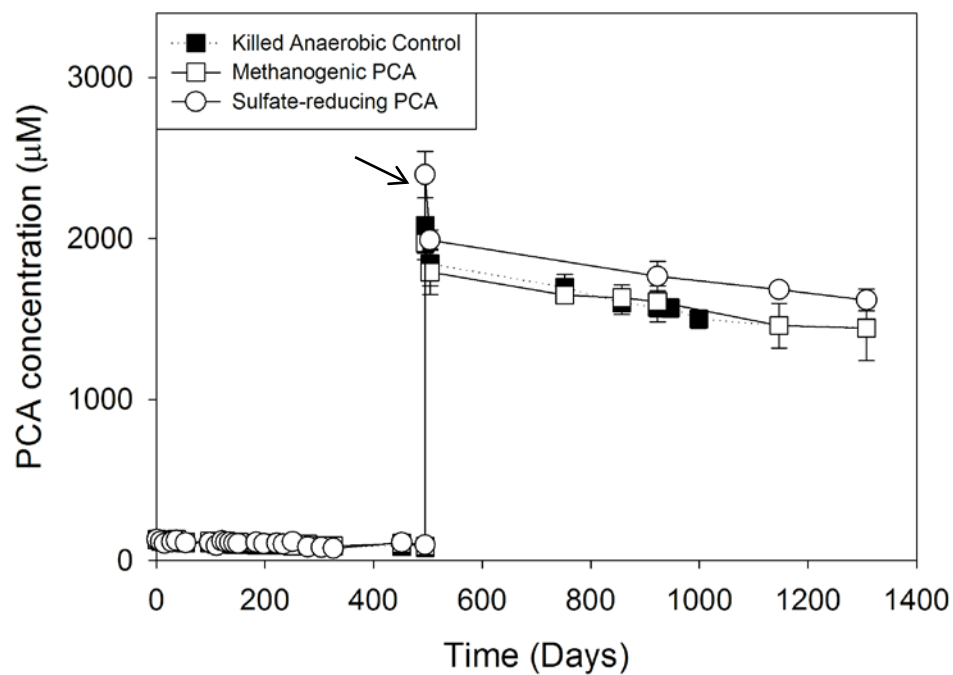are averages of triplicates and error bars represent one standard deviation.

Figure B. 10. Aniline in nitrate-amended and killed anaerobic aquifer microcosms from

the highly contaminated groundwater aquifer location, day 0 to 1400.

Symbols are averages of triplicates and error bars represent one standard

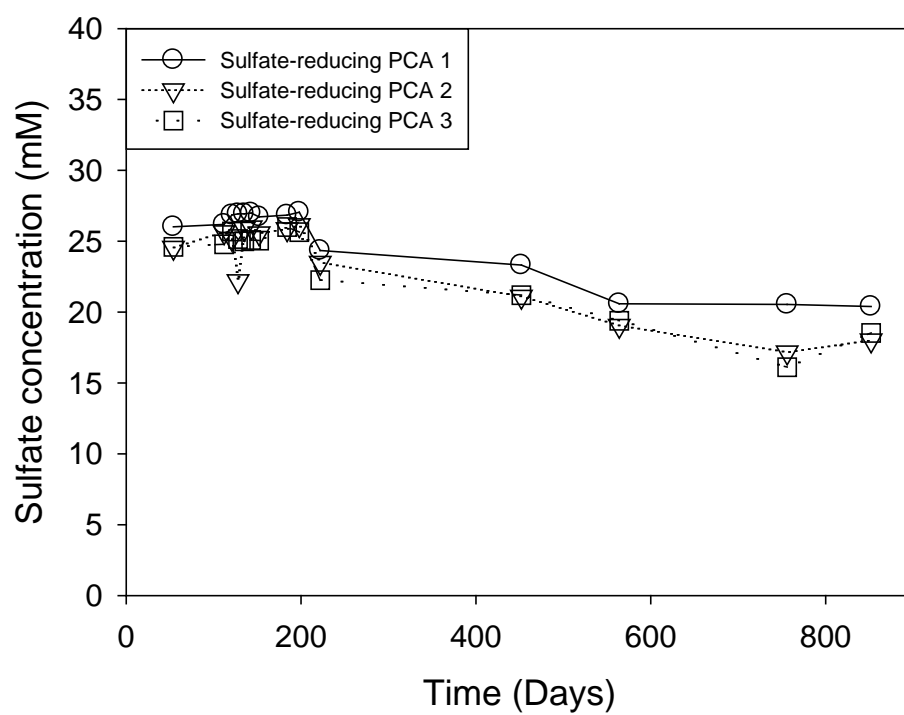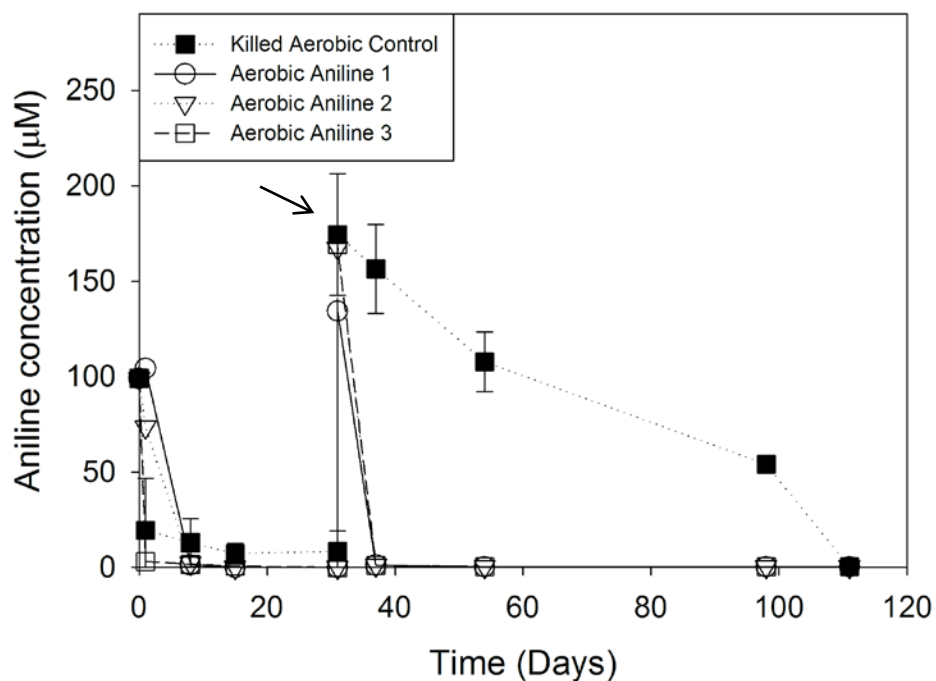deviation. The arrow indicates the re-amendment of substrate.

Figure B. 11. Nitrate concentration in aniline-nitrate-amended aquifer microcosms from

the highly contaminated groundwater aquifer location.

Figure B. 12. Aniline in Fe(III)-amended and killed Fe(III)-amended aquifer microcosms

from the highly contaminated groundwater aquifer location, day 0 to 350. For

the killed controls, symbols are averages of triplicates and error bars

represent one standard deviation. The arrow indicates the re-amendment of

substrate.

Figure B. 13. Aniline in Fe(III)-amended and killed Fe(III)-amended aquifer microcosms

from the highly contaminated groundwater aquifer location, day 0 to 1300.

For the killed controls, symbols are averages of triplicates and error bars

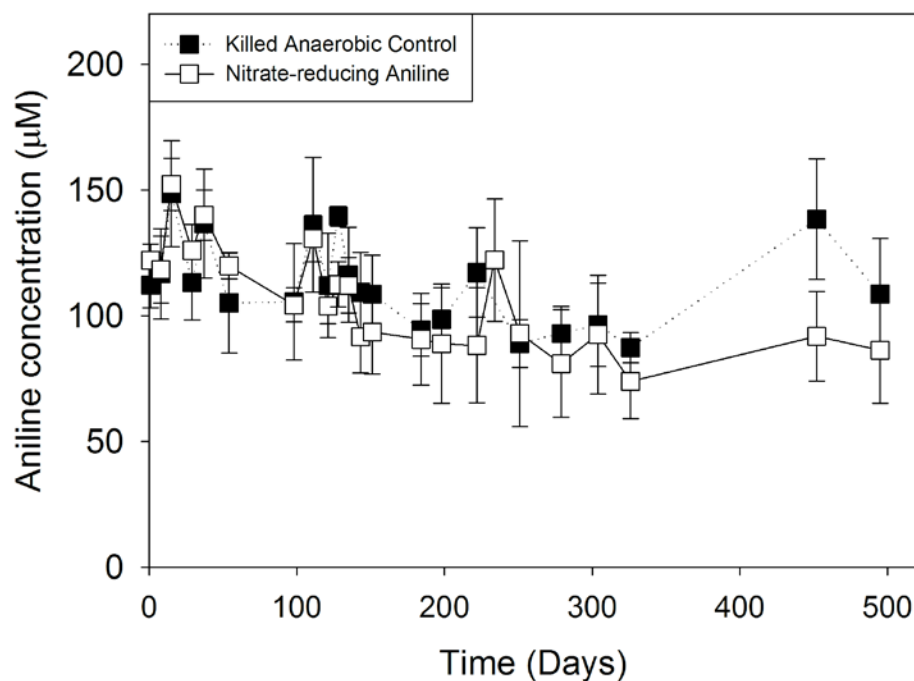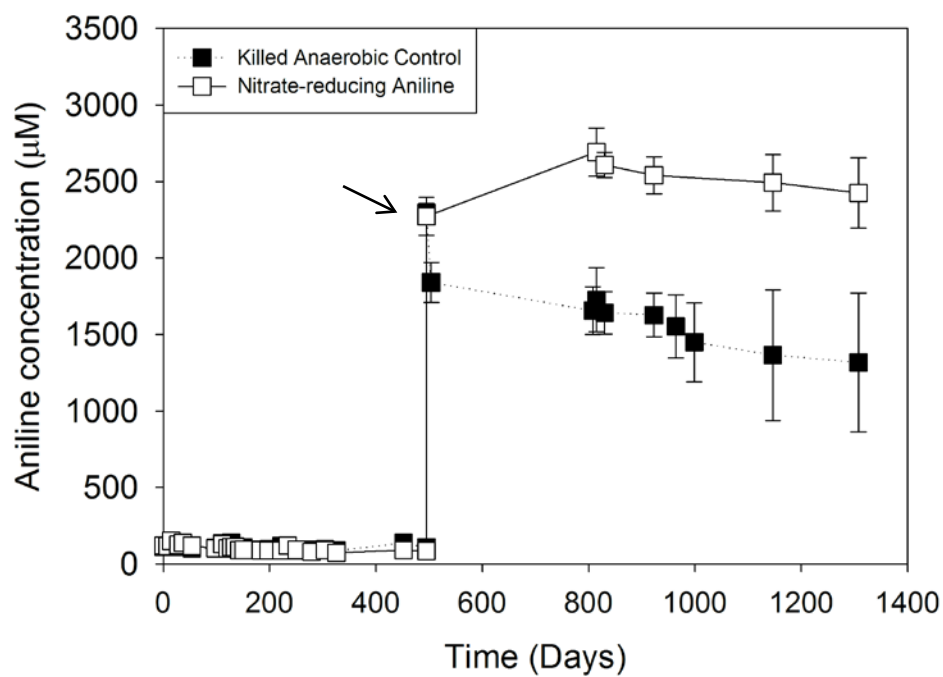represent one standard deviation. The arrow indicates the re-amendment of

substrate.

Figure B. 14. Aniline in sulfate-amended and killed anaerobic aquifer microcosms from

the highly contaminated groundwater aquifer location, day 0 to 1400. For the

killed controls, symbols are averages of triplicates and error bars represent

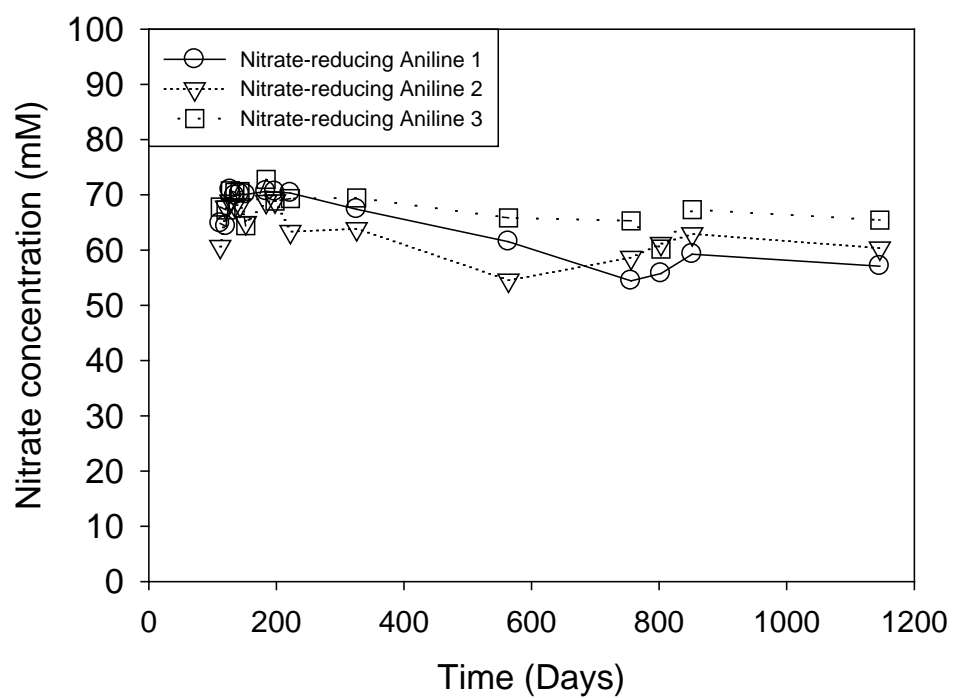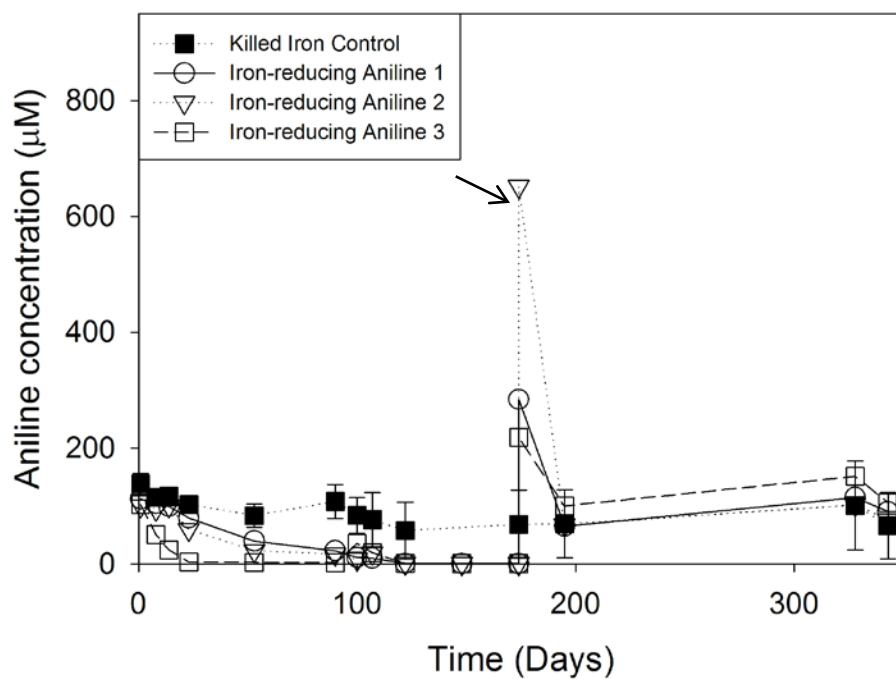one standard deviation. The arrow indicates the re-amendment of substrate.

Figure B. 15. Sulfate concentration in aniline-sulfate-amended aquifer microcosms from the highly contaminated groundwater aquifer location.

Figure B. 16. PCA in aerobic and killed aerobic aquifer microcosms from the highly

contaminated groundwater aquifer location, day 0 to 120. For the killed

controls, symbols are averages of triplicates and error bars represent one

standard deviation.

