BIODEGRADATION OF AROMATIC COMPOUNDS AT TWO CONTAMINATED SITES IN NEW JERSEY, USA

By

HAIDER SALMAN AWAID ALMNEHLAWI

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ABSTRACT OF THE DISSERTATION

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Dissertation Director

Donna E. Fennell

This dissertation describes studies of biodegradation of two groups of aromatic compounds which are major contaminants of concern at contaminated sites in New Jersey. Chlorinated dibenzo-*p*-dioxins pollute the Passaic River in the north, and aniline and para-chloroaniline (PCA) pollute a chemical manufacturing site in the south. All the chemical compounds in this study are harmful to living organisms and the environment. They are carcinogenic to humans. Sediments from the Passaic River were enriched with dibenzofuran (DF) (as an analog for dibenzo-*p*-dioxin) and three bacterial strains, *Janibacter* sp.PR1, *Agromyces* sp. PR2, and *Arthrobacter* sp. PR3, were isolated. The strains could use DF as a carbon and energy source. *Agromyces* sp.PR2 and *Arthrobacter* sp. PR3 were further examined and found to transform dibenzo-*p*-dioxin (DD) and 2-monochlorodibenzo-*p*-dioxin (2MCDD), but neither of them could transform 2,7-dichlorodibenzo-*p*-dioxin (2,7DCDD), even after

prolonged incubation. Functional gene characterization was performed for all three strains. *Janibacter* sp. PR1 and *Agromyces* sp. PR2 had identical angular dioxygenases which are closely related to previously characterized angular dioxygenases. We were unable to identify an angular dioxygenase from *Arthrobacter* sp. PR3. Isolation of native bacterial strains which degrade contaminants is an essential step needed for consideration in any future bioremediation technology of the site.

Biodegradation of aniline and PCA under different redox conditions was examined in aquifers underlying a chemical manufacturing site. A detailed set of site data was established by collaborators. The data were evaluated with respect to the sediment geochemistry, groundwater contaminant concentrations, and electron acceptor presence, then sediments from different depths were selected to establish specific microcosm tests. In the case of targeted microcosms, soils from specific depths were used, while for composited microcosms, soils from combined depths were used. Contaminants of concern, aniline and/or PCA were added as carbon sources. Targeted microcosms showed no substantial loss in aniline and PCA concentrations. Composited microcosms showed loss in aniline and PCA concentrations, especially under aerobic and sulfate-reducing redox conditions. The aniline and PCA concentrations were depleted several times after reamendment. Bacterial community analysis showed a shift in the bacterial community of the aerobic microcosms presumably reflecting growth on aniline. A pure isolate was obtained from the aerobic culture which could use aniline as a carbon and nitrogen source. The strain, along with another aniline degrading strain isolated from the Passaic River site, were used together to bioaugment one bottle of an inactive targeted microcosm set. Aniline was depleted quickly after addition of the cells, indicating active biomass is missing in the

targeted microcosms. Stable isotope probing (SIP) of active aerobic microcosms showed there was an incorporation of ^{13}C aniline. Analysis of the DNA fractions from the SIP experiment showed that a phylotype related to *Knoellia* dominated two of the microcosms, while phylotypes related to *Aquabacterium* and *Brevundimonas* also appeared to be important in aniline degradation. The outputs of the project are identification of biodegradation processes and specific active organisms at the two sites. These findings are important and useful for eventual application of bioremediation technologies at the sites.

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Table of Contents

Lists of Tables

Lists of Figures

List of Acronyms

DF - Dibenzofuran

DD - Dibenzo-*p*-dioxin

2MCDD – 2-monochlorodibenzo-*p*-dioxin

2,7DCDD - 2,7-dichlorodibenzo-*p*-dioxin

CDD/Fs - Chlorinated dibenzo-*p*-dioxins and furans

PCA - *para*chloroaniline

EPA - Environmental Protection Agency

MCDD - Monochlorodibenzo-*p*-dioxin

DiCDD - Dichlorodibenzo-*p*-dioxin

TrCDD - Trichlorodibenzo-*p*-dioxin

TCDD - Tetrachlorodibenzo-*p*-dioxin

PeCDD - Pentachlorodibenzo-*p*-dioxin

HeCDD - Hexachlorodibenzo-*p*-dioxin

HpCDD - Heptachlorodibenzo-*p*-dioxin (HpCDD)

OCDD - Octochlorodibenzo-*p*-dioxin

MCDF - Monochlorodibenzofuran

DiCDF - Dichlorodibenzofuran

TrCDF - Trichlorodibenzofuran

TCDF - Tetrachlorodibenzofuran

PeCDF - Pentachlorodibenzofuran

HeCDF - Hexachlorodibenzofuran

HpCDF - Heptachlorodibenzofuran

OCDF - Octochlorodibenzofuran

K_{ow} - Octanol/Water Partition Coefficient

TEFs - Toxicity Equivalent Factors

AhR - Aryl Hydrocarbon Receptor

CANs - Chloroanilines

CDDs - Chlorodibenzo-*p*-dioxins

CDFs - Chlorinated dibenzofurans

NJDEP - New Jersey Department of Environmental Protection

NJDOH - New Jersey Department of Health

PCBs - Polychlorinated biphenyls

LPR - Lower Passaic River

GC-MS - Gas Chromatography - Mass Spectrometry

HPLC - High-Performance Liquid Chromatography

HOBB - 2-hydroxy-4-[3'-oxo-3'H-benzofuran-2'-yliden] but-2-enoic acid

OD - Optical Density

PAHs - Polyaromatic hydrocarbons

PCE - Perchloroethylene

OTUs - Operational taxonomic units

NCBI - National Center of Biotechnology Information

RDPII - The Ribosomal Database Project

SIP - Stable Isotope Probing

CFMM - Carbon free Minimal Medium

CNFMM - Carbon and Nitrogen free Minimal Medium

Chapter 1

Introduction

1.1 Rationale

The removal of aromatic pollutants from contaminated sites remains a problematic issue for remediation. This work addressed biodegradation of aromatic pollutants at two contaminated sites in New Jersey. One, the Passaic River, New Jersey, is a dynamic estuarine river whose sediments have been contaminated for over seventy years with chlorinated dibenzo-p-dioxins and furans (CDD/Fs), among many other organic and inorganic pollutants. The other is a large complex chemical manufacturing site in southern New Jersey that has been in continuous operation for at least 180 years. The groundwater and sediments of the highly stratified subsurface beneath this site are contaminated with aniline and *para*chloroaniline (PCA), among many other organic and inorganic contaminants.

Dibenzo-*p*-dioxin, (DD) dibenzofuran (DF) and their chlorinated congeners (CDD/F) are generally produced in trace levels as side products in chemical synthesis reactions, or through combustion and incineration. The Passaic River, specifically the lower 8.3 miles before entering Newark Bay, is heavily contaminated with CDD/Fs (EPA 2014). These compounds accumulated in the river sediments due to industrial activities including chemical manufacturing of herbicides. CDD/Fs are toxic to humans and other living organisms and harm the environment. They are persistent in the environment and

could accumulate and biomagnify in living tissues (van den Berg et al., 2013). They are carcinogens, teratogens, and are mutagenic (Fiedler, 2003)

Aniline and PCA are widely used in large quantities in the chemical industry and large inadvertent releases have resulted in their spread in the environment. They are used in manufacturing of dyes, pesticides and pharmaceutical products. Aniline and PCA are carcinogens and genotoxic (Greenwood et al., 1979; Poste et al., 2014). These compounds were long used as chemical precursors for manufacture of dyes and other products at the southern New Jersey manufacturing site.

The removal of aromatic pollutants from contaminated environments remains problematic. Chemical and physical methods have been studied and applied in cleanup, but these methods may be expensive or not completely effective. Biological treatment is a useful technology which allows use of naturally occurring or bioaugmented bacteria as part of a remediation strategy. DD, DF, and CDD/F are biotransformed, mostly to less or non-toxic forms, aerobically and anaerobically using different types of pure or mixed microbial cultures (Fennell et al., 2004; Wittich et al., 1992). Highly chlorinated CDD/F congeners could be subject to dechlorination under anaerobic conditions and lightly or non-chlorinated congeners will be formed from that process. This work focused on the biotransformation of lightly and non-chlorinated congeners. This is especially important since a separate study in our laboratory found these compounds were released as a result of anaerobic dechlorination of more highly chlorinated congeners in Passaic River sediments (Dean et al. 2020).

In the case of aniline and PCA, various studies have shown that they could be transformed by microorganisms under different redox conditions (Kazumi et al., 1995;

Liu et al., 2002; Schnell et al., 1989). A 60 ft core was retrieved from beneath a detailed study area of the industrial site and characterized by a multi-institutional effort. The geochemical composition of the core showed different redox zones through the depth. The combined data set including geochemistry, microbial community analysis and groundwater levels of contaminants of concern contributed by our collaborators was used to form hypotheses about the potential for biotransformation of aniline and PCA at different depth horizons at the site. Laboratory microcosms were established to attempt to mimic the *in-situ* environment by providing some essential requirements to determine whether bacteria in the subsurface could degrade aniline and PCA.

Presence and identities of active bacterial communities at contaminated sites should be investigated so that informed decisions for choosing and applying the suitable bioremediation technology can be made. The findings from this study provided important information about what enhances or limits biodegradation at both these sites.

1.2 Hypotheses

1- DD and mono- and di-CDD can be biodegraded aerobically by bacteria isolated from Passaic River surficial sediments using DF as the carbon source.

2- All metabolic processes require specific functional genes.

3- Aniline and PCA can be biodegraded under different redox conditions through use as carbon and/or nitrogen sources by bacteria from the subsurface of the chemical manufacturing facility.

4- Active aniline-degrading bacteria can be identified by isolation and stable isotope probing.

5- The combined geochemical, groundwater and microbial community data from the chemical manufacturing facility Detailed Study Area help predict where and under what redox conditions specific active zones of aniline and PCA biodegradation occur.

1.3 Goals and Objectives

The overall goal of this study is assessing biodegradation of DD, DF, and CDDs in the Passaic River; and aniline and PCA in the subsurface of a chemical manufacturing site. To test the hypotheses the following specific objectives were followed:

1- Developed microcosms and enrichments from two contaminated sites in New Jersey using DF and aniline/PCA, respectively.
2- Isolated and characterized DF and aniline degraders from the sites.
3- Determined the activity of isolates on other contaminants of interest.
4- Used ^{13}C-aniline in stable isotope probing to identify the active bacteria in aniline degrading microcosms.
5- Used molecular genetic approaches to characterize the angular dioxygenase genes in DF isolates.
6- Used aniline degrading isolates to bioaugment non-active microcosms from the chemical manufacturing site.

1.4 Dissertation Overview

The dissertation contains six chapters. Chapter 1 is the Introduction. Chapter 2 is the Literature Review. Chapters 3-5 describe each study performed to address the research hypotheses and objectives to reach the goals. Chapter 6 is the Summary of the entire dissertation and includes discussion of the Environmental Implications of the results. Finally, all of that is followed by References and Appendices.

Chapter 2 Literature Review

2.1 Introduction

Many organic chemicals utilized in human activities have entered the environment in large quantities all over the world. They are detected in all environmental compartments in different concentrations. Different pesticides, herbicides, and industrial organic chemicals have been added to the environment. Most of these organic chemicals have toxic effects on humans and ecosystems. Some of them are carcinogens and mutagens, while others have toxic effects on the immune system.

Polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (CDD/Fs) are two major groups of toxic organic chemicals which are of concern because they are persistent in the environment and toxic to living organisms and ecosystems (Schecter et al., 2006). CDD/Fs cause serious problems for humans and the environment because they not only have toxic effects but are also difficult for microorganisms to biodegrade (Kuokka et al., 2014), and thus they persist for long periods of time.

There are two major sources of CDD/Fs to the environment. The first is anthropogenic such as release to the environment as byproducts from different chemical manufacturing processes including synthesis of pesticides and herbicides, and through waste incineration (Fiedler, 2007, Hites 2011). The second is natural sources. In the past many scientists believed there were no natural sources of CDD/Fs but in fact natural sources do occur

(Ferrario et al., 2000) and may be the result of the natural chlorine cycle in the environment (Häggblom and Bossert, 2004). According to several geological studies, 60 Kg per year of CDDs are added to the environment from forest fires in Canada alone (Sheffield, 1985), furthermore, they can be added from biological processes such as peroxidase catalyzed reaction of chlorophenols (Häggblom and Bossert, 2004).

Aniline, para-chloroaniline or 4-chloroaniline (PCA) and chloroanilines (CANs) are organic amines considered to be contaminants of concern due to their toxicity. Amines in general could enter the environment from natural or anthropogenic sources, then spread widely to different environmental systems. Aniline is widely used in different industries such as production of dyes, plastics pesticides, herbicides, resins, and pharmaceutical compounds, they also could be released from oil waste, pulp, coal processing wastewater and agriculture runoff (Poste et al., 2014). Naturally, aniline can be produced by microbial transformation of other aromatic compounds, for example nitrobenzene (Zhu et al., 2012), Aniline is toxic to aquatic, terrestrial and human systems (Poste et al., 2014; Tanaka et al., 2009) It could affect the central nervous system and disrupts the endocrine system. According to EPA, aniline is a probable human carcinogen (Poste et al., 2014; US EPA, 2011).

Chemical and physical approaches have been used to remove hazardous chemicals from the environment. Oxidation is used to transform these compounds to less toxic or less hazardous forms. Many oxidizing agents have been utilized in remediation including ultraviolet light and chemical oxidants including ozone, permanganate, persulfate, chlorine dioxide, and hydrogen peroxide (Siegrist et al., 2011). Fenton's reagent (hydrogen peroxide in conjunction with ferrous iron) has been evaluated for removal of CDDs (Kao et al.,

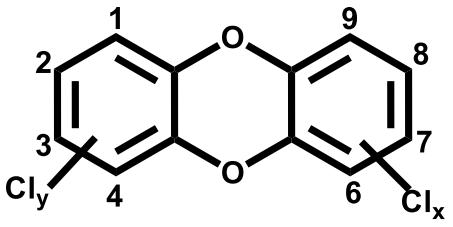
2001). Physiochemical methods are useful for some chemicals, but less effective or non-effective for highly toxic and persistent chemicals such as CDD/Fs (Kao and Wu, 2000). Incineration is another method used to destroy CDD/Fs, but this method may not be economical compared to other alternatives (Sakaki and Munetsuna, 2010).

It is necessary to find alternative methods to remove these toxic compounds from the environment and biological methods are promising techniques. They are cost potentially effective and environmentally friendly (Narihiro et al., 2010). Microbes can often use the contaminants of concern as a sole source of carbon and energy—for example dibenzofuran (Jin et al., 2006). Aerobic and anaerobic approaches have been used for biodegradation of CDD/Fs and bacteria are the most important microbes that are used in biodegradation processes (Field and Sierra-Alvarez, 2008). Aerobic and anaerobic biodegradation of aniline by bacteria have been studied by using pure or mixed cultures and several isolates have been obtained including *Delftia, Pseudomonas,* and *Rhodococcus* (Aoki et al., 1983;Konopka et al., 1989;Chengbin et al., 2009; Kahng et al., 2000; Shen et al., 2011).

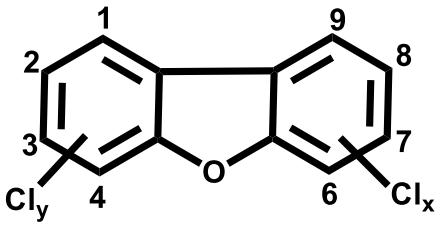
2.2 Structures

2.2.1 CDD/Fs

CDD/Fs are chlorinated organic chemicals that consist of two benzene rings linked by oxygen bridges. Dibenzo-*p*-dioxin is the precursor or the parent of the polychlorinated dibenzo-*p*-dioxins. For dibenzofuran, the parent of the polychlorinated dibenzofurans, the two benzene rings are linked by a furan ring (Figure 2.1) (Sakaki and Munetsuna, 2010, Hites, 1990).



Polychlorinated dibenzo-*p*-dioxins
PCDDs



Polychlorinated dibenzofurans
PCDFs

Figure 2.1. Structures of polychlorinated dibenzo-*p*-dioxin and polychlorinated dibenzofuran, structures constructed using ChemDraw Professional 17 software.

There is a possibility for eight chlorine atoms to be in the structure of CDD/Fs on eight positions of their structure, positions 1-4 on the first benzene ring and 6-9 on the second ring. Thus, 75 congeners of CDDs can be formed depending on the number of chlorine atoms and the position on the benzene rings, while 135 congeners can be formed for CDFs. The structure of CDD/Fs plays an important role in the reactivity of these compounds. Absence of reactive groups in their structure makes them stable and hard to react or biodegrade (Hong et al., 2004). The number of chlorine atoms influences bioavailability of congeners in the system, with the highly chlorinated congeners having a high tendency to be adsorbed on solid materials (Bunge and Lechner, 2009). Position of the chlorines influences the toxicity of congeners. Congeners having chlorine atoms on lateral positions

are more toxic than congeners that have chlorine on other positions or non-lateral positions. The lateral positions are 2, 3, 7, and 8. There are 17 congeners among the total 210 congeners that have chlorines in the lateral position (Öberg et al., 1990; Wagner et al., 1990). 2,3,7,8-Tetrachloro dibenzo-*p*-dioxin (herein denoted as TCDD), is often called the most toxic known compound (Patrizi et al., 2014).

2.2.2 Aniline and PCA

Aniline is an aromatic amine and it consists of a benzene ring with an amine group. PCA is an aniline with chlorine on carbon number four on the phenyl ring (Figure 2.2) (Niu and Boggs, 1984; Trotter et al., 1966). The amine group in the structure plays an important role in their toxicity.

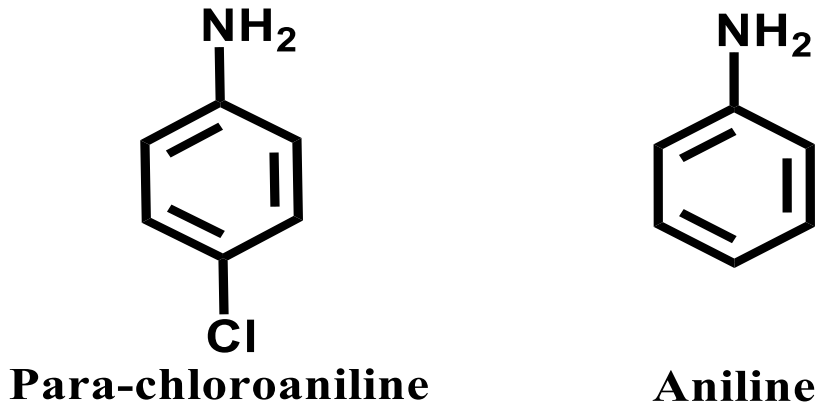


Figure 2.2 Chemical structures of aniline and PCA, structures constructed using ChemDraw Professional 17 software.

2.3 Characteristics

2.3.1 Physical and Chemical Characteristics

2.3.1.1 CDD/Fs

Physical and chemical properties of chemical compounds are related to their structure. If these properties are known, the behavior of specific compounds can be predicted. CDD/Fs in general have low vapor pressure, so they are semi volatile. The vapor pressure values of these compounds range between 8.2×10^{-13} mm Hg as a lowest value and 4×10^{-8} mm Hg as a highest value. The solubility of CDD/Fs is low. The highest concentration in water is 419 ng/L for the most soluble congener while the lowest solubility is 19.3 ng/L for the least soluble congener. This extremely low solubility therefore limits the availability of these compounds to microbes and affects their ability to biodegrade them.

CDD/Fs have high values of octanol-water coefficient (K_{ow}). The log K_{ow} values range between 5.6 and 8.2 making them very soluble in lipid and fatty acids. At the same time, they have high values of organic carbon coefficient (log K_{oc}), ranging between 4.6 and 7.6, that will lead them to be adsorbed strongly to solid materials (Mackay et al., 2006). In general, CDD/Fs have high molecular weights, TCDD has a highest value 321.974 g/mol which also has an effect on its partitioning among solid, liquid, and gas phases (Kao et al., 2001). All the above properties have to be considered by researchers when they design or model a CDD/Fs biodegradation process (Table 2.1) (Bunge and Lechner, 2009) and (Table 2.2) (Urbaniak, 2013).

Table 2.1 Physical and chemical properties for selected CDDs (Bunge and Lechner, 2009).

Congener	Water solubility at 25°C (µM)	Vapour pressure at 25°C (Pa)	Henry´s law constant at 25°C (Pa - m^3/mol)	log K_{ow}
2MCDD	1.35	0.017	14.8	5
2,3DiCDD	0.059	0.0004	6.6	5.6
1,2,4TrCDD	0.026	0.0001	3.8	6.4
1,2,3,4TCDD	0.002	6×10^{-6}	3.8	6.6
2,3,7,8TCDD	6×10^{-5}	1×10^{-7}–5×10^{-6}	1.6- 10.3	6.8
1,2,3,4TCDD	4.6×10^{-4}	8.8×10^{-8}	0.264	7.4
1,2,3,4,7,8HxCDD	2×10^{-5}	5.1×10^{-9}	4.52	7.8
1,2,3,4,6,7,8HpCDD	6×10^{-6}	7.5×10^{-10}	0.13	8.0
OCDD	1.6×10^{-7}	1.1×10^{-10}	0.68	8.2

Table 2.2 Physical and chemical properties for selected CDFs (Urbaniak, 2013).