Figure B. 17. PCA in nitrate-amended and killed anaerobic aquifer microcosms from the

highly contaminated groundwater aquifer location, day 0 to 500. Symbols are

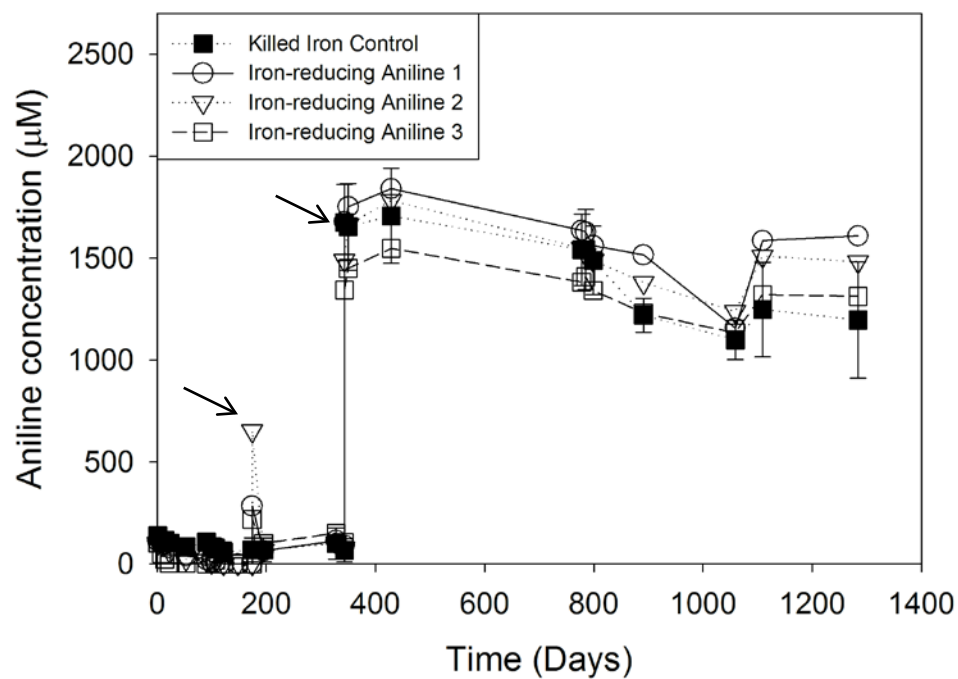averages of triplicates and error bars represent one standard deviation.

Figure B. 18. PCA in nitrate-amended and killed anaerobic aquifer microcosms from the

highly contaminated groundwater aquifer location, day 0 to 1400. Symbols

are averages of triplicates and error bars represent one standard deviation.

The arrow indicates the re-amendment of substrate.

Figure B. 19. Nitrate concentration in PCA-nitrate-amended aquifer microcosms from
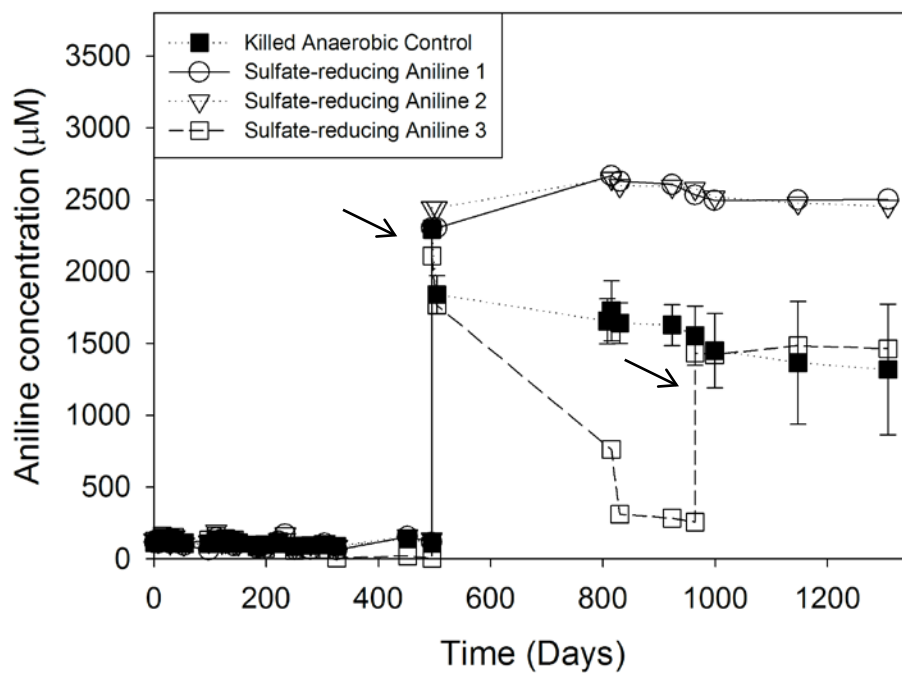
the highly contaminated groundwater aquifer location.

Figure B. 20. PCA in Fe(III)-amended and killed Fe(III)-amended aquifer microcosms

from the highly contaminated groundwater aquifer location, day 0 to 400.

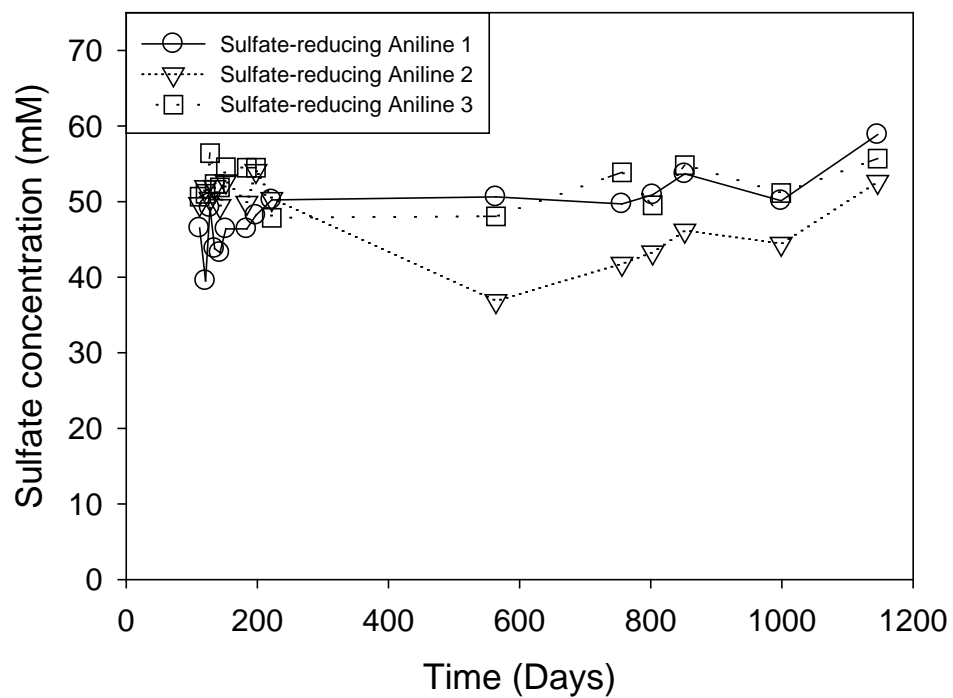Symbols are averages of triplicates and error bars represent one standard

deviation.

Figure B. 21. PCA in Fe(III)-amended and killed Fe(III)-amended aquifer microcosms

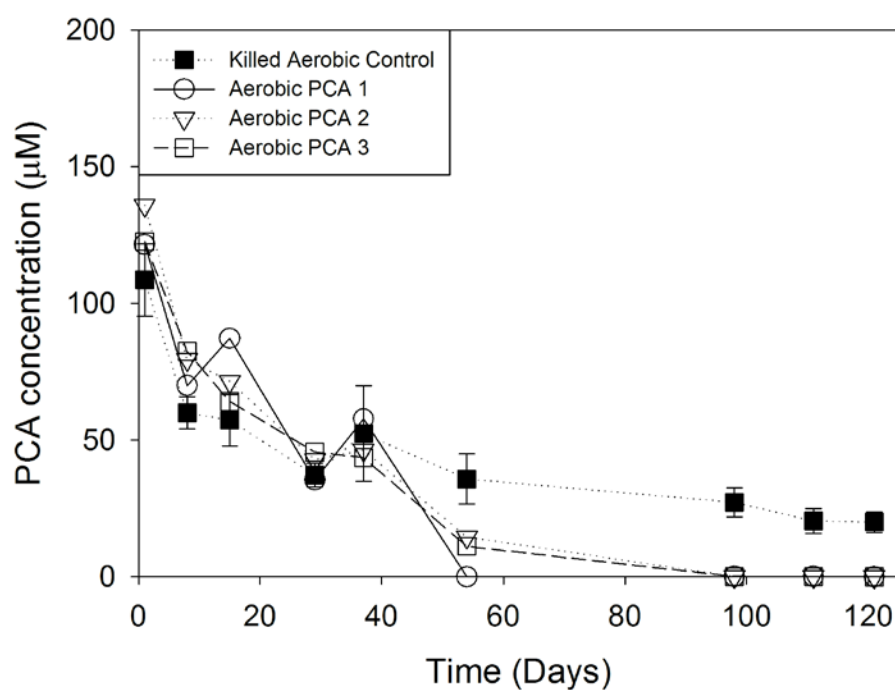from the highly contaminated groundwater aquifer location, day 0 to 1400.

Symbols are averages of triplicates and error bars represent one standard

deviation. The arrow indicates the re-amendment of substrate.

Figure B. 22. PCA in sulfate-amended and killed anaerobic aquifer microcosms from the highly contaminated groundwater aquifer location, day 0 to 500. Symbols are averages of triplicates and error bars represent one standard deviation.
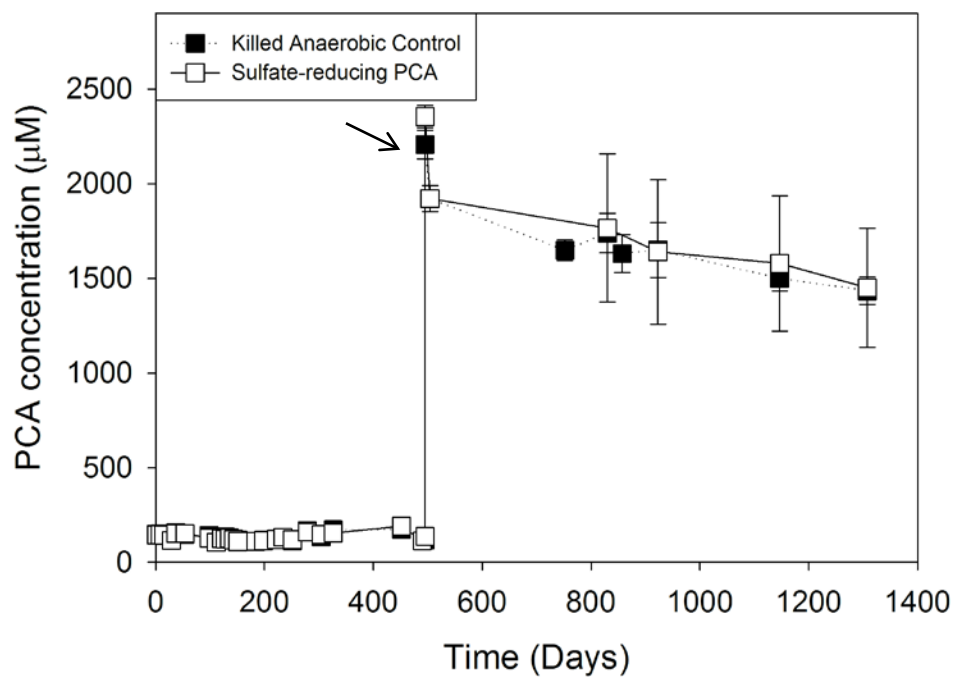
Figure B. 23. PCA in sulfate-amended and killed anaerobic aquifer microcosms from the

highly contaminated groundwater aquifer location, day 0 to 1400. Symbols

are averages of triplicates and error bars represent one standard deviation.
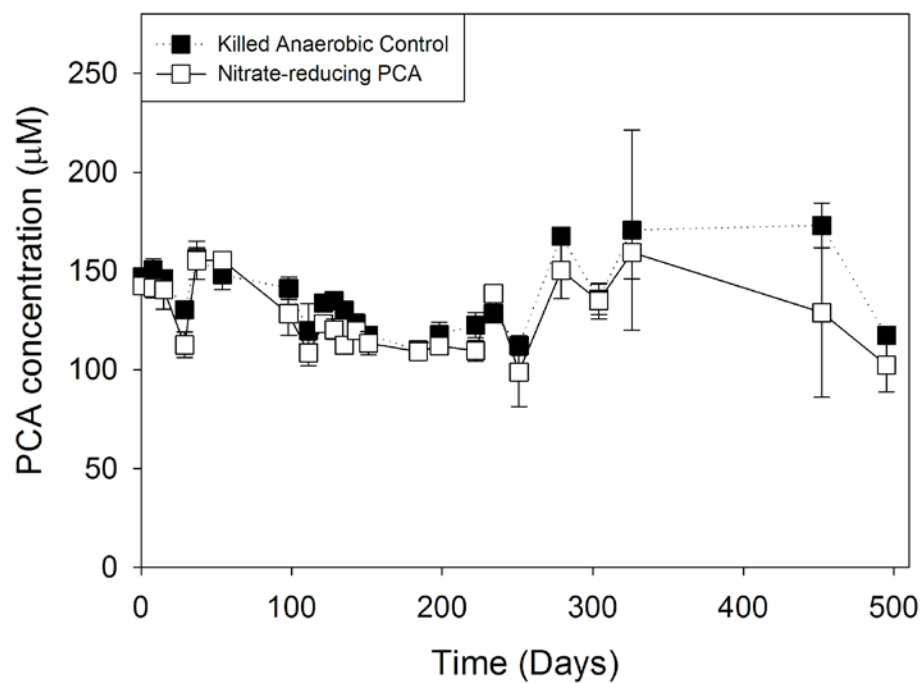
The arrow indicates the re-amendment of substrate.

Figure B. 24. Sulfate concentration in PCA- sulfate-amended aquifer microcosms from
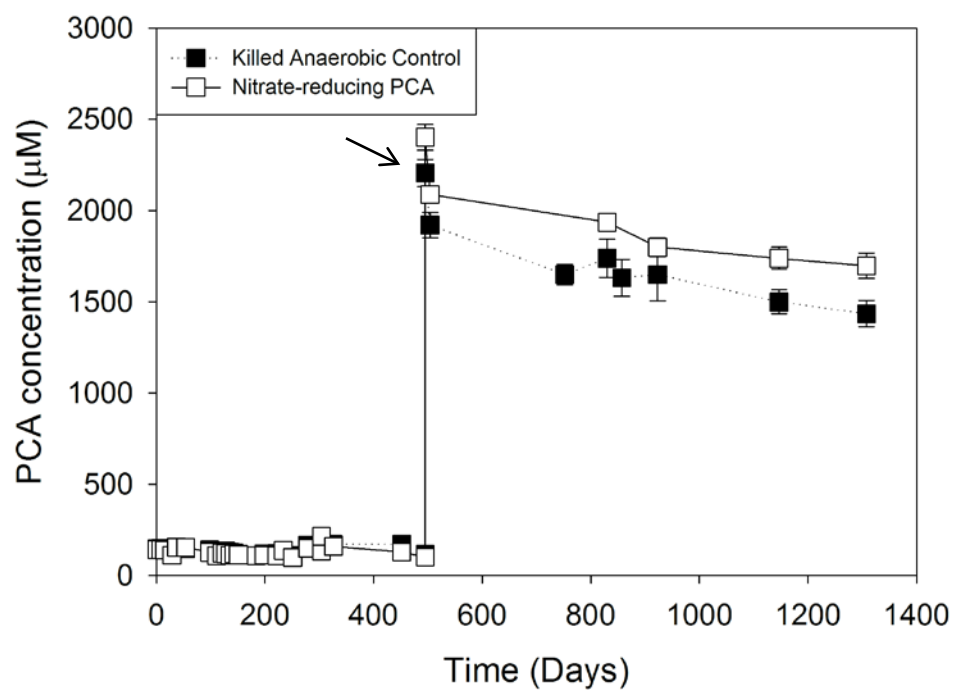
the highly contaminated groundwater aquifer location.