Congener	Melting point	Solubility in water in mg/L	Vapour pressure (Pa) in 25°C	Log K_{ow}
2,3,7,8-TCDF	227-228	4.19×10^{-4}	2.0×10^{-6}	6.53
1,2,3,7,8-PeCDF	225-227	4.19×10^{-4}	2.3×10^{-7}	6.79
2,3,4,7,8-PeCDF	196-196.5	2.36×10^{-4}	3.5×10^{-7}	6.92
1,2,3,4,7,8-HxCDF	225.5-226.5	8.25×10^{-6}	3.2×10^{-8}	6.92
1,2,3,6,7,8-HxCDF	232-234	1.77×10^{-6}	2.9×10^{-8}	6.92
1,2,3,7,8,9HxCDF	246-249	1.77×10^{-6}	12.4×10^{-8}	6.92
2,3,4,6,7,8-HxCDF	239-240	1.77×10^{-6}	2.6×10^{-8}	6.92
1,2,3,4,6,7,8-HpCDF	236-237	1.35×10^{-6}	4.7×10^{-9}	7.92
1,2,3,4,7,8,9-HpCDD	221-223	1.35×10^{-6}	6.2×10^{-9}	7.92
OCDF	258-260	1.16×10^{-6}	5×10^{-9}	8.78

2.3.1.2 Aniline and PCA

Aniline is a colorless, oily liquid with relatively high solubility in water. It has a moderate to low octanol/water partition coefficient (log K_{ow} = 0.90) ((Lide, 1991) (EPA website2) (Table 2.3). PCA is a white, colorless to slightly yellow solid with a sweet odor and slight solublility in water. PCA tends to partition to the organic phase, it has a higher octanol/water partition coefficient (log K_{ow} = 1.83) ((Lide, 1991) (NJ.gov website) (Table 2.3).

Table 2.3 Physical and chemical properties of Aniline and PCA (PubChem website).

Probertites	**Aniline**	**PCA**
Molecular Weight	93.129 g/mol	127.571 g/mol
Boiling Point	184.1 °C	232°C
Melting Point	-6 °C	72.5°C
Flashing Point	76°C	123°C
Solubility in water	36,000 mg/L (at 25 °C)	3,900 mg/L (at 25 °C)
Vapor pressure	0.6 mm Hg	0.027 mm Hg
Log K_{ow}	0.9	1.83
Stability	Not stable (air and light)	Stable

2.4 Toxicity

2.4.1 CDD/Fs

Toxicity is the harmful effect of substances on the living organisms. CDD/Fs have a wide range of toxicity. TCDD is the most toxic congener to humans among the 210 congeners of CDD/Fs therefore the Toxicity Equivalent Factors (TEFs) for other congeners are compared to the toxicity of TCDD (Niittynen et al., 2007). The range of TEFs of CDD/Fs start from 0.0003 as a lowest value for 1,2,3,4,6,7,8,9 - octachlorodibenzo-*p*-dioxin) to 1.0 for the highest toxicity congeners, TCDD and 1,2,3,7,8-PeCDD. (Van den Berg et al., 2006).

Toxicity is related to the structure of the congener including the number and position of the chlorines. Congeners that have lateral chlorines are more toxic than congeners do not, therefore if a lateral chlorine is removed that will decrease the toxicity, or, if a non-lateral chlorine is added that also decreases the toxicity (Zhao et al., 2008).

The affinity of binding between CDD/F congeners and cell receptors effects their toxicity. Congeners that have high affinity to bind to the Aryl Hydrocarbon Receptor (AhR) have more toxicity effects and vice versa. AhR is a cell receptor responsible for binding with hydrocarbons (Figure 2.3). The affinity of 1,2,3,4,7,8 hexachloro-dibenzo-*p*-dioxin to bind to Ahr receptor is only 10% of the affinity of TCDD, and that is the reason behind differences in their toxicities. TCDD and Ahr complex could enter the nucleus after binding with another receptor, then effect on the of gene expression process (Niittynen et al., 2007; Sorg, 2014).

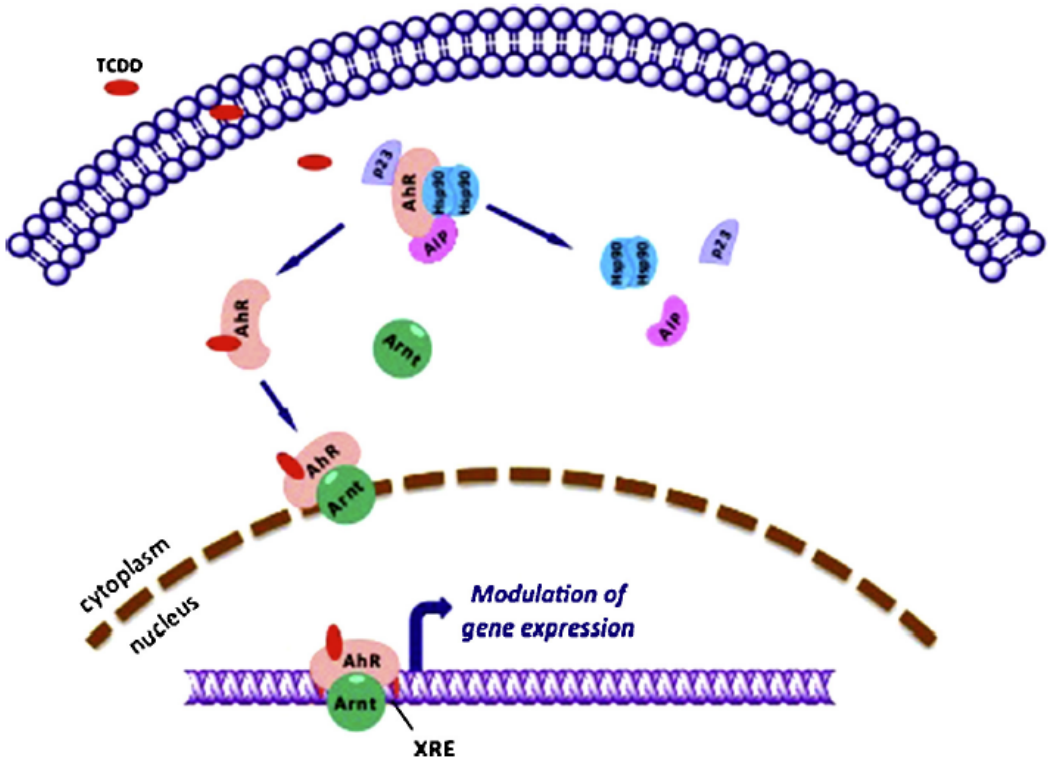


Figure 2.3 AhR signaling pathway TCDD as an example.

The most toxic congeners of the CDD/Fs are the 17 congeners having all lateral chlorines (2,3,7,8 positions). Seven of these belong to the CDDs while ten of them belong to the CDFs (Van den Berg et al., 2006). All these congeners have lateral chlorines in their structure, and they can accumulate through the food chain (Table 2.4). (Patrizi et al., 2014).

Table 2.4 Lateral chlorinated CDD/Fs and their TEFs (Van den Berg et al., 2006).

CDD/Fs CONGENER	WHO 2005 TEF
2,3,7,8-TCDD	1
1,2,3,7,8-PeCDD	1
1,2,3,4,7,8-HxCDD	0.1
1,2,3,6,7,8-HxCDD	0.1
1,2,3,7,8,9-HxCDD	0.1
1,2,3,4,6,7,8-HpCDD	0.01
OCDD	0.0003
2,3,7,8-TCDF	0.1
1,2,3,7,8-PeCDF	0.03
2,3,4,7,8-PeCDF	0.3
1,2,3,4,7,8-HxCDF	0.1
1,2,3,6,7,8-HxCDF	0.1
1,2,3,7,8,9-HxCDF	0.1
2,3,4,6,7,8-HxCDF	0.1
1,2,3,4,6,7,8-HpCDF	0.01
OCDF	0.0003

The major human intake pathway for CDFs is ingestion with food, while other pathways are considered minor such as inhalation and skin contact. More than 95% of CDD/Fs exposure occurs via ingestion (Fiedler, 2003).

There are different toxicological effects of CDD/Fs on many living organisms ranging from microbes to humans. Toxic intensity depends on congener type, dose, and period of exposure.

Carcinogenicity is the most concerning toxicity effect. TCDD is the most carcinogen congener, it causes liver tumors in laboratory animals at low concentrations. CDD/Fs induce tumor formation or development. They disrupt many cell functions such as growth hormones, apoptosis and communication systems, that encourage tumor formation (Fiedler, 2003).

Toxicity effects of CDD/Fs are variable from species to species, for example rodent cells are more sensitive than human cells (Schecter, 2013).The acute lethal dose for 50% of the animal cohort (LD50%) is 1 μg/kg for guinea pigs, while it is 5100 μg/kg for hamsters, indicating evidence for substantial differences in toxicity to different species. There is a difference within the species itself, two strains of rat have different LD50% 10 μg/kg for the first one and 9600 μg/kg for the second one for the same congener. Furthermore, there is a gender difference in toxicity response to CDD/Fs, female rats are more sensitive than males, while mice and guinea pig females are more resistance (Pohjanvirta et al., 2012; Pohjanvirta et al., 1993).

CDD/Fs have effects on the number and gender distribution of offspring. For example, Vietnamese communities that were exposed to TCDD have more girls than boys, and the numbers of offspring have decreased in Ufa, Russia due to exposure to these compounds (Fiedler, 2003). There is also a relationship between the concentration of CDDs in mother's milk and sister chromatid exchange frequency. (Suzuki et al., 2014).

CDD/Fs effects on different human systems such as the immune system, nervous system, reproductive system, cardiovascular system, and digestive system, result in increases in diabetes, development delay, weight loss, behavior disorder, skin lesions, liver damage and hearing loss.(Chastain Jr and Pazdernik, 1985; Fiedler, 2003). Exposure to CDD/Fs have effects on fish, for example the body weight and size, shape and development of short nose and Atlantic fish have been affected by exposure to TCDD. (Chambers et al., 2012).

Many countries assign acceptable risk levels of CDD/Fs. Germany set 1 pg/kg, the World Health Organization assigned 10 pg/kg, while Scandinavian countries set a limit of 5 pg/kg, as acceptable levels for daily intake per body weight (Fiedler, 2003). These values are only estimated since experimental data are from laboratory animal experiments, therefore these limits may or may not reflect the real limit for human exposure (Fiedler, 2003).

2.4.2 Aniline and PCA

Aniline and PCA are toxic to different living organisms including humans. Aniline is one of the few compounds with an effect on the spleen and erythrocytes. It causes splenic tumors in laboratory animals and several compounds of similar structure to aniline are considered carcinogens (e.g., toluidine and diaminodiphenyl sulfone (Dapsone) (Bus and Popp, 1987). The presence of the amine group in the structure of aniline leads to methemoglobinemia and an effect on erythrocytes, and then on spleen weight and physiology. Aniline is toxic to the aquatic ecosystem, as well. It effects many aquatic animals in embryo development, hatching, lifetime, and it is lethal at high concentrations (Abe et al., 2001; Abram and Sims, 1982). Aniline may cause headaches, dizziness, and nausea in humans (Jenkins et al., 1972; Khan et al., 1997). EPA reported that aniline is a

probable carcinogen. PCA may concentrate and transform in fish tissue and cause pigmentation and abnormal development (Burkhardt-Holm et al., 1999). It causes significant changes in the liver and kidney of zebrafish (Oulmi and Braunbeck, 1996).

2.5 Environmental Fate and Transport

2.5.1 CDD/Fs

CDD/Fs are hydrophobic and therefore likely to be sorbed on solid materials such as organic carbon, soils, and sediments after their release to the environment. They have high values for K_{ow} and tend to accumulate in biota, fatty acids or adipose tissues. This increases their effects on humans because they do not leave the human body with urine. They have low values of solubility; and may be less available to microbes for biodegradation. Chlorine number and position effects the fate and transport of CDD/F congeners. Congeners with fewer chlorines and non-lateral chlorines are more soluble and have higher vapor pressure than others (Bunge and Lechner, 2009; Field and Sierra-Alvarez, 2008; Kao et al., 2001). Five to ten days are required for CDD/Fs to reach equilibrium on solid phase, which effects their migration in the environment (Fan et al., 2006). They are clearly persistent compounds in the environment.

CDD/Fs are semi volatile compounds, but atmospheric transport is one of the major methods of their transport. They can transport by air for long distances and they also undergo photochemical reactions (photolysis) or radical mediated reactions. However, these reaction rates are slower than their subsequent accumulation in different environmental matrices. After transport, these compounds may be deposited or sorbed on

plants and pass through food chain (Fiedler, 2003). CDD/Fs have been found long distances from their original sources in different concentrations. For example, they were detected in several km downwind from a closed pentachlorophenol plant in China (Li et al., 2012). They have been detected more than 100 km away from the original generated sources for example municipal solid waste incinerators, medical waste incinerators (Lohman and Seigneur, 2001). Communities near sources of CDD/F contamination may be at risk of exposure (Figure 2.4) (Urbaniak, 2013).

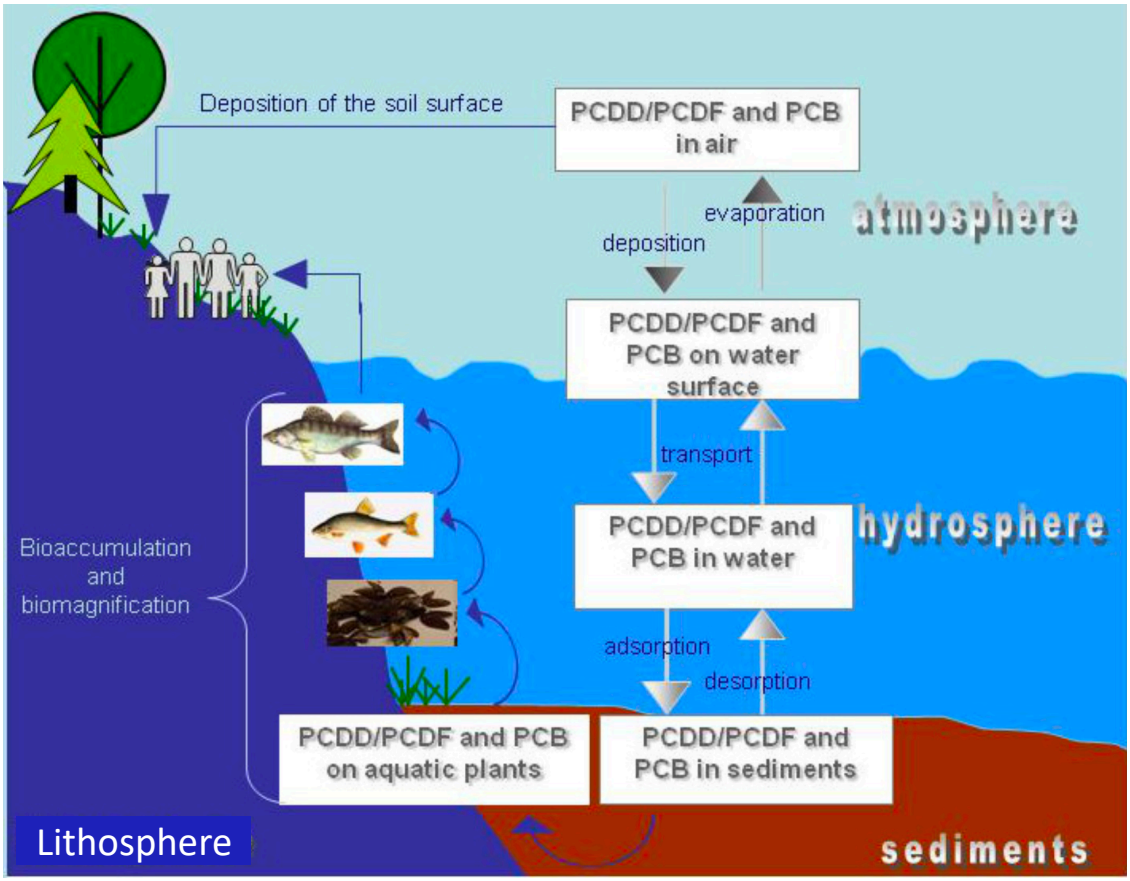


Figure 2.4 Transport and circulation of CDD/Fs (Urbaniak, 2013).

It has been reported that the deposition and weathering of CDD/Fs has a significant role in changing the soil nature and signature over time because of the CDD/Fs addition. Urban lake sediment samples have higher CDD/Fs concentration than suburban and remote lakes samples. Thirty-two km from the source, atmospheric deposition was the major mechanism of CDD/Fs transportation (Loyola-Sepúlveda et al., 2018).

2.5.2 Aniline and PCA

Aniline is soluble in water and tends to be in the water phase rather than the solid or organic phase. Aniline can move from the liquid to the solid phase over the long term by two processes: reversible mass transfer and irreversible reactions. Aniline reactions are pH dependent. Aniline may partition to the solid phase at low pH by ion exchange (reversible) or by covalent binding reaction (irreversible) at high pH values. (Fábrega-Duque et al., 2000; Li et al., 1999).

PCA is more hydrophobic than aniline and less volatile, therefore it tends to sorb to the solid or organic phases. The presence of chlorine in the structure makes it harder to biodegrade. Photolysis plays an important role in the fate of both compounds and it is faster than biodegradation in some cases. Aniline is more sensitive to degradation by light than PCA (Hwang et al., 1987).

2.6 Biodegradation

2.6.1 CDD/Fs

Biodegradation is the process of microbes using organic compounds and transforming them to simpler or less toxic compounds. CDD/Fs are subject to both aerobic and anaerobic biodegradation. Each type of biodegradation requires specific conditions. Initial substrates and end products may be different for each type, but in general the major difference between them is the presence or absence of oxygen, which serves as the terminal electron acceptor under aerobic conditions.

2.6.1.1 Aerobic Biodegradation

In the presence of oxygen as a terminal electron acceptor some CDD/Fs can be degraded to less toxic forms or even mineralized to carbon dioxide and water. Certain aerobic bacteria can use CDD/Fs as either as a sole source of carbon and energy, or mediate biotransformation in a co-metabolic process. Aerobic biodegradation is an effective technique for non-chlorinated or lightly chlorinated congeners (1-3 chlorine atoms) to be removed from the environment. Heavily chlorinated congeners are not subject to aerobic biodegradation (with some exceptions noted below), and aerobic biodegradation increases as number of chlorine decreases (Field and Sierra-Alvarez, 2007). There are many bacterial genera that have been reported as aerobic CDD/F - degraders. *Sphingomonas* and *Pseudomonas* are the most common genera that have been studied in aerobic biodegradation.

Sphingomonas wittichii strain RW1 was isolated by enrichment cultures inoculated with Elbe River water samples. It can use DD and DF as carbon and energy sources with doubling times of 8 and 5 hrs, respectively. That was the first report for a strain which

could degrade DD completely to carbon dioxide (mineralization) (Wittich et al., 1992). The same strain was found to degrade several mono and di-chlorinated-DDs, but it did not degrade highly chlorinated congeners (Wilkes et al., 1996). A later study demonstrated that strain RW1 could transform 2,7 DiCDD and 3,4,5,6 TeCDD, and formed several metabolites such as chlorocatechols and chlorophenols, but it did not carry out complete mineralization (Hong et al., 2002). Interestingly, the strain could also transform 1,2,3,4,7,8, HxCDD and 1,2,3 TriCDD releasing chlorocatechols and chlorophenols but it could not attack 1,2,3,7,8 PeCDD or 2,3,7 TriCDD. This work improved the understanding of the importance of substitution patterns in the biodegradability of chlorinated CDD/Fs (Nam et al., 2006). *S wittichii* strain RW1 also expressed specific patterns of genes in response to exposure to the DD and CDD substrates and the presence of sorptive clay (Chai et al., 2016).

Pseudomonas strain HH69 was isolated from soil samples by using DF as a sole carbon and energy source. During biodegradation, salicylic acid, gentisic acid and catechol were formed as metabolites. It could metabolize 1 g/L of DF in 120 hrs, and it could be used in pure and mixed cultures (Fortnagel et al., 1990).

Pseudomonas sp. RW10 was used along with *Sphingomonas* sp. RW16 in a mixed culture. The results showed that the two organisms could degrade 2- and 3MCDF through a *meta* cleavage pathway. Interestingly neither strain alone could degrade these compounds, indicating that there is a synergetic effect to the degradation processes—*Sphingomonas* cells attacked the non-chlorinated ring and used chlorotrihydroxybiphenyls to support their growth, while another metabolite (chlorosalicylate) was degraded further by *Pseudomonas* cells to support their growth (Wittich et al., 1999). *Pseudomonas putida* PH-01 could

grow aerobically on DF and transformed it, but catechol accumulated in the medium (Hong et al., 2000). Soil slurry samples inoculated with *Pseudomonas resinovorans* strain CA10 enhanced the biodegradation rate of 2,3DiCDD and reached non-detected concentrations within 14 days (Widada et al., 2002). *Pseudomonas veronii* PH-03 degraded DD, 1- and 2MCDD but grew poorly on 1,2,3,4TeCDD. It formed a toxic metabolite while growing on the tetra congener (Hong et al., 2004).