Figure B. 25. PCA in methanogenic and killed anaerobic aquifer microcosms from the

highly contaminated groundwater aquifer location, day 0 to 500. Symbols are

averages of triplicates and error bars represent one standard deviation.

Figure B. 26. PCA in methanogenic and killed anaerobic aquifer microcosms from the

highly contaminated groundwater aquifer location, day 0 to 1400. Symbols

are averages of triplicates and error bars represent one standard deviation.

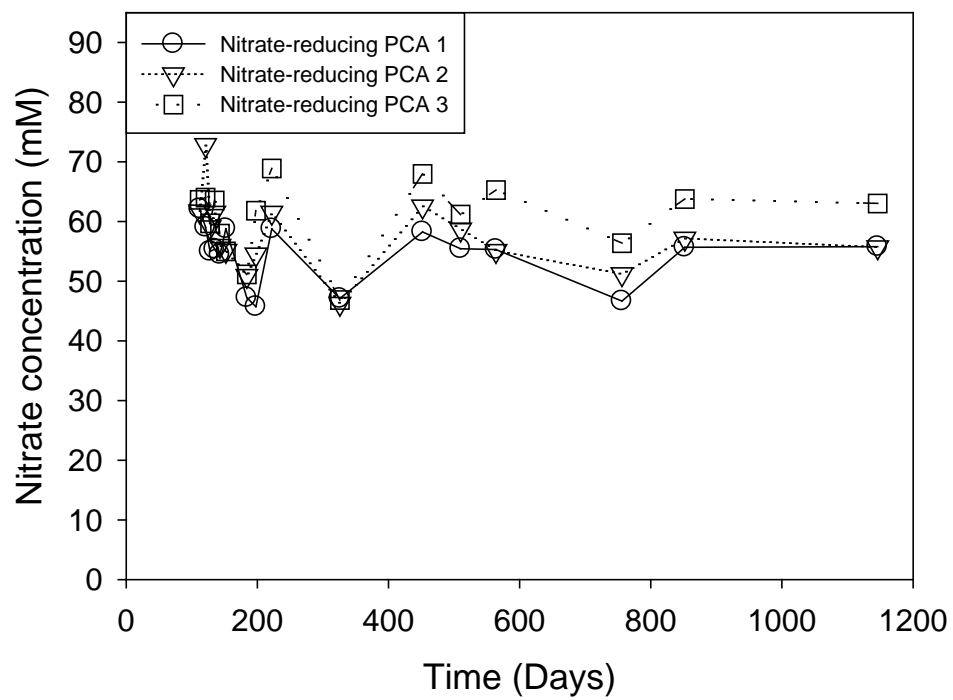The arrow indicates the re-amendment of substrate.

Figure B. 27. PCA in nitrate-amended and killed anaerobic sediment microcosms from

the lightly contaminated freshwater canal location, day 0 to 350. Symbols are

averages of triplicates and error bars represent one standard deviation. The
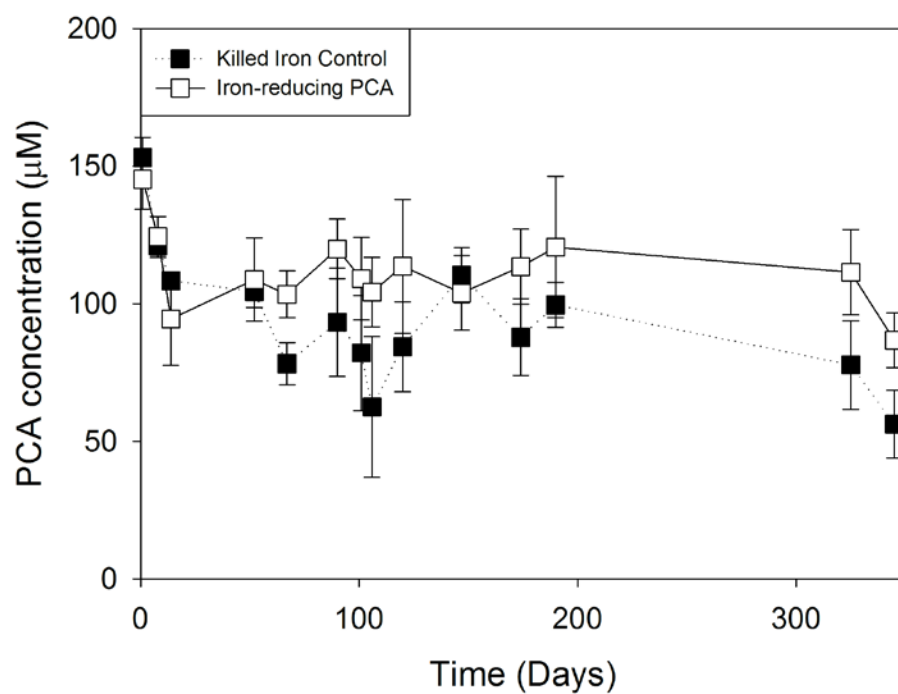
arrow indicates the re-amendment of substrate.

Figure B. 28. PCA in nitrate-amended and killed anaerobic sediment microcosms from

the lightly contaminated freshwater canal location, day 0 to 1300. Symbols

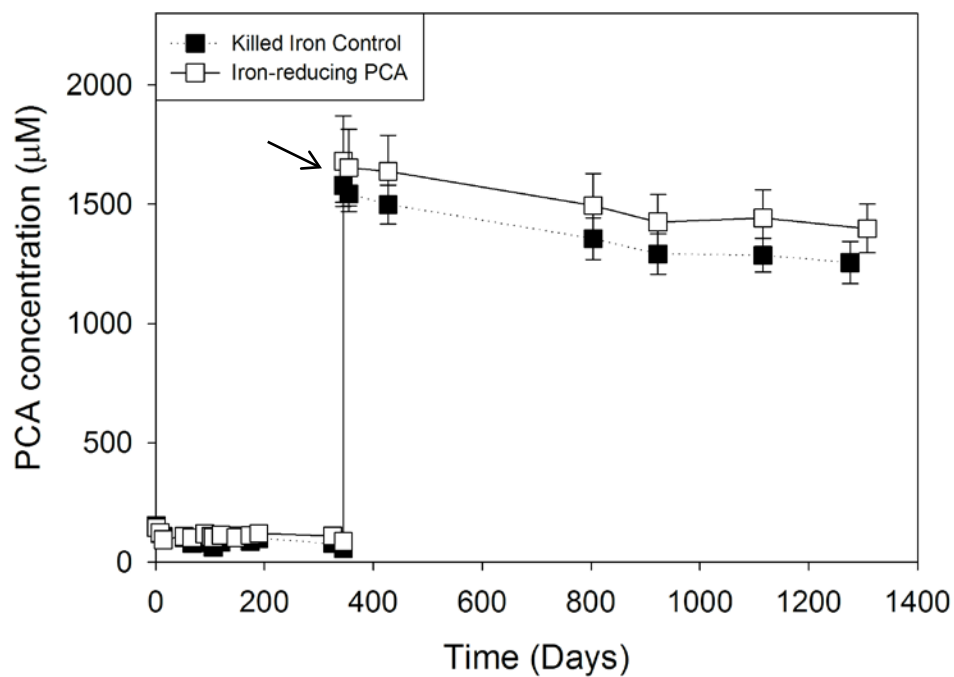are averages of triplicates and error bars represent one standard deviation.
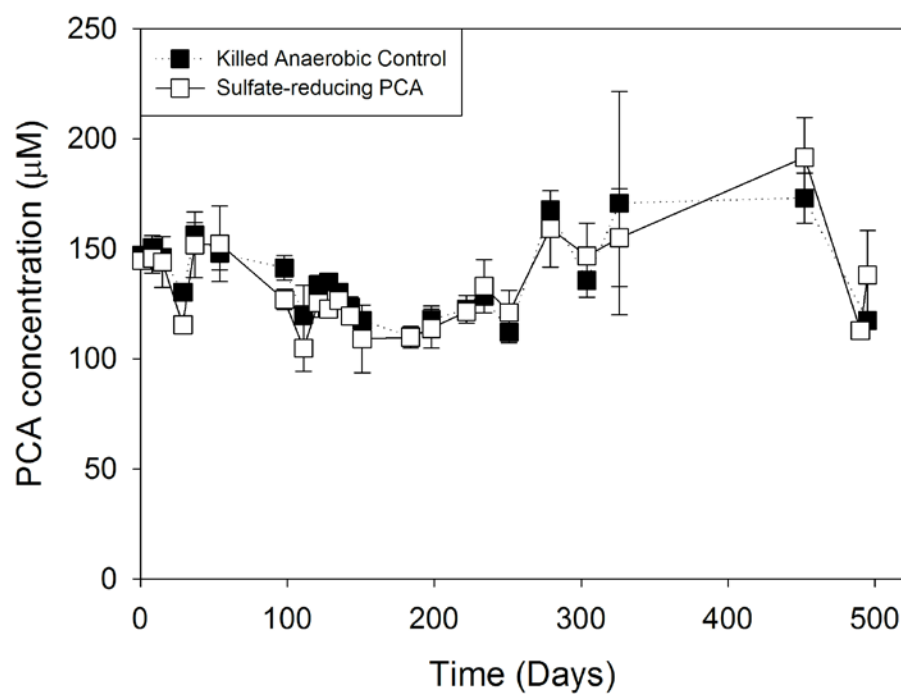
The arrow indicates the re-amendment of substrate.
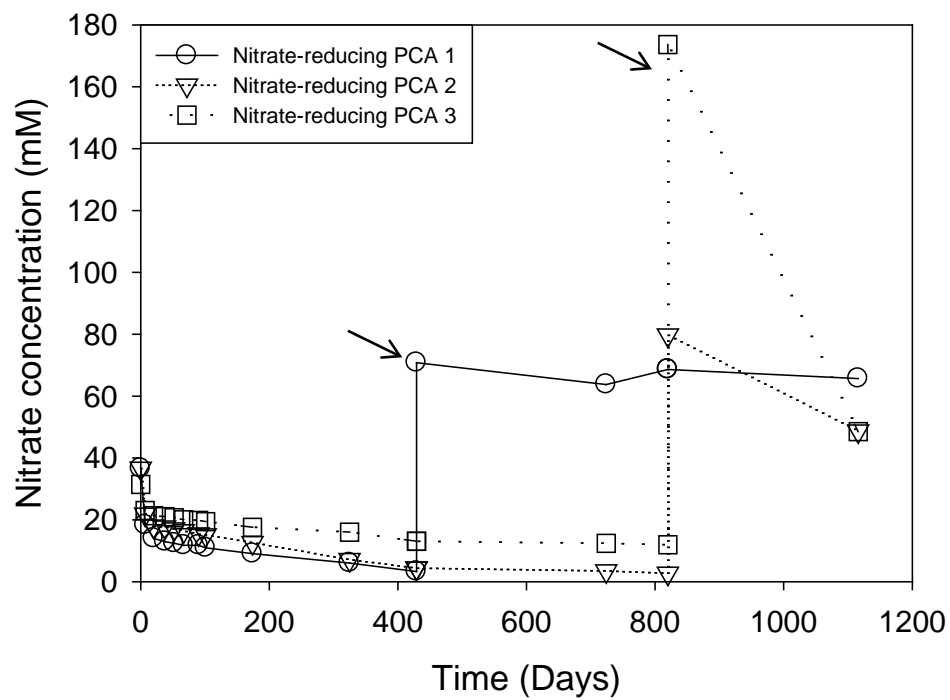
Figure B. 29. Nitrate concentration in PCA-nitrate-amended sediment microcosms from

the lightly contaminated freshwater canal location. The arrow indicates the

re-amendment of nitrate.

(b)


Figure B. 30. PCA in Fe(III)-amended and killed Fe(III)-amended sediment microcosms

from the lightly contaminated freshwater canal location, day 0 to 350.

Symbols are averages of triplicates and error bars represent one standard
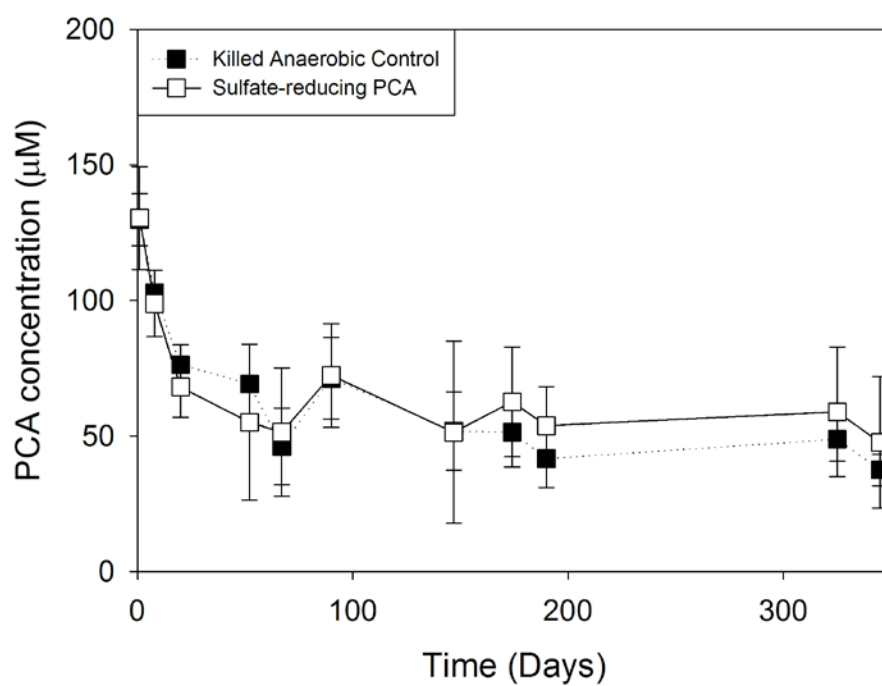
deviation.

Figure B. 31. PCA in sulfate-amended and killed anaerobic sediment microcosms from

the lightly contaminated freshwater canal location, day 0 to 350. Symbols are

averages of triplicates and error bars represent one standard deviation.

Figure B. 32. Aniline in nitrate-amended and killed anaerobic aquifer microcosms from

the highly contaminated freshwater canal location, day 0 to 350. Symbols are

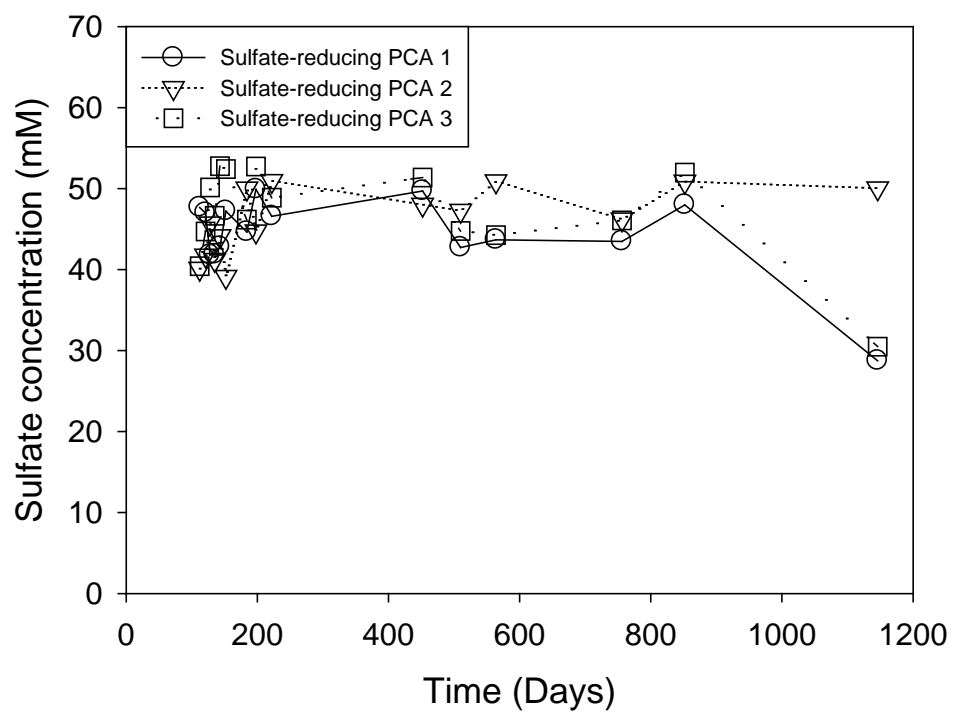averages of triplicates and error bars represent one standard deviation. The

arrow indicates the re-amendment of substrate.