Rhodococcus strain HA01 could degrade DF and 2- and 3MCDF, but the activity was low in transforming DD (Aly et al., 2008). While *Rhodococcus* strain p52 could remove 500 mg/L of DF in 48 hr and 70 mg/L of 2MCDF in 96 hr, as well as metabolizing other aromatic compounds such as biphenyl, phenanthrene, xanthine, fluorene, and naphthalene, among others (Peng et al., 2013).

Janibacter XJ-1 and YA were isolated from contaminated sediments and they showed ability to degrade DF with accumulation of a yellow intermediate. Strain YA could also degrade 1, and 2MCDD (Iwai et al., 2005; Jin et al., 2006). *Terrabacter* is very closely related to *Janibacter* and could also degrade DF and lightly chlorinated DD (Habe et al., 2002a).

Other genera have also been found capable of aerobic biodegradation of various DD, DF, and CDD/Fs including *Paenibacillus, Nocardioides, Klebsiella,* and *Staphylococcus* (Fukuda et al., 2002; Iida et al., 2006; Kubota et al., 2005; Monna et al., 1993).

The ideal and desired outcome of degradation CDD/Fs is mineralization. In mineralization bacterial cells use CDD/Fs (food) and metabolites will be formed, enter into the central metabolic pathway, and eventually released as carbon dioxide and water. On the other hand, if the compounds are metabolized in a co-metabolic process, end products should be

further degraded by other community members because these products may be more toxic than the parent compounds. In co-metabolism, bacteria do not gain energy from using the compounds, but enzymes produced during other metabolic processes fortuitously mediate partial degradation (Urbaniak, 2013).

The enzymatic system of aerobic biodegradation consists of several enzymes, with each enzyme responsible for a step in the aerobic degradation. Understanding the enzymatic system and the genetic basis behind it is essential to improving the biodegradation process and could perhaps lead to increasing the degradation rate via genetic engineering.

The initial step in aerobic biodegradation is oxygenation. A dioxygenase is responsible for this important step. The angular dioxygenase has multiple components: terminal oxygenase, ferredoxin and reductase (Chang, 2008). Angular dioxygenase attacks one of the aromatic rings of CDD/Fs at the position adjacent to the connection bridge (4,4a position) by adding molecular oxygen (Chakraborty and Das, 2016b). Results of the first step lead to formation of an unstable compound that spontaneously transforms to 2,2,3-trihydroxybiphenyl, in the case of DF, and 2,2,3-trihydroxydiphenyl ether, in the case of DD. Both intermediates are subject to a extradiol ring cleavage step by trihydroxybiphenyl dioxygenase, followed by a hydrolysis step, which is achieved by a hydrolase, to form catechol and salicylic acid from DD and DF, respectively. Catechol and salicylic acid should be further degraded via meta or ortho cleavage to form simpler compounds that could enter the central metabolic pathway. In the case of chlorinated congeners, chlorinated intermediates will be formed, such as chlorocatechol and chlorosalicylic acid (Bünz and Cook, 1993; Chakraborty and Das, 2016a; Chang, 2008; Happe et al., 1993; Nojiri and Omori, 2002; Wilkes et al., 1996; Wittich et al., 1992) (Figure 2.5).

Another dioxygenase called lateral dioxygenase attacks lateral positions of the compounds (1,2; 2,3; or 3,4 carbon atoms). Aerobic biodegradation by lateral dioxygenase attack is an incomplete process and could lead to formation of non-degradable intermediates. *Pseudomonas putida* and *Sphingobium yanoikuyae* have a lateral dioxygenase. The initial step converts DD and DF to cis-dihydroxylated intermediates which are difficult to biodegrade further (Klečka and Gibson, 1979; Klečka et al., 1980). *Pseudomonas* sp. strain HH69 and *Rhodococcus* sp. strain HA01 have both lateral and angular dioxygenases (Aly et al., 2008; Fortnagel et al., 1990).

Many genetic studies have been done to investigate CDD/F dioxygenases in different genera. *Sphingomonas wittichii* strain RW1 has all the genes needed for dioxin mineralization (Wilkes et al., 1996; Wittich et al., 1992), and it has been used as a model organism in comparison with other microbial strains or systems (Figure 2.6). Its angular dioxygenase, 2,2,3 trihydroxybiphenyl dioxygenase, was characterized as an extradiol dioxygenase and its gene (dbfB) was cloned (Happe et al., 1993). In 2010 the whole genome of RW1 was sequenced (Miller et al., 2010). *Terrabacter* sp. strain YK3 has an angular dioxygenase gene on a large circular plasmid and *Janibacter terrae* strain XJ-1 was found to have an almost identical gene (Iida et al., 2002; Jin et al., 2006). A novel oxygenase gene which encoded an angular dioxygenase was isolated and characterized from *Terrabacter* sp. strain DBF63 (Kasuga et al., 2001; Kasuga et al., 2013). In *Nocardioides* sp. strain DF412 two gene clusters were found, the first cluster encoding for a ring hydroxylation dioxygenase located on a plasmid, the second cluster encoding for ring cleavage dioxygenase located on the chromosome (Miyauchi et al., 2008).

Gene clusters for two angular dioxygenases and one lateral dioxygenase were identified in *Rhodococcus* sp. strain HA01 (Aly et al., 2008).

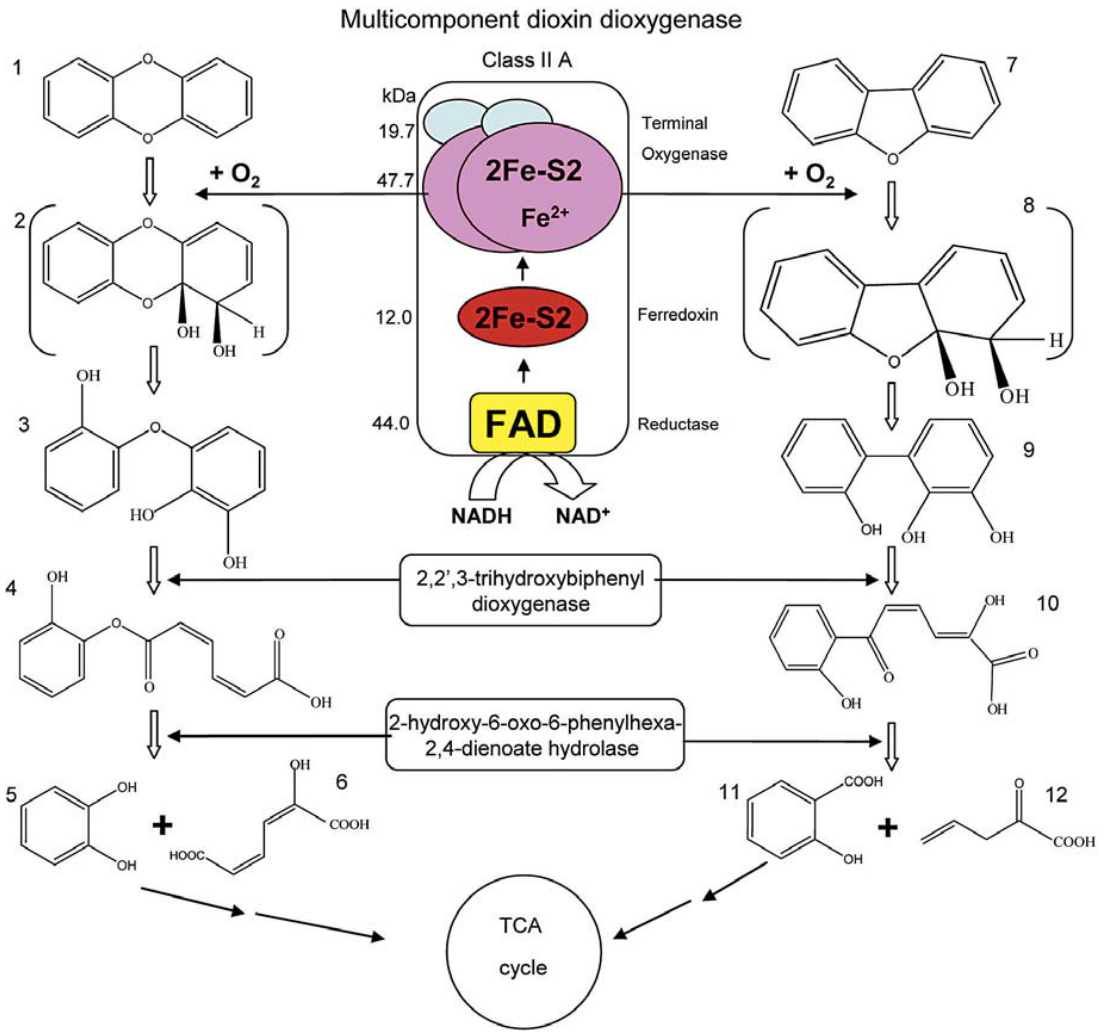


Figure 2.5 Degradation pathway of dibenzofuran and dibenzo-*p*-dioxin. (Chang et al., 2008). (1) Dibenzo-*p*-dioxin; (2) 4,4a-dihydro-dihydroxydibenzo-*p*-dioxin; (3) 2,2 _,3-trihydroxydiphenyl ether; (4) 2-hydroxy-6-oxo-6-(2-hydroxyphenoxy)hexa-2,4-dienoate; (5) catechol; (6) 2- hydroxy-muconate; (7) dibenzofuran; (8) 4,4a-dihydro-dihydroxydibenzofuran; (9) 2,2 _ ,3-trihydroxybiphenyl; (10) 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa-2,4-dienoate; (11) salicylicacid; (12) 2-oxopent-4-enoate.

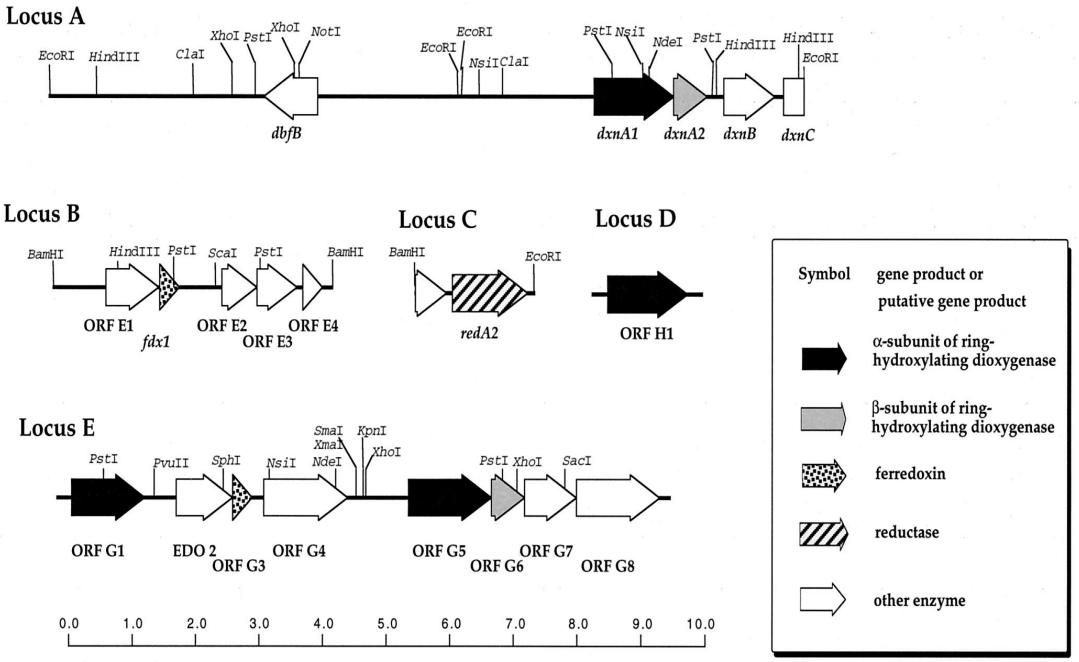


Figure 2.6. *Sphingomonas wittichii* RW1 gene organization (Armengaud et al., 1998).

2.6.1.2 Anaerobic biodegradation

In the absence of oxygen bacteria need an alternative electron acceptor to grow and reproduce. CDD/Fs can be used by some bacteria as electron acceptors. The major mechanism of anaerobic transformation of the CDD/Fs is reductive dechlorination. This process results in formation of lightly or non-chlorinated congeners that may be less toxic and could be subject to aerobic biodegradation. Dechlorination could happen even a few centimeters below the water level if anaerobic conditions are present (Field and Sierra-Alvarez, 2008; Zanaroli et al., 2015).

There are many factors affecting the dechlorination rate, including pH, temperature, congener type and concentration, and presence of effective bacterial species (Urbaniak, 2013). When CDD/Fs serve as electron acceptors other organic or inorganic compounds must be present to serve as electron donors to complete the redox couple for production of energy during dechlorination (Bunge and Lechner, 2009). The chlorine number and position effect the rate of biotransformation. Congeners that have unflanked lateral or a higher number of chlorines may be less susceptible to biotransformation. The benefit of this process is in transforming the heavily chlorinated congeners to lightly chlorinated congeners, which may be less toxic and less stable in the environment (Urbaniak, 2013).

There are other reports of transformation of CDD/Fs in a variety of environments. (Kao et al., 2001) reported that 86% of TCDD was removed in anoxic soils established under conditions to enhance dechlorination. Other redox conditions, such as iron reduction and methanogenesis, were found not to be effective by the same group. Similarly, different reducing condition cultures were established by using sediments from New York/New Jersey Harbor to detect the dechlorination of 1,2,3,4TetraCDD. Only methanogenic

microcosms showed activity, with other conditions showing no dechlorination (Vargas et al., 2001).

Several bacterial genera are capable of reductive dechlorination, and some are capable of coupling this process to energy generation via organohalide respiration. One widely studied genus is *Dehalococcoides*. The type strain, *Dehalococcoides mccartyi* strain 195, has an ability to dechlorinate different congeners of CDD/Fs (Fennell et al., 2004). Sediment enrichment cultures from the Kymijoki River could dechlorinate 1,2,3,4TetraCDD/F and OCDF (Liu et al., 2014). Reductive dechlorination is a slow process requiring months or years to see activity. In that study 10 to 29 months were required to dechlorinate some congeners. On the other hand, the time required depends on the type of congeners and environmental conditions. Anaerobic dechlorination of 1,2,3,4 TCDD was faster than 1,2,3,4, TCDF (Liu et al., 2014), for example. Different pathways of degradation can occur, depending on which chlorine will be removed first (Figure 2.7) (Bunge and Lechner, 2009) and ? (Figure 2.8) (Kuokka et al., 2014).

Finally, mixed conditions between aerobic and anaerobic conditions were designed by using bioreactors or by only changing the incubation conditions over time. For example, starting with anaerobic conditions, followed by transition to aerobic conditions to combine dechlorination with complete mineralization. The results were promising despite the degradation rates being low, and there are many difficulties to maintain stable conditions (Binh et al., 2016; Narihiro et al., 2010).

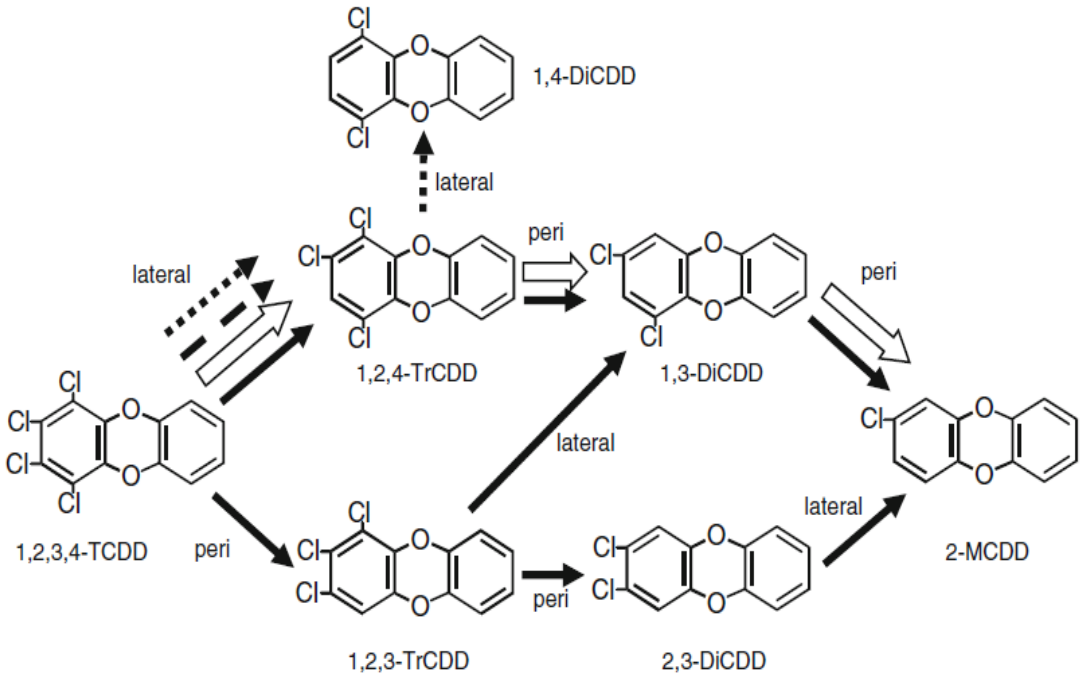


Figure 2.7 Biodegradation pathway of 1,2,3,4-TCDD by reductive dehalogination mechanisms (Bunge and Lechner, 2009).

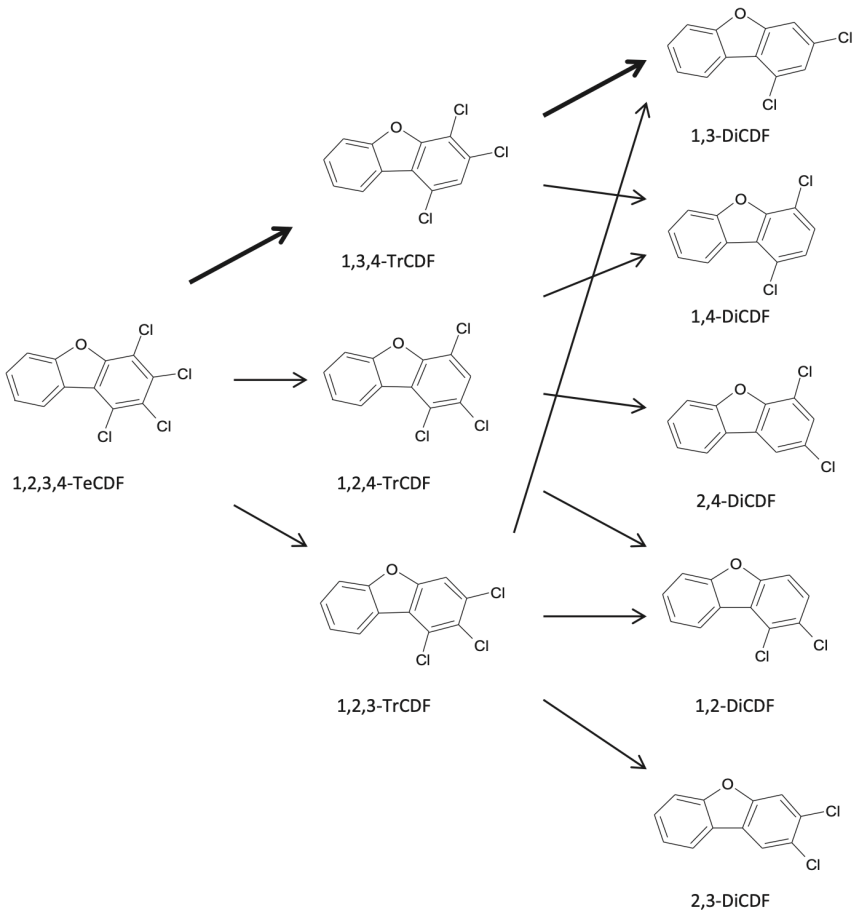


Figure 2.8 Biodegradation pathway of 1,2,3,4-TCDF by reductive dehalogination mechanisms (Kuokka et al., 2014).

2.6.2 Aniline and PCA

A variety of chemical and physical methods have been used to degrade and remove aniline and PCA from the environment. For example, pyrite was studied, and the efficiency was highly dependent on pyrite particle size and dosages, pH, and oxygen concentration (Zhang et al., 2015). Radiation (Sanchez et al., 2002) and electrochemical cells (Brillas et al., 1995) were investigated as a method to degrade aniline. Degradation of PCA in a water body was

investigated by using sun light, various complex products were formed due to photolysis of PCA (Gosetti et al., 2010). Activated persulfate alone and in combination with copper sulfide were also useful for degrading aniline (Liang et al., 2013). Chemical and physical methods can be expensive, and in some cases, these methods produce complex compounds or intermediates (Gosetti et al., 2010). Therefore, biodegradation is a promising way to degrade aniline and PCA.