Figure B. 33. Aniline in nitrate-amended and killed anaerobic aquifer microcosms from

the highly contaminated freshwater canal location, day 0 to 1200. Symbols

are averages of triplicates and error bars represent one standard deviation.

The arrow indicates the re-amendment of substrate.

Figure B. 34. Nitrate concentration in aniline-nitrate-amended sediment microcosms

from the highly contaminated freshwater canal location.

Figure B. 35. Aniline in Fe(III)-amended and killed Fe(III)-amended sediment

microcosms from the highly contaminated freshwater canal location, day 0 to

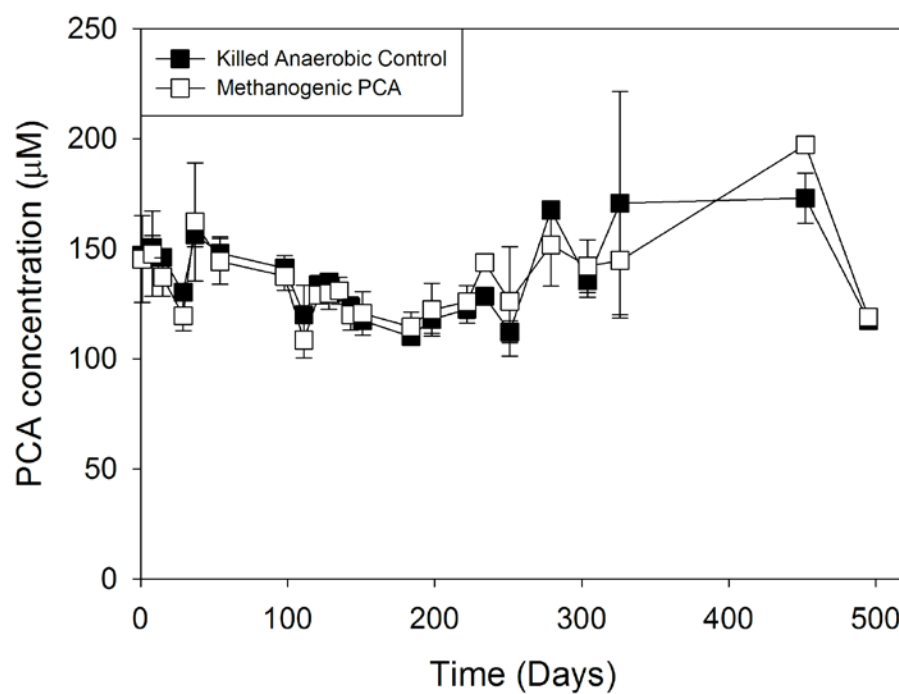350. Symbols are averages of triplicates and error bars represent one standard

deviation.

Figure B. 36. Aniline in sulfate-amended and killed anaerobic aquifer microcosms from the highly contaminated freshwater canal location, day 0 to 300. Symbols are averages of triplicates and error bars represent one standard deviation. The arrow indicates the re-amendment of substrate.
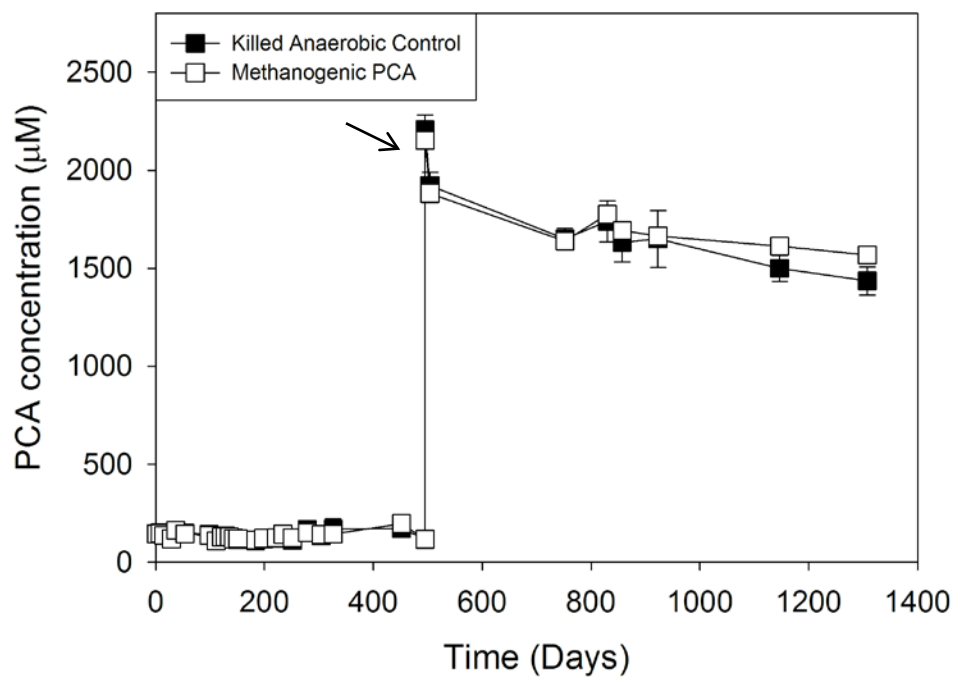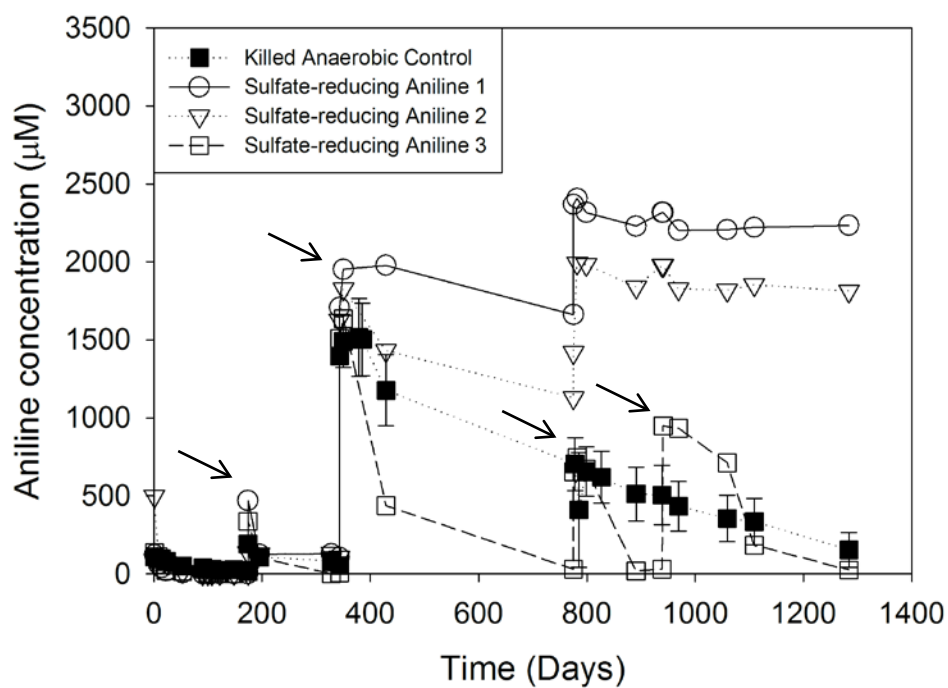
Figure B. 37. Aniline in sulfate-amended and killed anaerobic aquifer microcosms from the highly contaminated freshwater canal location, day 0 to 1300. For the killed control symbols are averages of triplicates and error bars represent one standard deviation. The arrow indicates the re-amendment of substrate.
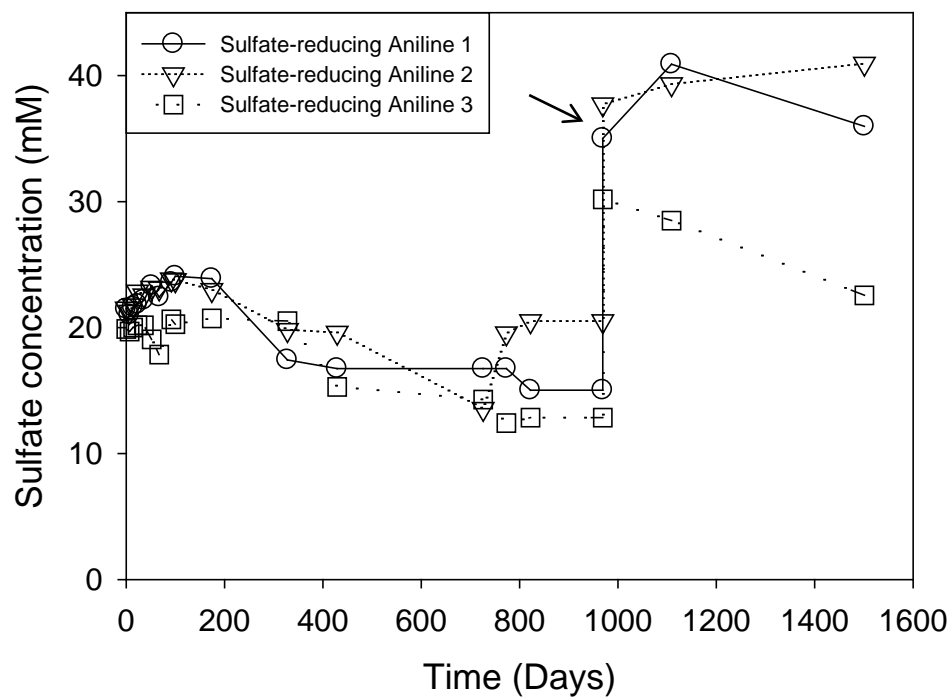
Figure B. 38. Sulfate concentration in aniline-sulfate-amended sediment microcosms

from the highly contaminated freshwater canal location. The arrows indicate
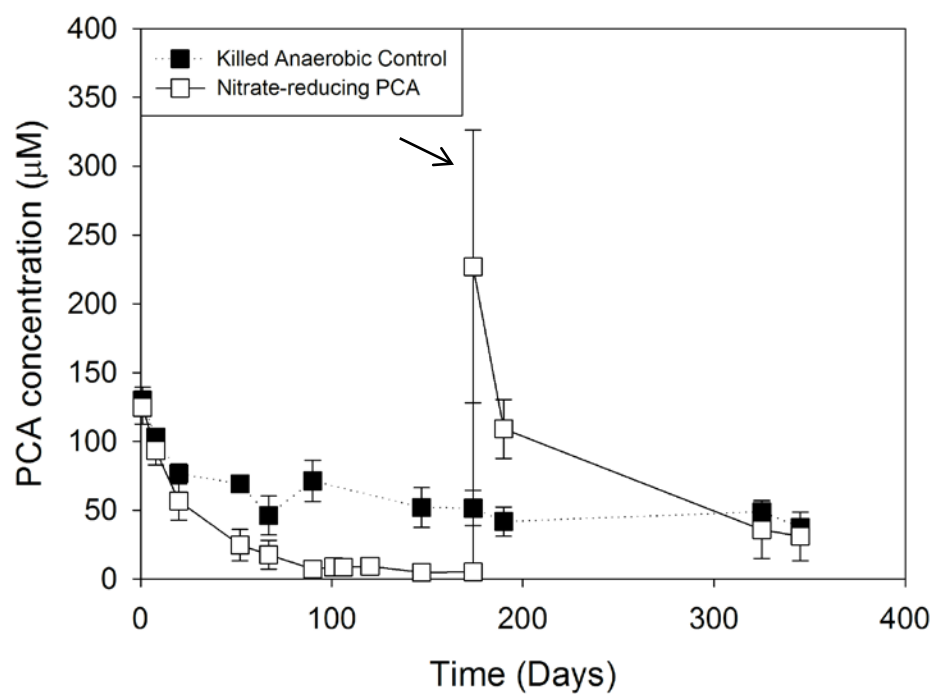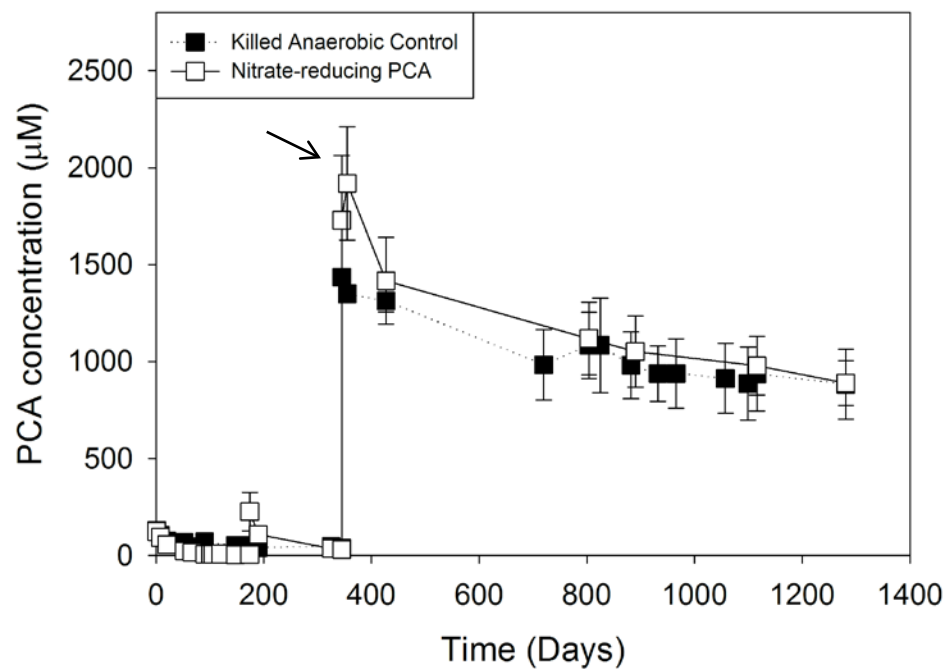
the re-amendment of sulfate.
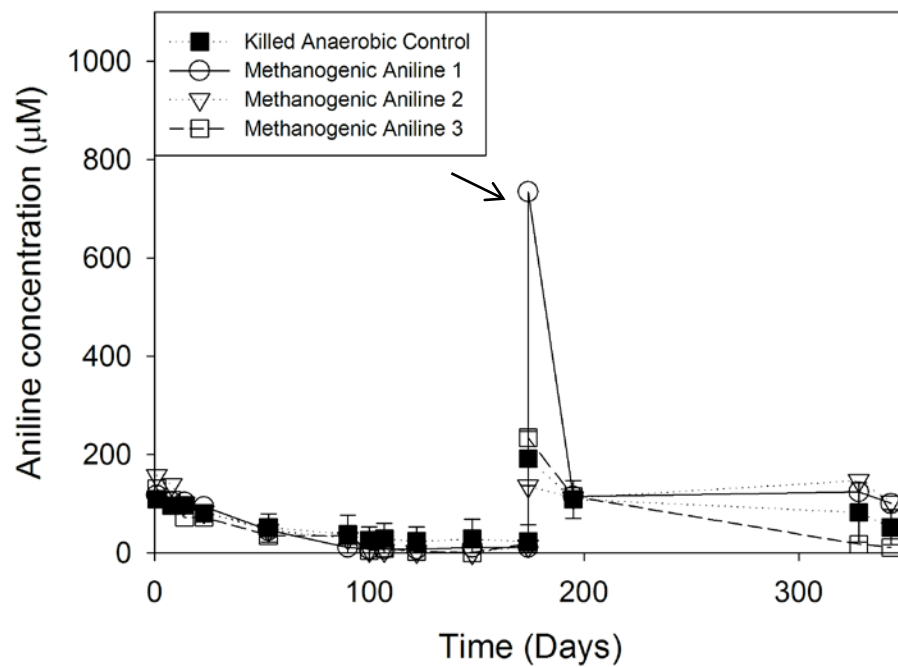
Figure B. 39. Aniline in methanogenic and killed anaerobic aquifer microcosms from the highly contaminated freshwater canal location, day 0 to 350. Symbols are averages of triplicates and error bars represent one standard deviation. The arrow indicates the re-amendment of substrate.
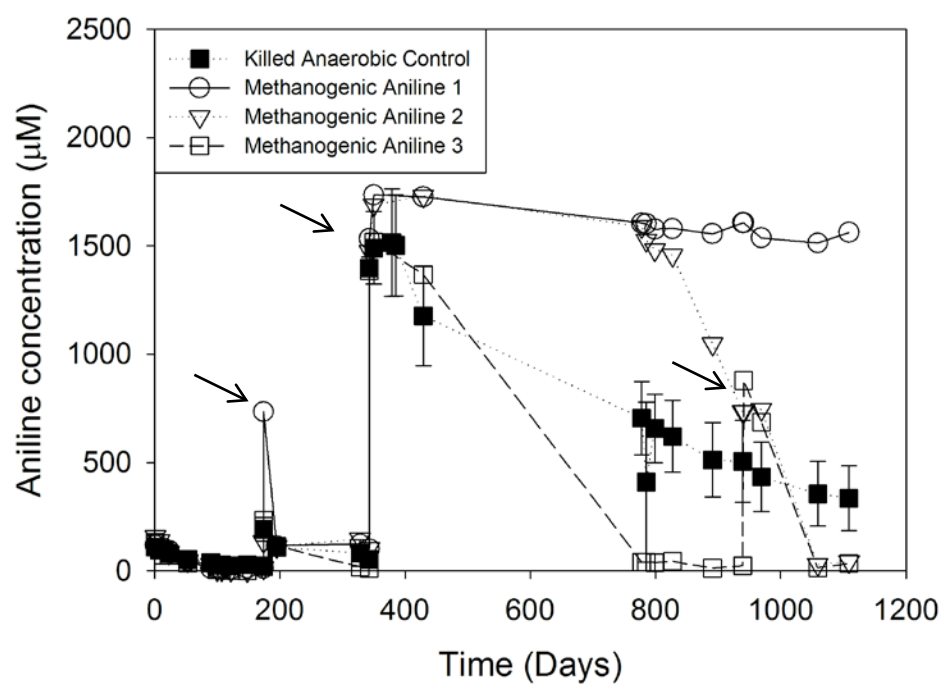
Figure B. 40. Aniline in methanogenic and killed anaerobic aquifer microcosms from the highly contaminated freshwater canal location, day 0 to 1200. For the killed control symbols are averages of triplicates and error bars represent one standard deviation. The arrow indicates the re-amendment of substrate.
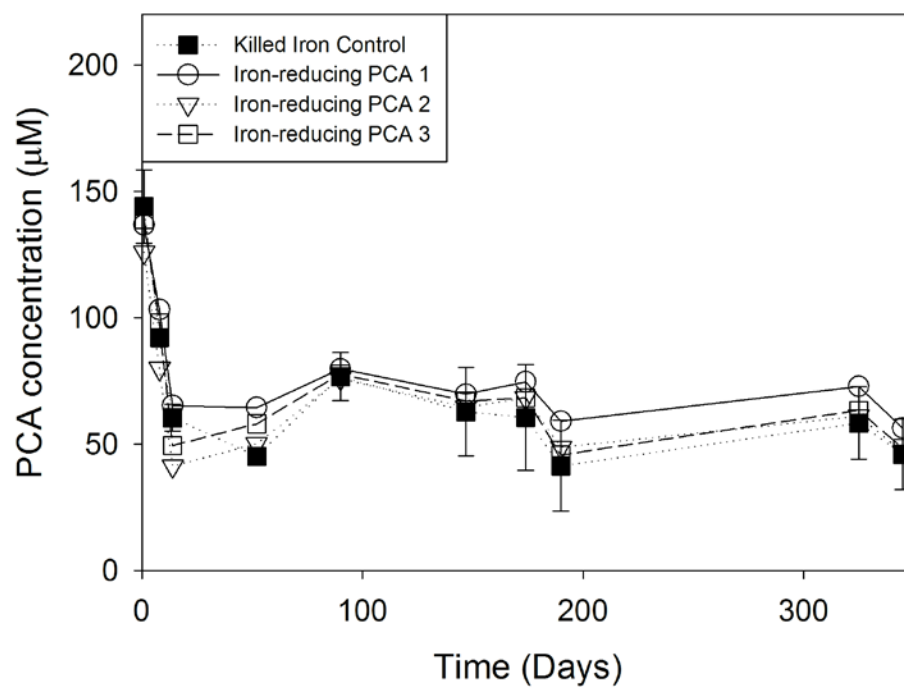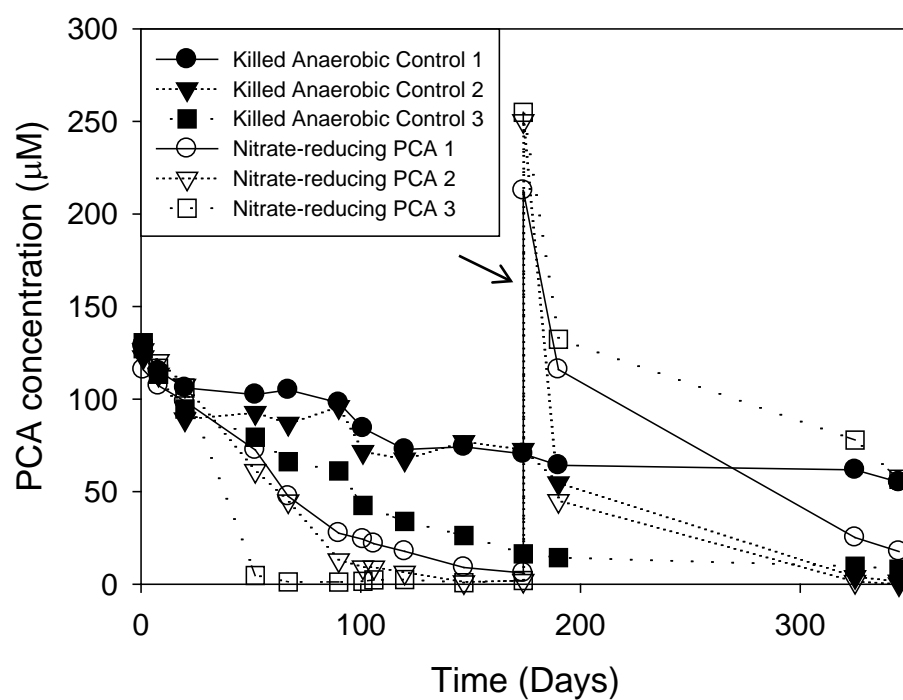
Figure B. 41. PCA in nitrate-amended and killed anaerobic aquifer microcosms from the

highly contaminated freshwater canal location, day 0 to 350. The arrows

indicate the re-amendment of substrate.
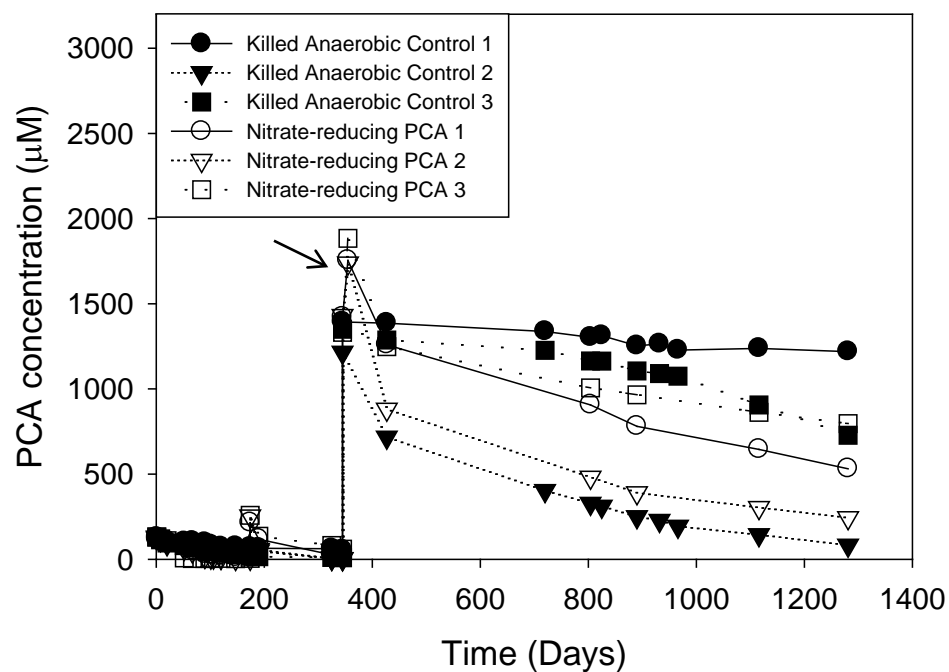
Figure B. 42. PCA in nitrate-amended and killed anaerobic aquifer microcosms from the highly contaminated freshwater canal location, day 0 to 1300. The arrow indicates the re-amendment of substrate.
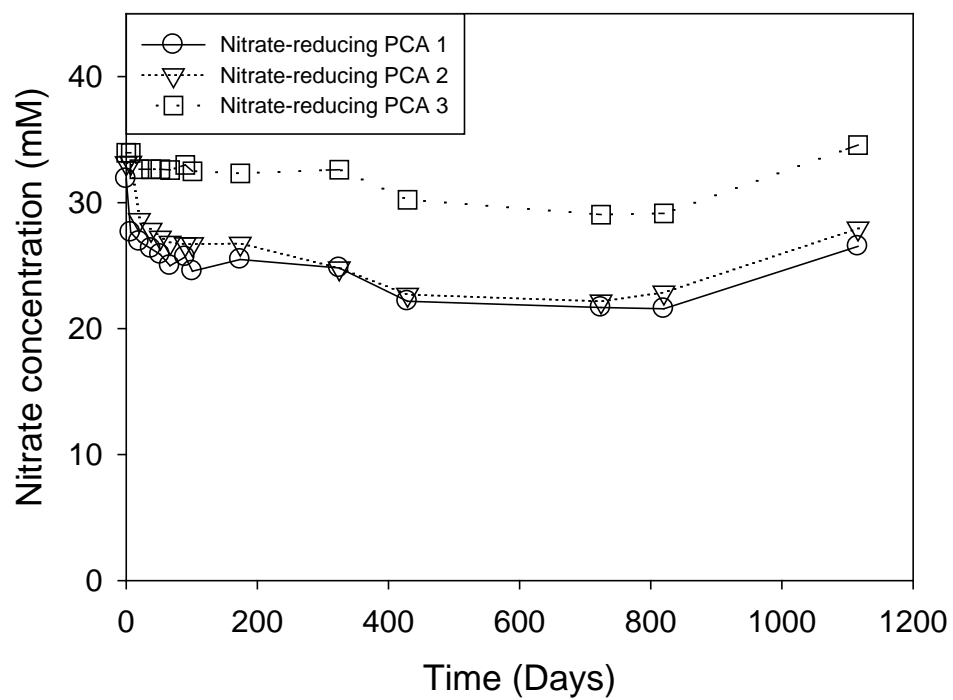
Figure B. 43. Nitrate concentration in PCA- nitrate-amended sediment microcosms from

the highly contaminated freshwater canal location.
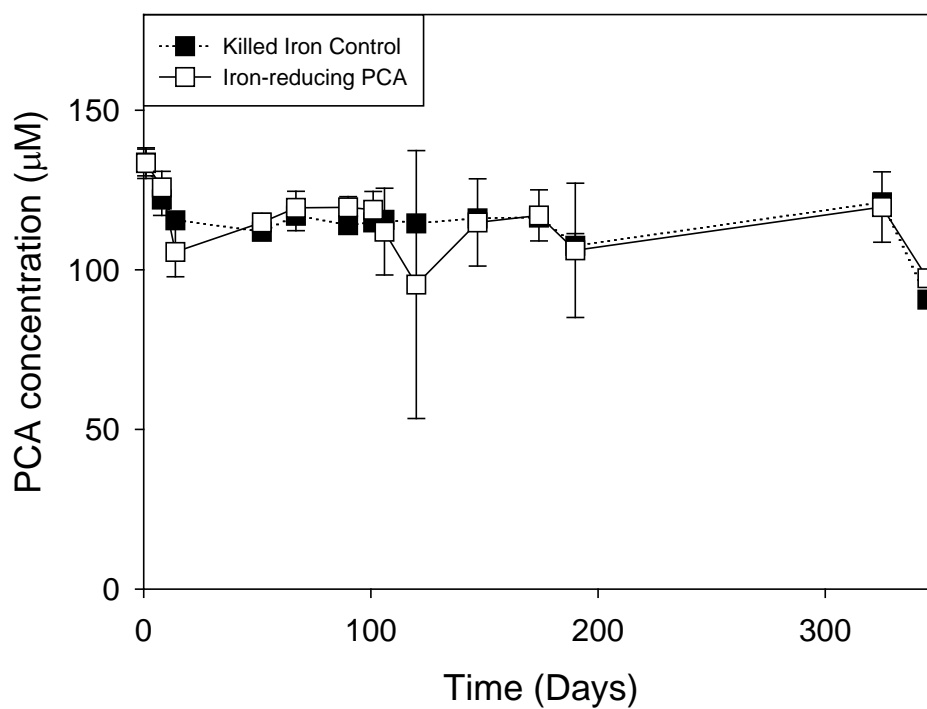
Figure B. 44. PCA in Fe(III)-amended and Fe(III)-amended killed aquifer microcosms

from the highly contaminated freshwater canal location, day 0 to 350.

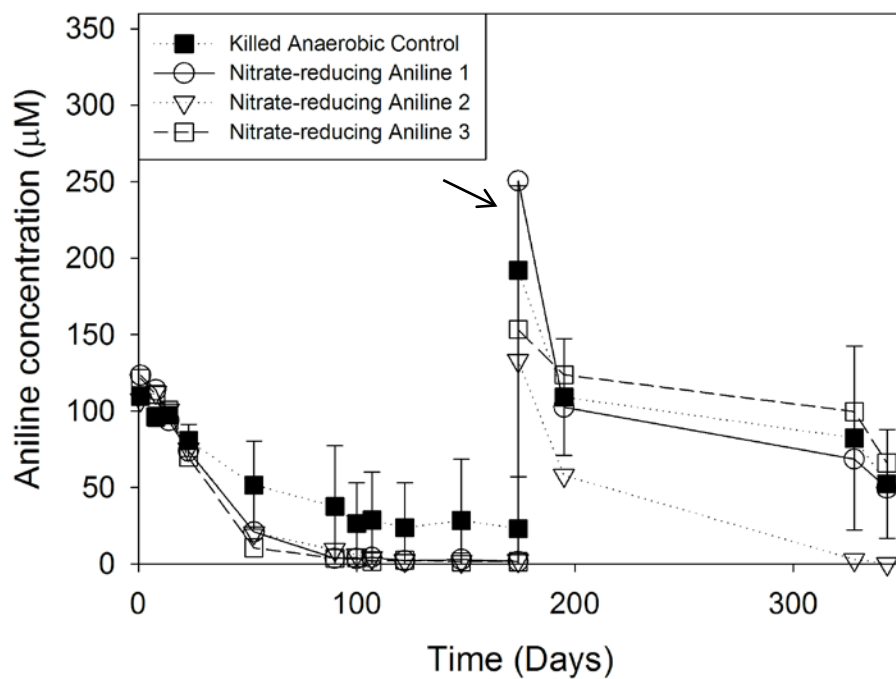Symbols are averages of triplicates and error bars represent one standard
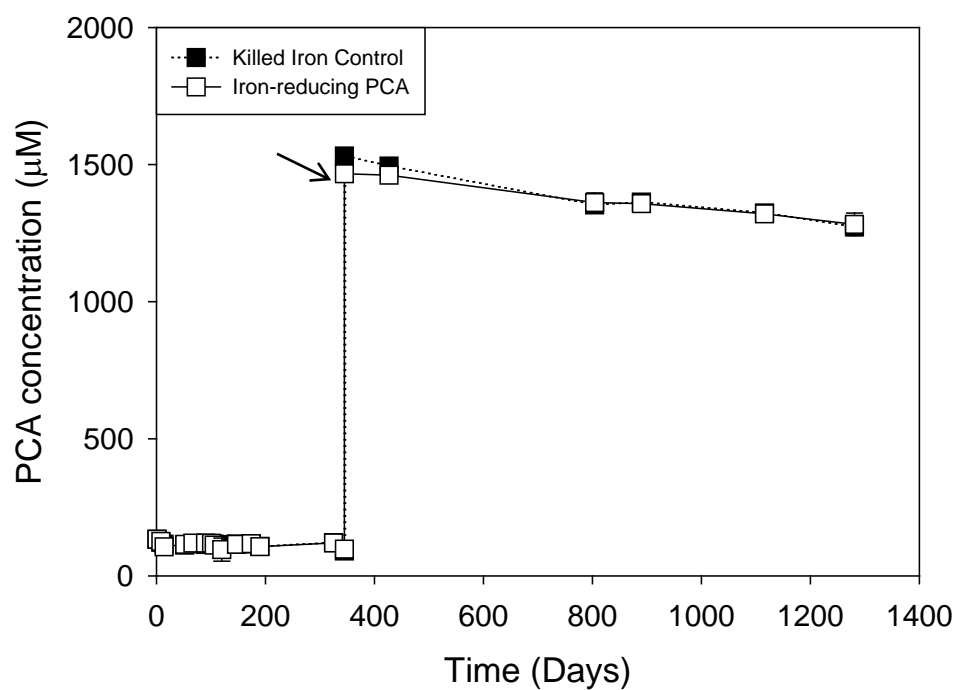
deviation.