2.6.2.1 Aerobic Biodegradation

Some bacterial strains have the ability to use aniline and PCA as major sources of carbon and nitrogen, while others could use aniline and PCA as a carbon source only. In both cases, in presence of oxygen, bacteria could degrade aniline to less or non-toxic forms or even via complete mineralization to carbon dioxide through central metabolism pathways. Aerobic degradation of aniline is a relatively a fast process and could remove aniline from the environment. Several pure strains have been reported as aniline degraders while mixed cultures are also used to enhance the degradation efficiency. *Pseudomonas* and *Delftia* are the most common genera investigated for aniline and PCA biodegradation. *Pseudomonas putida* was one of the first species reported as an aniline and chloroaniline (CANs) degrader, in 1982. This strain has an ability to mineralize 3,4-DiCA to succinate and acetate with liberation of CO_2 and Cl^- (You and Bartha, 1982). *Pseudomonas* strain K1 showed the same growth rate on aniline and lactate with a 2 hr doubling time; it metabolized aniline even in presence of lactate (Konopka et al., 1989). *Pseudomonas* strain AK20 used 2-methylaniline as a carbon and nitrogen source. It grew faster on aniline and preferred aniline in a multiple substrate mixture (Konopka, 1993). *Pseudomonas putida* CA16 could

use PCA as sole carbon and nitrogen source and chlorine was released in the process, it showed a good growth rate with 75% degradation of PCA (Vangnai and Petchkroh, 2007). *Delftia* can use high concentrations of aniline to grow. *Delftia* AN3 was isolated from activated sludge, could grow on aniline as a carbon, nitrogen and energy source. Interestingly, it could tolerate aniline at approximately 54 mM (5000 mg/L) and the maximum growth rate of the organism occurred at an aniline concentration of 32 mM. Growth ceased at concentration of 64 mM. In this process aniline was degraded to acetaldehyde and pyruvate, then entered the central metabolic pathway (Liu et al., 2002). The isolate could not grow on 2,3dichloroaniline or on PCA.

Delftia tsuruhatensis grew on 3200 mg/L of aniline and could degrade 1000 mg/L in continuous culture when cells were fixed on polycaproamide fiber. It does not have the ability to degrade CA or other relative compounds (Sheludchenko et al., 2005). PCA and several CANs were degraded separately or in mixture by another strain, *Delftia tsuruhatensis* H1. Adding another carbon source, such as yeast extract, promoted the degradation process while the degradation of chloroanilines was highly inhibited by adding aniline (Zhang et al., 2010).

Rhodococcus is also a common genus important in aniline degradation. *Rhodococcus erythropolis* AN-13 from soil utilized aniline as a carbon and nitrogen source and ammonia was liberated in this process; it preferred aniline even in the presence of glucose. Addition of other carbon sources promoted its growth and the degradation rates (Aoki et al., 1983). Other species were also found important in aerobic degradation of CANs, including members of *Acinetobacter, Nocardia, Comamonas,* and *Moraxella.* (Liu et al., 2002).

Aerobic activated sludge, as a mixed microbial community, was used in an aniline biodegradation study. Up to 350 mg/L aniline was degraded, but nitrification was inhibited significantly during aniline degradation (Gheewala and Annachhatre, 1997). Mixed cultures were also studied for aniline biodegradation. Recently, three types of bacteria were used separately and in mixed batch cultures. The results showed that the biodegradation rate increased significantly in the case using mixed cultures and approximately 1500 mg/L was degraded completely (Cui et al., 2017).

Aerobic biodegradation of aniline could be mediated by two major mechanisms: *ortho* or *meta* cleavage. Dechlorination of CA could happen as a first step of aerobic biodegradation. Aniline and PCA could be mineralized to CO_2 and water through the TCA cycle but, sometimes endproducts persisted and further degradation is needed. Catechol and chlorocatechol are common intermediates in this prosses. *Meta* cleavage processes, especially in case of CANs, formed recalcitrant intermediates (Hongsawat and Vangnai, 2011; Lyons et al., 1984; Nitisakulkan et al., 2014; Zeyer et al., 1985). The general aerobic biodegradation pathways of aniline are summarized in (Figure 2.9).

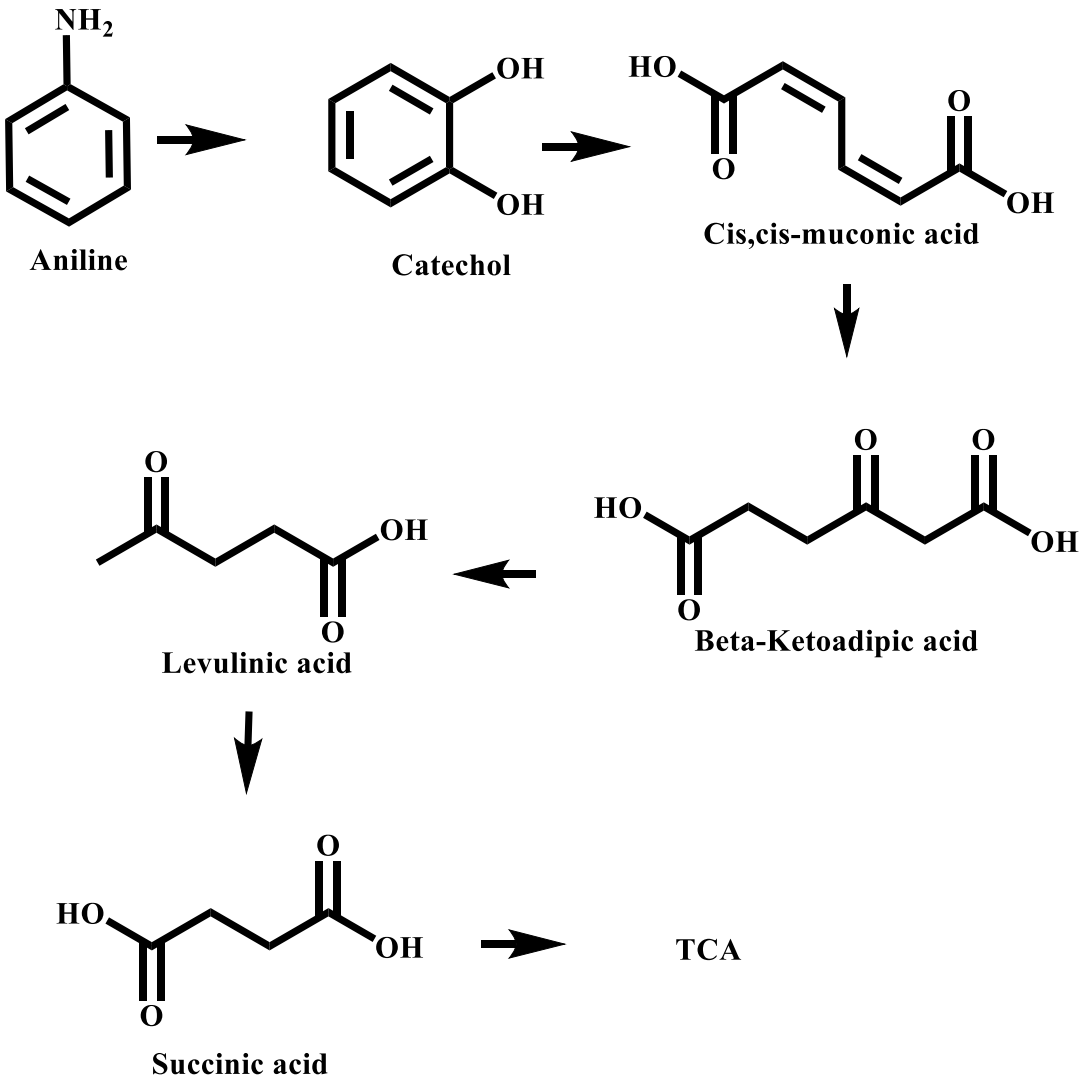


Figure 2.9 General pathway of aerobic biodegradation of aniline, structures constructed using ChemDraw Professional 17 software.

The universal first step in aerobic aniline and PCA biodegradation is oxidative deamination. Aniline dioxygenase is the responsible enzyme for this step which produces catechol or chlorocatechol. Aniline dioxygenase could be induced as a response for wide range of substrate specificity. For example, toluene dioxygenase in *Pseudomonas putida* T57 could also be induced in presence of aniline and could degrade several types of CANs including PCA. Hydrolase and ring cleavage (at the *meta* or *ortho* positions) enzymes are involved in the next steps of aniline biodegradation to form less complex compounds which enter the central metabolism pathway. *Meta* ring cleavage leads to formation of alpha keto acids while the *ortho* pathway leads to formation of beta ketoadipate. (Fukumori and Saint, 1997; Nitisakulkan et al., 2014; Urata et al., 2004). Genes and gene clusters were cloned from *Pseudomonas* and *Delftia* and identified as aniline aerobic degradation genes (Fukumori and Saint, 1997; Nitisakulkan et al., 2014; Urata et al., 2004; Zhang et al., 2008). An entire plasmid, 100 kilobase, involved in aniline degradation was isolated from *Pseudomonas* (Anson and Mackinnon, 1984).

2.6.2.2 Anaerobic Biodegradation

In the absence of oxygen, biodegradation of aniline and PCA could happen in conjunction with use of an alternative electron acceptor. Aniline and PCA could serve as an electron donor and carbon source under different anoxic conditions with complete or partial aniline degradation. The most common mechanism of the anaerobic aniline and CANs degradation is conversion to catechol and chlorocatechol then meta or ortho ring cleavage. *Desulfobacterium anilini* from marine sediments was the first anaerobic pure culture

isolated on aniline under sulfate reducing conditions. Complete mineralization of aniline was achieved, and the end products were CO_2 and NH_3 (Schnell et al., 1989). *Delftia acidovorans* HHY99 could degrade aniline aerobically, and also under nitrate reducing conditions (Kahng et al., 2000). In soil and sludge enrichments, aniline was completely degraded under denitrifying conditions and the degradation rate was faster in the presence of bicarbonate with liberation of N_2 and CO_2. On the other hand, there was no change in aniline concentration under methanogenic conditions (Alexandra De et al., 1994). Aniline was degraded under sulfate reducing and methanogenic conditions with 37 and more than 100 days lag phases, respectively (Sun et al., 2015). Methanogenic conditions could be very important because highly chlorinated aniline could be converted to di- or mono-CANs or, aniline, via reductive dechlorination mechanisms (Kuhn and Suflita, 1989). PCA was mineralized by using sediment enrichments and Fe^{3+} was the terminal electron acceptor in iron reducing conditions, however dechlorination was not observed (Kazumi et al., 1995). Reductive deamination is anaerobic mechanism could convert halogenated aniline to halobenzenes. The first report of this mechanism was under nitrate reducing conditions by *Rhodococcus* sp. (Travkin et al., 2002).