Figure B. 45. PCA in Fe(III)-amended and Fe(III)-amended killed aquifer microcosms

from the highly contaminated freshwater canal location, day 0 to 1300.

Symbols are averages of triplicates and error bars represent one standard
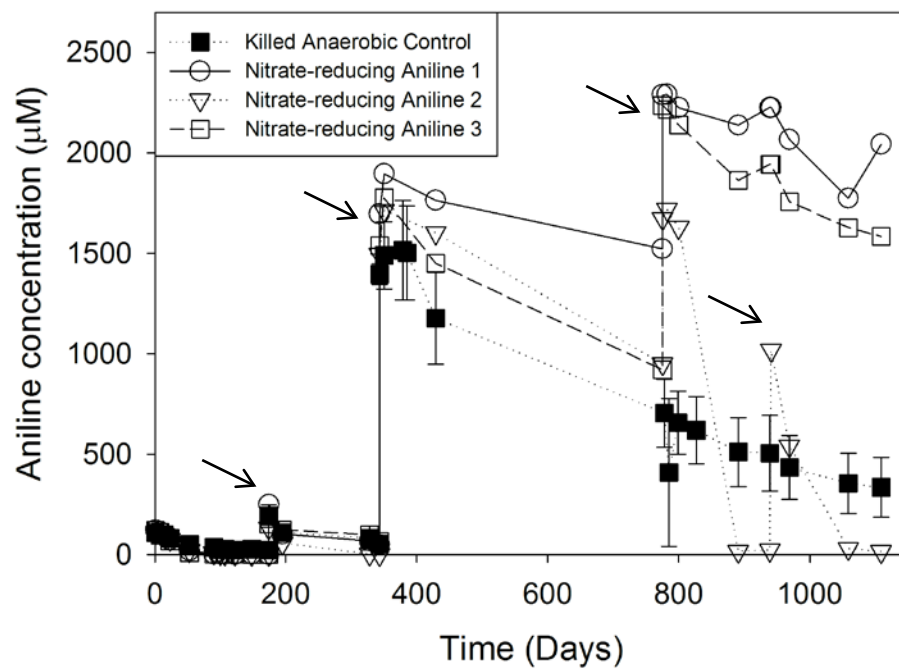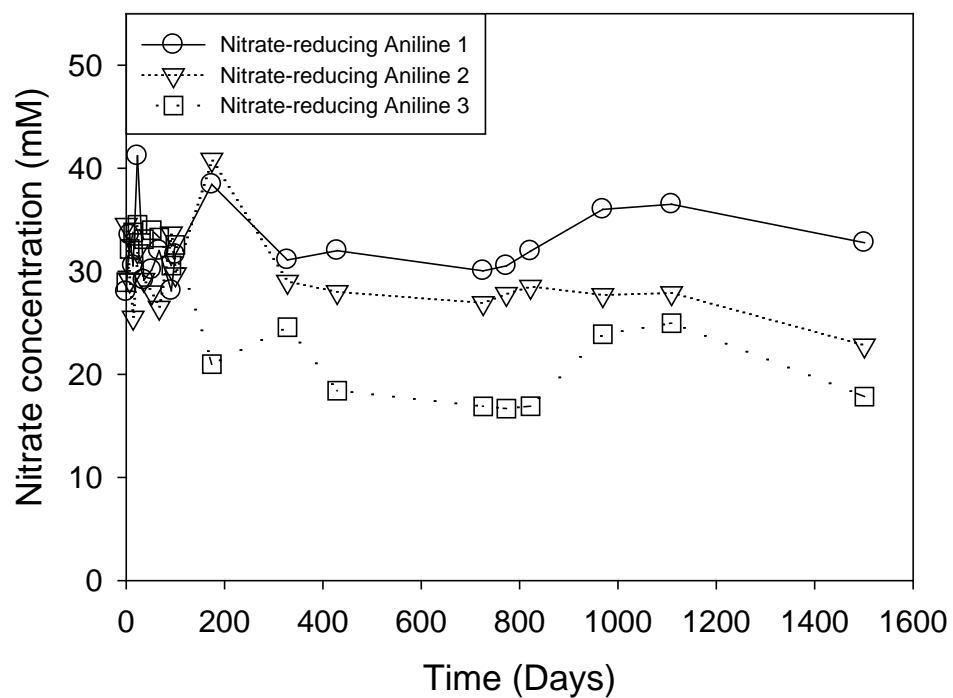
deviation. The arrow indicates the re-amendment of substrate.
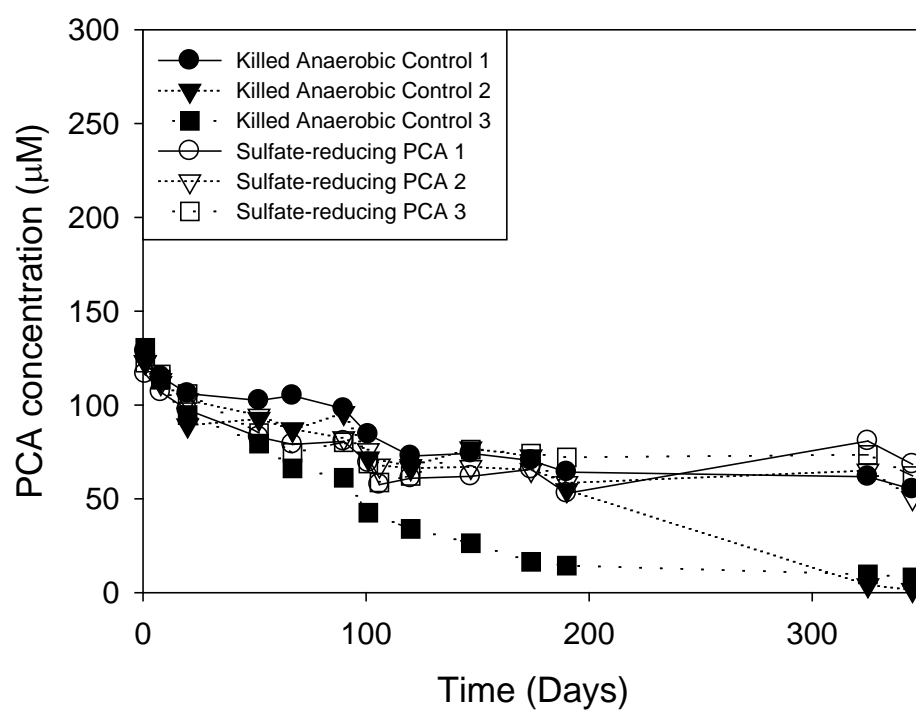
Figure B. 46. PCA in sulfate-amended and killed anaerobic aquifer microcosms from the

highly contaminated freshwater canal location, day 0 to 350.
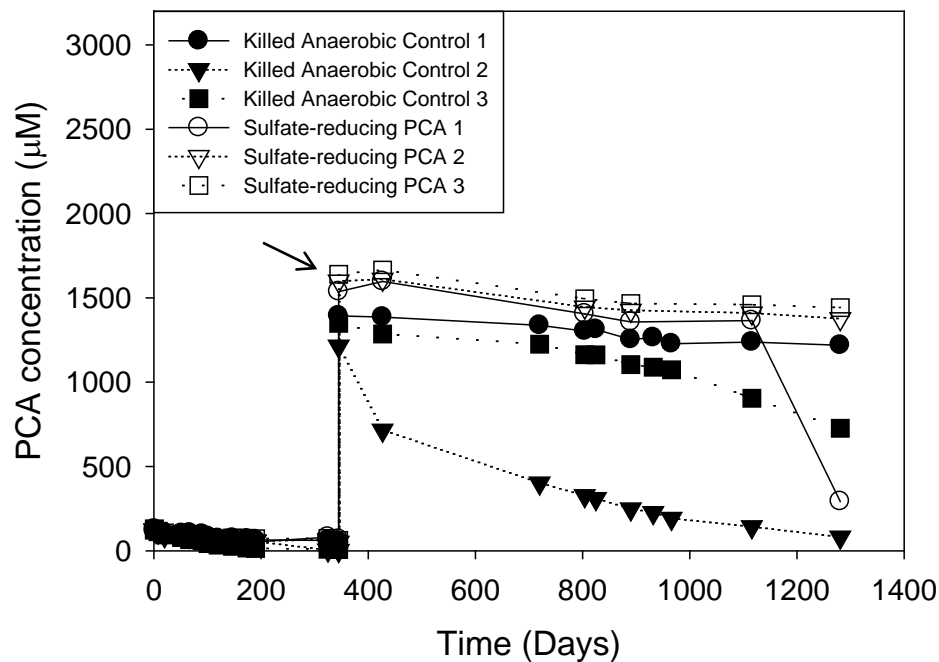
Figure B. 47. PCA in sulfate-amended and killed anaerobic aquifer microcosms from the

highly contaminated freshwater canal location, day 0 to 1300. The arrow

indicates the re-amendment of substrate.
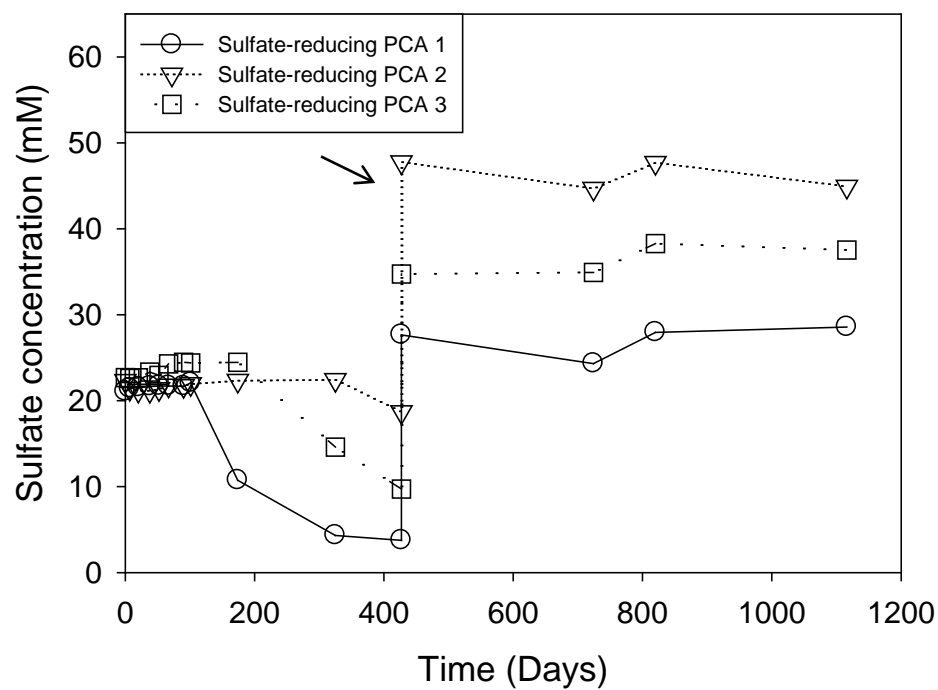
Figure B. 48. Sulfate concentration in PCA-sulfate-amended sediment microcosms from

the highly contaminated freshwater canal location. The arrow indicates the

re-amendment of sulfate.
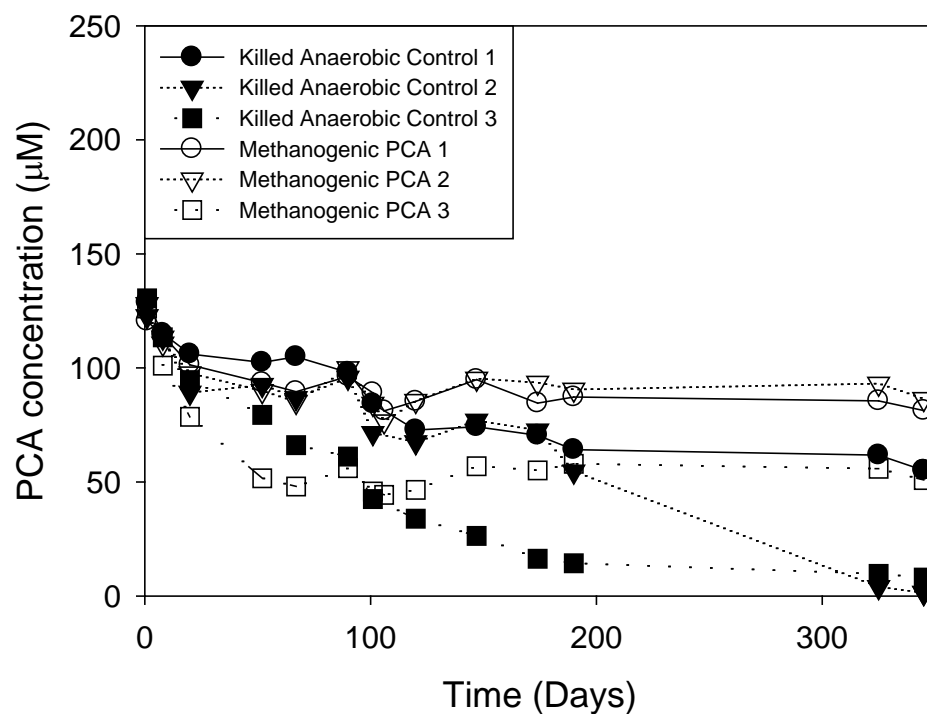
Figure B. 49. PCA in methanogenic and killed anaerobic aquifer microcosms from the

highly contaminated freshwater canal location, day 0 to 350.
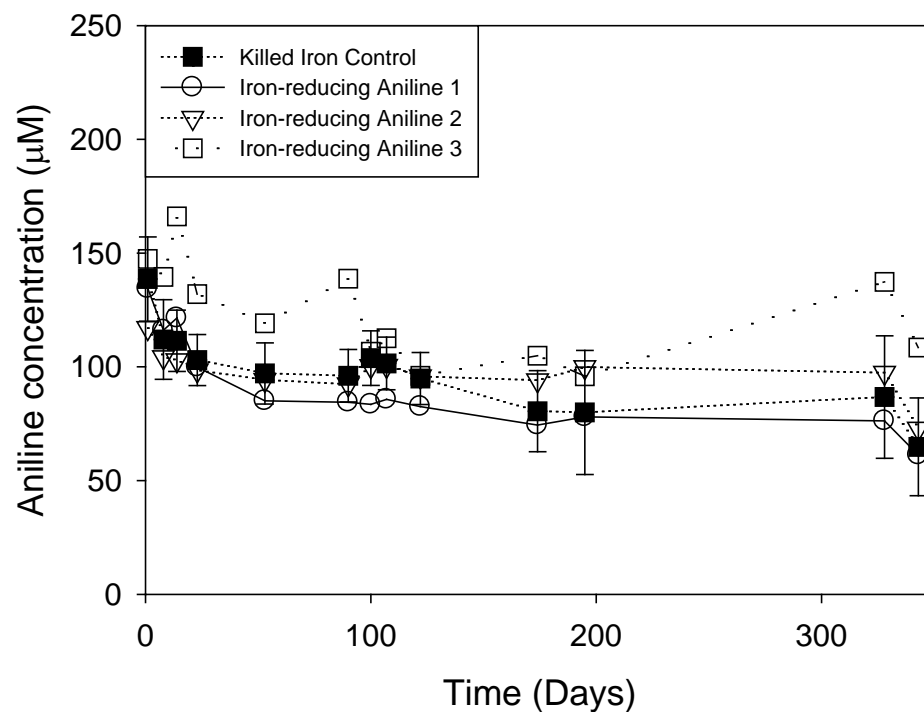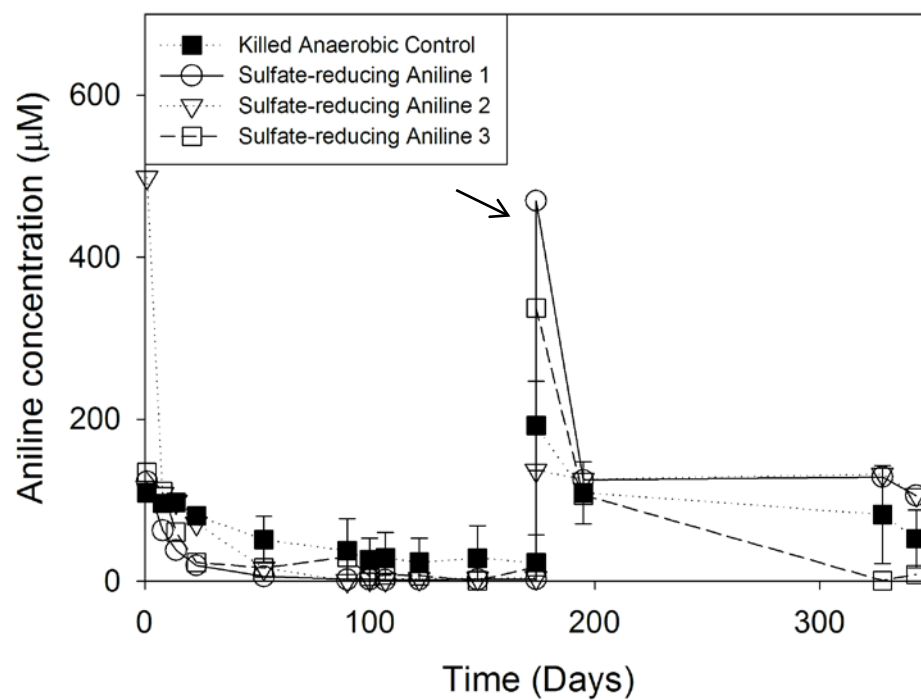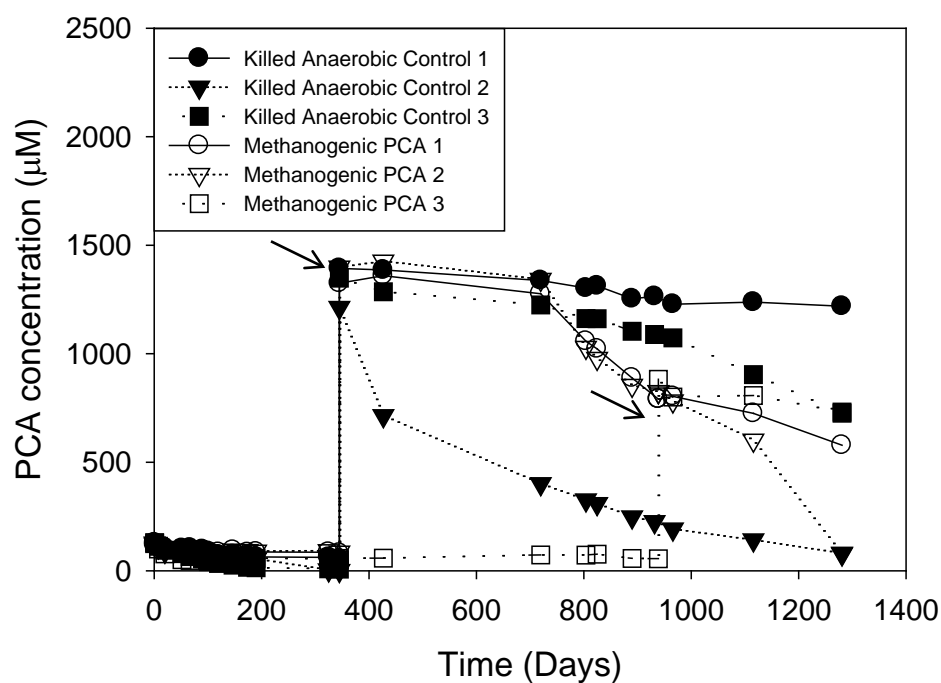
Figure B. 50. PCA in methanogenic and killed anaerobic aquifer microcosms from the

highly contaminated freshwater canal location, day 0 to 1300. The arrow

indicates the re-amendment of substrate.
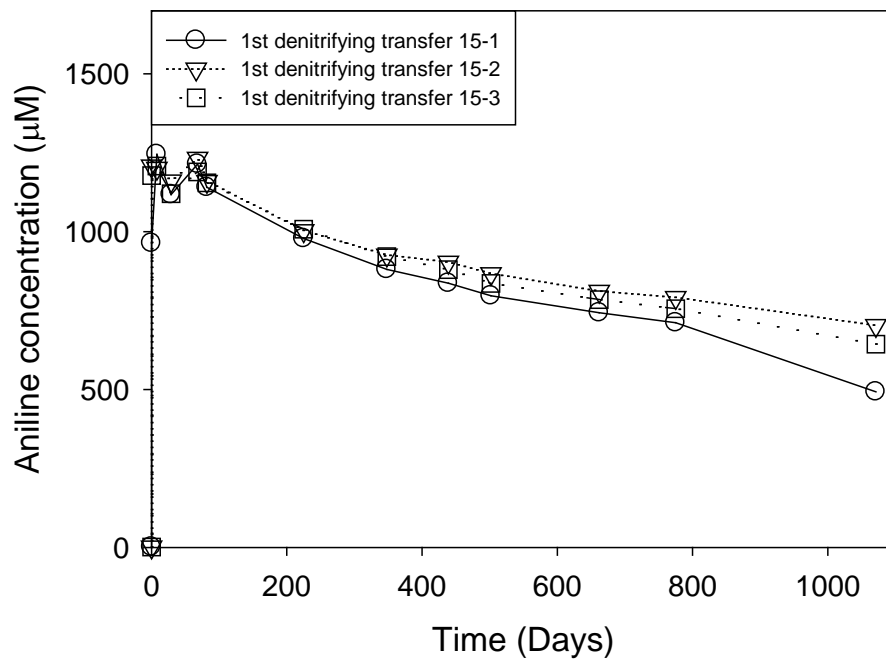
Figure B. 51. Aniline in nitrate-amended enrichment (with NH$_4$Cl, without reducing

agents) transferred from one of the triplicate microcosms from the lightly

contaminated groundwater aquifer location.
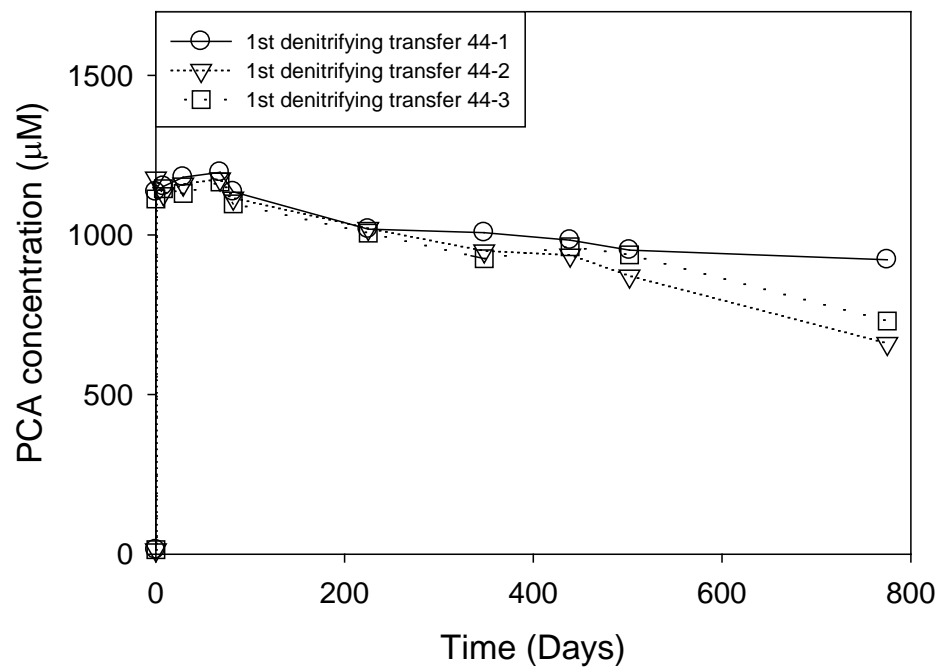
Figure B. 52. PCA in nitrate-amended enrichment (with NH$_4$Cl, without reducing agents) transferred from one of the triplicate microcosms from the lightly contaminated groundwater aquifer location.
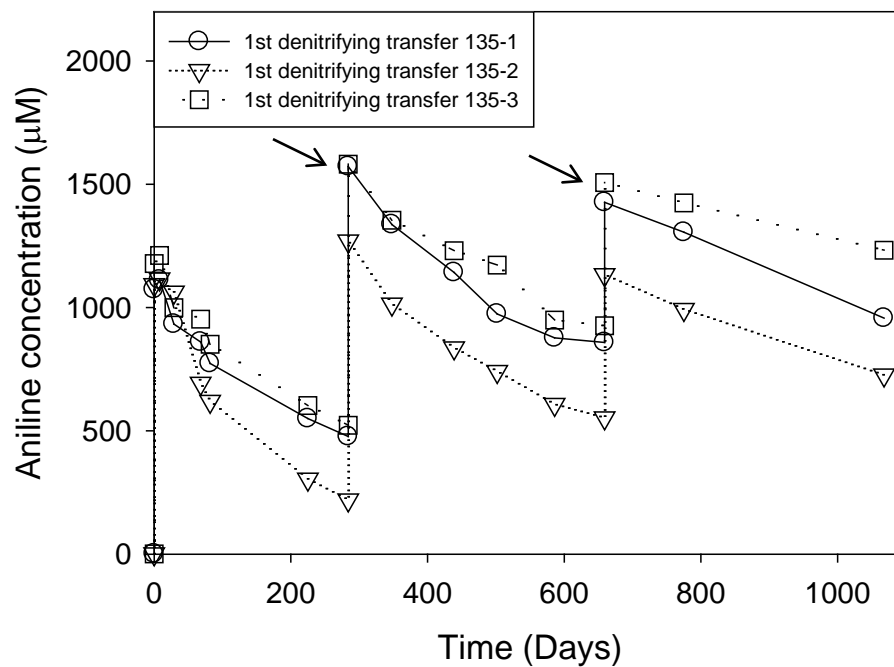
Figure B. 53. Aniline in nitrate-amended enrichment (with NH₄Cl, without reducing

agents) transferred from one of the triplicate microcosms from the lightly

contaminated freshwater canal location. The arrow indicates the re-

amendment of substrate.
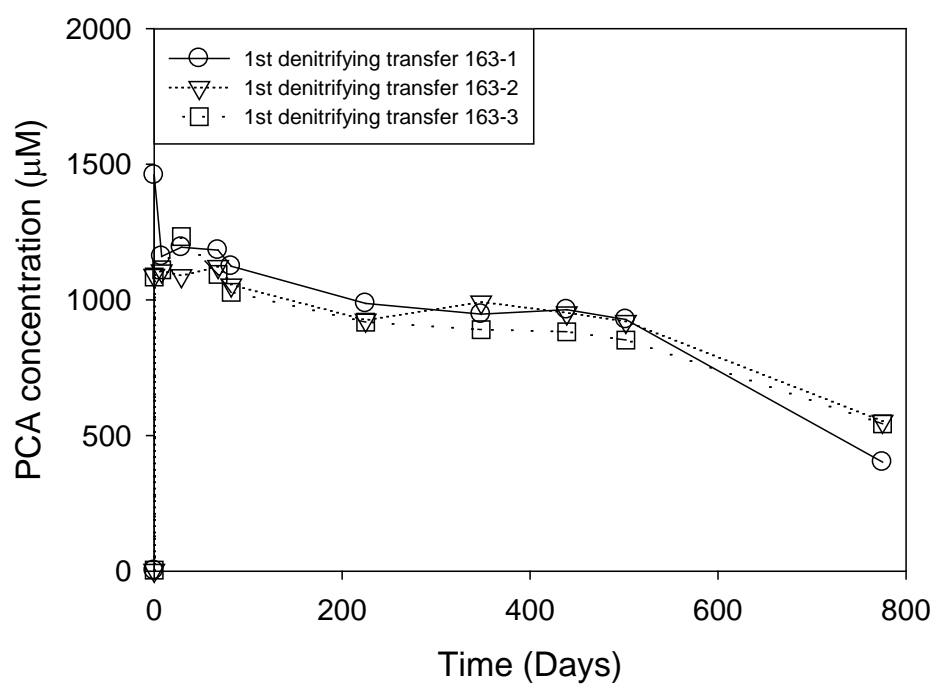
Figure B. 54. PCA in nitrate-amended enrichment (with NH$_4$Cl, without reducing agents) transferred from one of the triplicate microcosms from the lightly contaminated freshwater canal location.
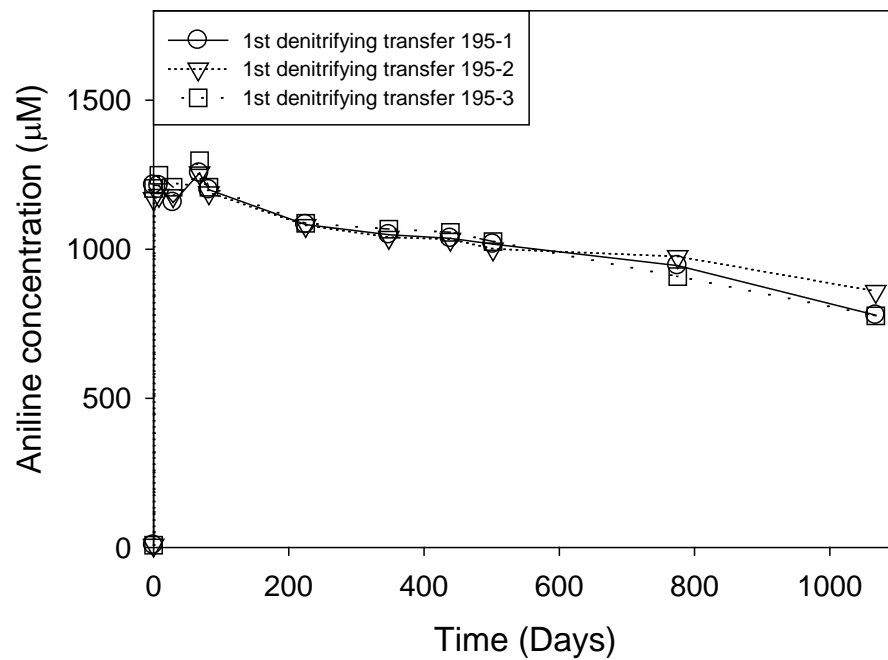
Figure B. 55. Aniline in nitrate-amended enrichment (with NH₄Cl, without reducing

agents) transferred from one of the triplicate microcosms from the highly

contaminated freshwater canal location.
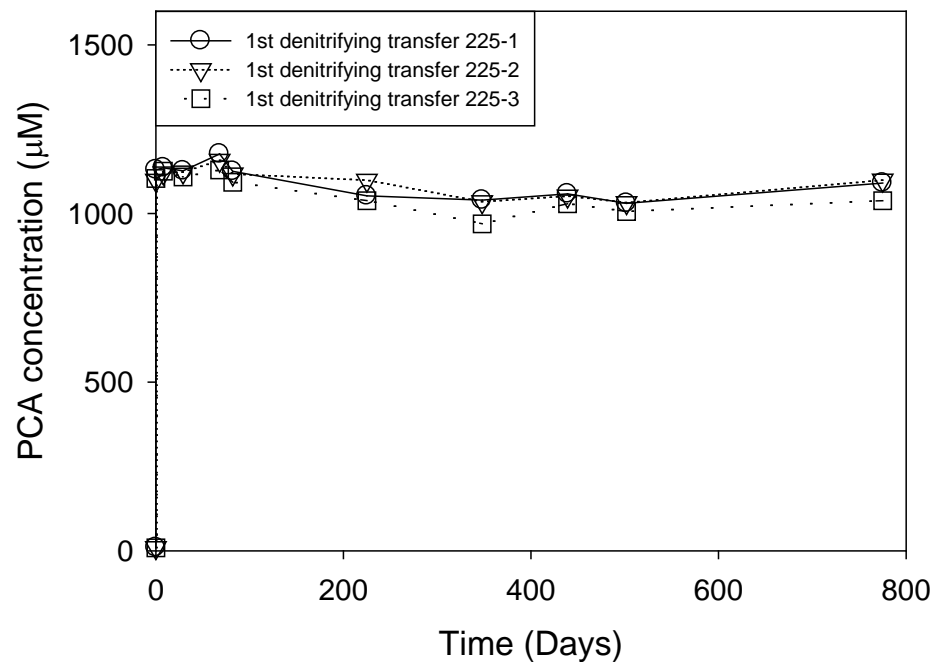
Figure B. 56. PCA in nitrate-amended enrichment (with NH$_4$Cl, without reducing agents)

transferred from one of the triplicate microcosms from the highly

contaminated freshwater canal location.
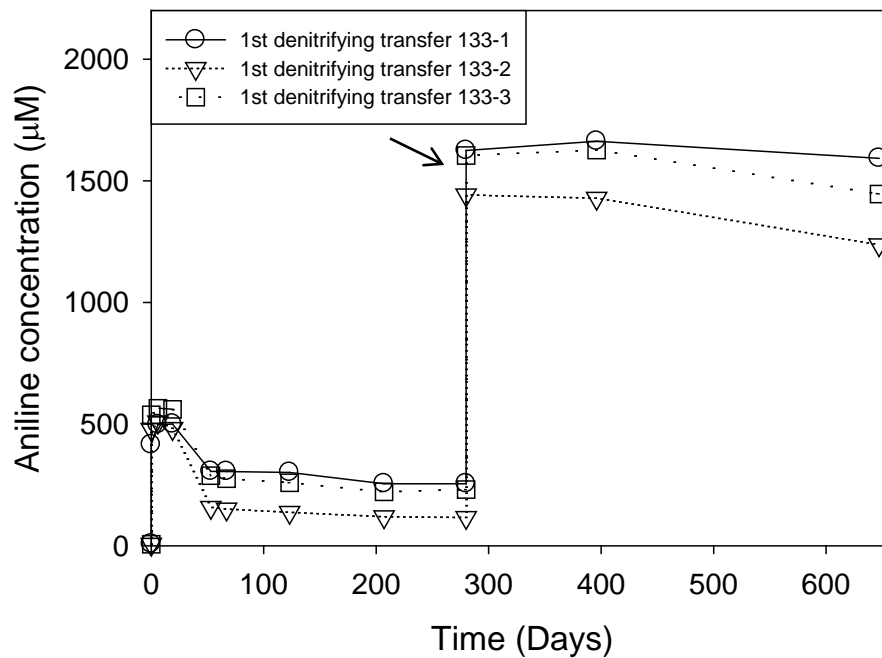
Figure B. 57. Aniline in nitrate-amended enrichment (with NH$_4$Cl and reducing agents) transferred from one of the triplicate microcosms from the lightly contaminated freshwater canal location. The arrow indicates the re-amendment of substrate.
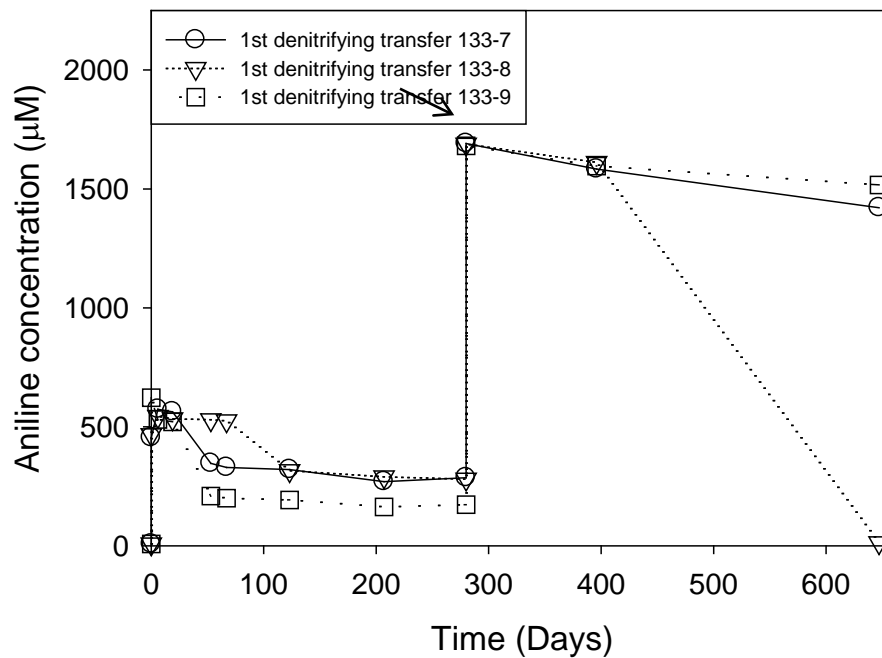
Figure B. 58. Aniline in nitrate-amended enrichment (with reducing agents, without

NH₄Cl) transferred from one of the triplicate microcosms from the lightly

contaminated freshwater canal location. The arrow indicates the re-

amendment of substrate.
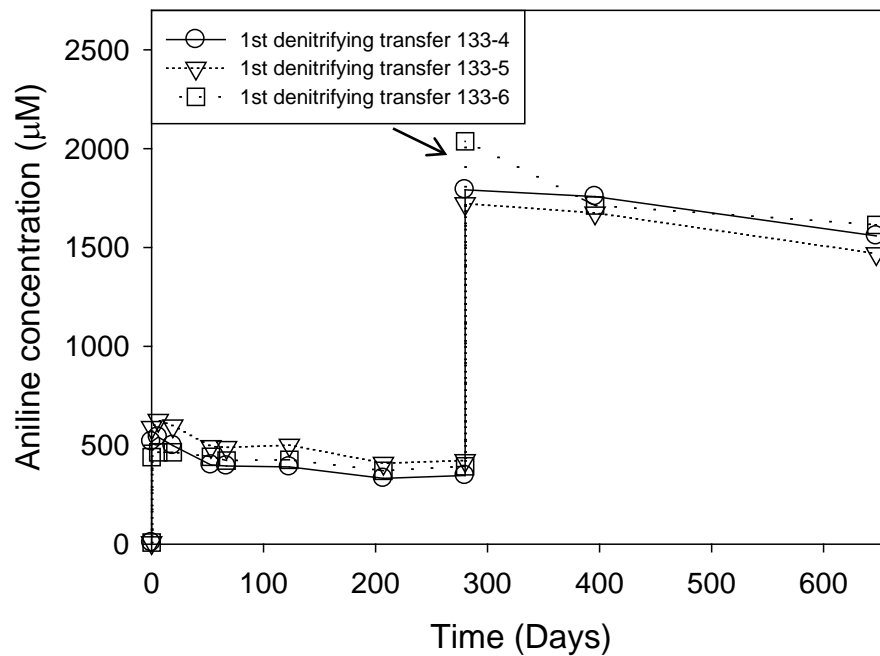
Figure B. 59. Aniline in nitrate-amended enrichment (without NH$_4$Cl and without

reducing agents) transferred from one of the triplicate microcosms from the

lightly contaminated freshwater canal location. The arrow indicates the re-

amendment of substrate.
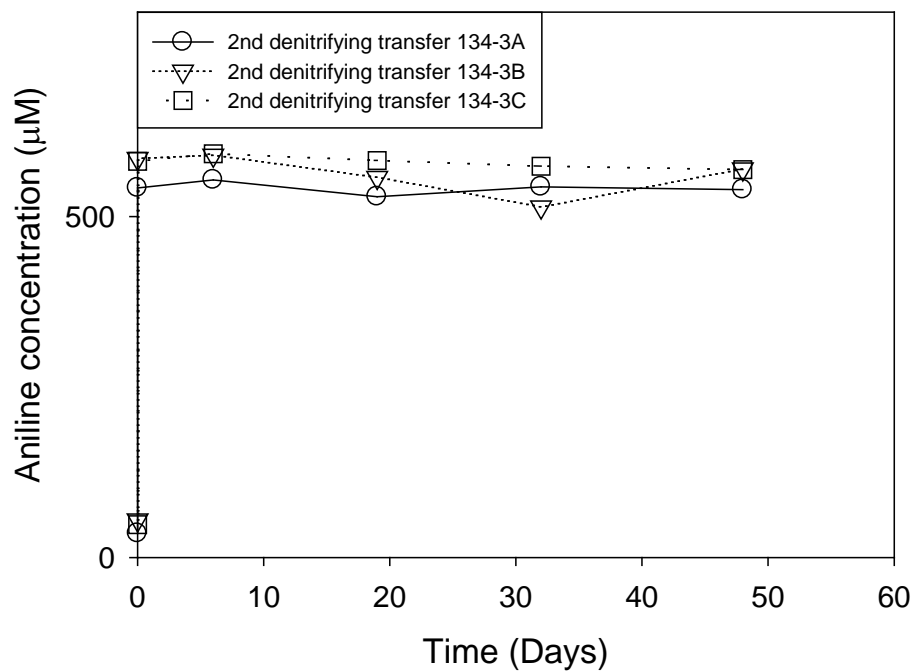
Figure B. 60. Aniline in the second nitrate-amended transfer enrichment (without NH$_4$Cl

and with reducing agents) transferred from one of the triplicate first nitrate-

amended transfer cultures from the lightly contaminated freshwater canal
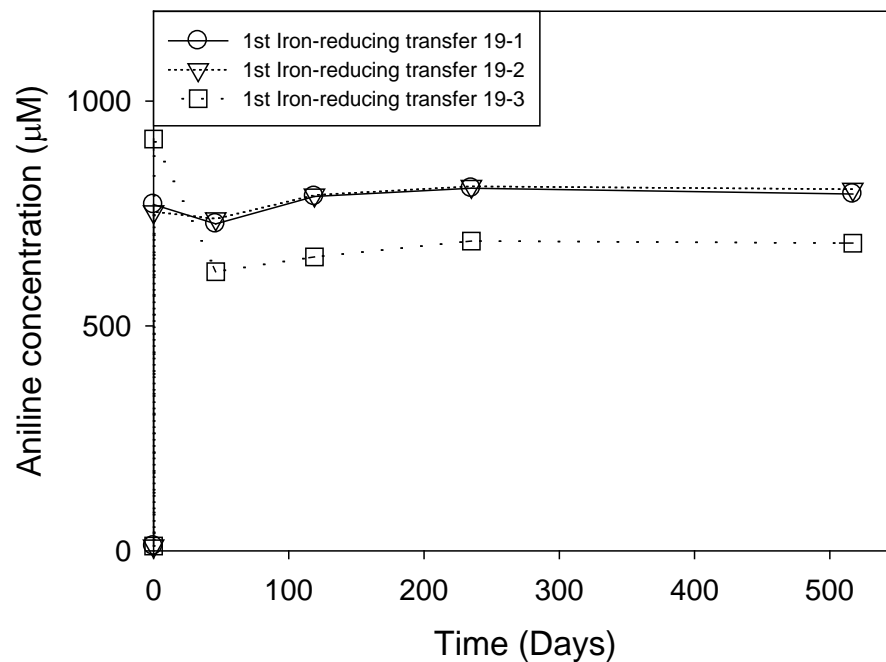
location.

Figure B. 61. Aniline in the Fe(III)-amended enrichment transferred from one of the

triplicate microcosms from the lightly contaminated groundwater aquifer
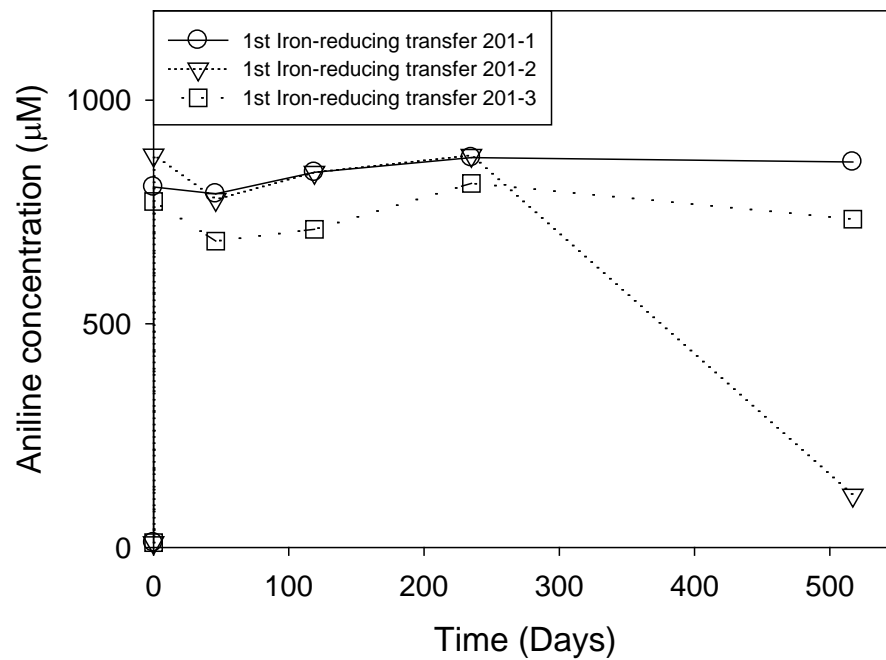
location.

Figure B. 62. Aniline in the Fe(III)-amended enrichment transferred from one of the

triplicate microcosms from the highly contaminated freshwater canal
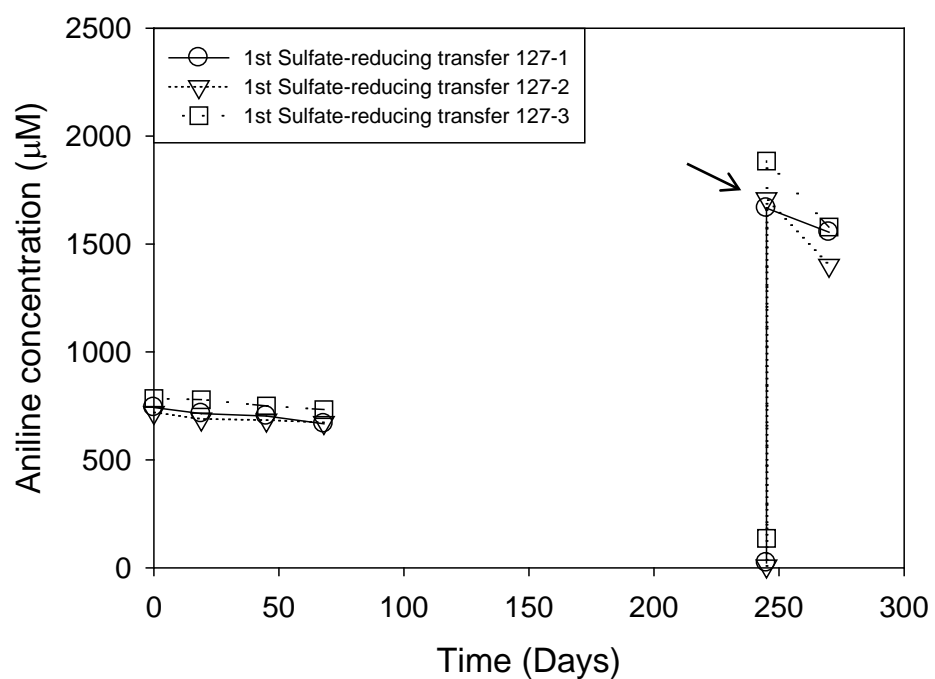
location.

Figure B. 63. Aniline in sulfate-amended enrichment transferred from one of the triplicate microcosms from the lightly contaminated freshwater canal location. The arrow indicates the re-amendment of substrate.

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