Chapter 3

Aerobic Degraders of Chlorodibenzo-*p*-dioxins and Dibenzofuran in the Sediments of the Passaic River, New Jersey, and Characterization of Their Functional Genes

[This chapter is submitted to: International biodeterioration and biodegradation]

Abstract

The Passaic River in New Jersey, USA is heavily polluted by hazardous contaminants including chlorodibenzo-*p*-dioxins (CDDs), polychlorinated biphenyls, and heavy metals. Highly chlorinated CDDs may be dechlorinated in anoxic aquatic sediments and the daughter products of these processes are lightly chlorinated congeners. These congeners could also be subject to aerobic biodegradation. We examined biotransformation of lightly and non-chlorinated dibenzo-*p*-dioxin congeners by aerobic bacteria isolated from Passaic River sediments using dibenzofuran as the sole carbon and energy source. Isolates were identified by 16S rRNA gene sequence analysis. Three bacterial isolates, PR1, PR2, and PR3 are closely related to *Janibacter terrae* (99.4%), *Agromyces mediolanus* (99.2%) and *Arthrobacter oryzae* (99.5%), respectively. PR1, closely related to known CDD degrader *Janibacter*, grew more rapidly on dibenzofuran than PR2 and PR3. Isolates PR2 and PR3 were further characterized since they are not closely related to genera known to degrade CDDs and showed the ability to transform non-chlorinated dibenzo-*p*-dioxin (DD) and 2-monochlorodibenzo-*p*-dioxin (2MCDD), with slight differences in transformation rates. Neither strain transformed 2,7-dichlodibenzo-*p*-dioxin (2,7DCDD). Molecular analysis revealed that *Janibacter* sp. PR1 and *Agromyces* sp. PR2 have angular dioxygenase genes

99.7% identical to the angular dioxygenase in *Terrabacter* sp. DBF63. The gene for *Arthrobacter* sp. PR3 was not characterized. The presence of indigenous bacteria that transform DD and MCDD suggest that metabolites formed from dechlorinated CDDs could be further aerobically degraded in Passaic River sediments. To our knowledge, however, this is a first report of isolates related to *Agromyces* and *A. oryzae* being identified as DF degraders.

3.1 Introduction

The Lower Passaic River (LPR) has been polluted with industrial contaminants over many decades (Bopp et al., 1991; Iannuzzi, 2002) (EPA, 2014). Contaminants of concern include heavy metals, polychlorinated biphenyls (PCBs), chlorinated dibenzo-*p*-dioxins (CDDs) and chlorinated dibenzofurans (CDFs). The Diamond Alkali Superfund site encompasses the lower 8.3 miles of the Lower Passaic River (LPR) and is characterized by high levels of 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD), which is one of the most toxic CDD congeners (van den Berg et al., 2013)(EPA 2016) and was produced inadvertently during the synthesis of 2,4,-D (2,4-dichlorophenoxyacetic acid) and other herbicides, mainly from 2,4,5-trichlorophenol purification process, the primary sources of CDD/Fs to the Passaic River have been chemical manufacturing to produce herbicides, deposition from incineration processes, and combined sewer overflows (Ehrlich et al., 1994; Iannuzzi et al., 1997; Parette et al., 2018; Tu et al., 2011). Despite cessation of industrial production that discharged much of the major contaminant, 2,3,7,8-TCDD, in the 1970s, high concentrations of this and other CDD/Fs are still detected (Bopp et al., 1991; Fiedler, 2007; Parette et al., 2018). CDDs are one class of pollutants that form the basis for fish and

shellfish advisories (NJDEP and NJDOH, 2019) and a ban on blue crab harvesting (NJDEP, 2012; NJDEP and NJDOH, 2019) for the tidal portion of the Passaic River. Further, gaseous emissions of CDDs from the water column of the New York Harbor were dominated by diCDD/Fs (Lohmann et al., 2000) indicating that many different congeners may be problematic in this system. The presence of CDD/Fs in Passaic River sediments imparts a specific sediment signature that is detected in the surrounding tidal area, depending on the distance from the original source (Loyola-Sepúlveda et al., 2018).

CDD/Fs are highly hydrophobic and have low aqueous solubilities (Fiedler, 2003). In addition, they do not have reactive groups; therefore, their biodegradation by microorganisms may be slow, if it occurs at all. The chlorine number and position affect the rate of biodegradation/biotransformation of CDD/Fs. Congeners that have laterally positioned or a higher number of chlorines are less likely or slower to aerobically biodegrade (Kao et al., 2001). Heavily chlorinated congeners are not readily susceptible to aerobic biodegradation, however lower chlorinated congeners are more susceptible. In the presence of oxygen as a terminal electron acceptor, lightly chlorinated CDD/Fs are transformed to simpler and less toxic forms or even mineralized. Further, some aerobic bacteria can use them as a sole source of carbon and energy (Field and Sierra-Alvarez, 2007).

Non-chlorinated dibenzofuran (DF) and dibenzo-*p*-dioxin (DD) are closely related compounds that can be used as carbon sources by aerobic bacteria, some of which can also degrade lightly chlorinated CDD/Fs (Sakaki and Munetsuna, 2010). There is a strong similarity in the biodegradation pathways for the aerobic degradation of DD and DF due to the similarity in their structures (Fiedler, 2003). The enzymes involved are implicated

both in DF and DD degradation processes (Nojiri and Omori, 2002). There are many bacterial genera that have been identified that aerobically biodegrade DD and lightly chlorinated CDDs; *Sphingomonas, Pseudomonas, Rhodococcus* and *Terrabacter* are the most common genera that have been identified from environmental enrichments (Aly et al., 2008; Fortnagel et al., 1990; Habe et al., 2002a; Wittich et al., 1992). *Sphingomonas* sp. strain RW1 is the most well-studied organism capable of CDD biodegradation (Habe et al., 2002a; Halden et al., 1999; Wittich et al., 1999; Wittich et al., 1992). Oxygenation is the key and initial step in the aerobic degradation process for DF, DD and lightly chlorinated CDDs (Armengaud et al., 1998). The dioxygenase responsible for this step most often attacks at the angular positions, i.e. the positions on the ring adjacent to the ether bridge, to produce trihydroxybiphenyl (THB) from DF and trihydroxybiphenylether from DD (Chakraborty and Das, 2016b).

Based on previous work that reported anaerobic dechlorination of CDDs in the Passaic River (Barkovskii and Adriaens, 1996; Fu et al., 2001), we hypothesize that DD and DF degrading bacteria may be present in LPR sediments. Further, some of these organisms may also degrade diCDDs. We isolated and identified bacteria from the sediments of the Passaic River that aerobically degrade non- and lightly-chlorinated DDs and examined functional genes in the DD mineralization pathway.

3.2. Materials and Methods

3.2.1 Chemicals

Dibenzofuran (DF, 98% purity, CAS 132-64-9), dibenzo-*p*-dioxin (DD, >99% purity, CAS 262-12-4), 2-monochlodibenzo-*p*-dioxin (2MCDD, 98% purity, CAS 39227-54-8), 2,7-dichlorodibenzo-*p*-dioxin (2,7DCDD, 98% purity, CAS 33857-26-0) and *p*-terphenyl-d14

solution (98.5% purity, CAS 1718-51-0) were purchased from Ultra Scientific (North Kingstown, RI, USA). Stock solutions for each congener (1000 mg/L) and the internal standard *p*-terphenyl-d14 (80 mg/L) were prepared. Analytical standards of 0, 0.05, 0.1, 0.25 0.5, 1, 2.5, 5 and 10 mg/L were also prepared. Hexane was used as a solvent to prepare the stocks and the dilutions (Honeywell Burdick and Jackson, MI, US, % purity, CAS 110-54-3). Silicon dioxide (floated silica 200 mesh and finer) was purchased from Fisher Scientific (Pittsburgh, PA, USA) and used as a carrier for the congeners for enrichment cultures (Adrian et al., 2009).

3.2.2 Study site and sample collection Sediment samples were collected by Ekman dredge from the Passaic River (Location: 40° 43.693'-74° 7.21'). Sediment was packed into acid cleaned jars and stored at 4°C until use. Temperature, pH, and salinity were measured using a calibrated handheld multiparameter water quality meter (556 MPS, YSI Inc., USA).

3.2.3 Minimal media preparation

Aerobic minimal medium was prepared as described previously (Fukuda et al., 2002) with modifications to ensure the trace metals did not precipitate. The final medium contained per L of milli-Q water: 6.4 g $Na_2HPO_4 \bullet 7H_2O$, 1.5 g KH_2PO_4, 0.3 g NaCl, 0.5 g NH_4Cl, 0.5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \bullet 7H_2O$, 0.02 g $CaCl_2$, and 0.0018 g $FeSO_4 \bullet 7H_2O$. The medium was prepared by preparing and then mixing four separate solutions in 0.25 L milli-Q water as follows: solution 1, 6.4 g $Na_2HPO_4 \bullet 7H_2O$, 1.5 g KH_2PO_4; solution 2, 0.3 g of NaCl, 0.5 g of NH_4Cl; solution 3, 0.5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \bullet 7H_2O$; and solution 4, 0.02 g $CaCl_2$, 0.0018 g $FeSO_4 \bullet 7H_2O$. The pH of each solution was adjusted to 7. Solutions were

autoclaved for 15 min at 121ºC then cooled to 50ºC before mixing under sterile conditions. Medium was stored at 4ºC until use.

3.2.4 Microcosm setup

Microcosms were formed by adding 250 mL of medium, 5 g of Passaic River sediment, and 250 mg DF crystals as the sole carbon source to sterile 500 mL Erlenmeyer flasks. Microcosms were prepared in triplicate including a live set, a killed set (autoclaved once per day over three days), abiotic (minimal medium without sediments) controls, and biotic (microcosms without DF) controls. Microcosms were incubated at 28ºC and shaken at 180 rpm for three weeks in the dark. Microcosm aliquots (0.25 mL) were transferred to 25 mL fresh media with 25 mg DF, three separate times as an enrichment process. Transferring was done every 7 days.

3.2.5 Isolation, purification and growth curves

Minimal media agar was prepared using minimal medium with 15 g/L noble agar (Affymetrix Inc. Cleveland, OH) and 50 mg/L of cycloheximide (Millipore sigma Louis, MO) as a fungal inhibitor. For isolation of single colonies, 100 µL of diluted (10^{-1} to 10^{-6}) culture was spread on triplicate agar plates. DF crystals were placed directly on the lid of the plates as a carbon source using methods described previously (Jin et al., 2006; Miyauchi et al., 2008) and modified by using the fungal inhibitor, serial dilution of the original enrichments, and prepared in triplicate. Triplicate plates were also prepared without DF and without inoculum to serve as controls. Plates were incubated at 28ºC until colonies developed. Finally, single colonies were streaked onto LB agar plates to ensure pure colonies. Pure strains were stored at -80ºC in 30% glycerol. Triplicate growth curves were performed for each strain using minimal medium with (1g/L) DF. Samples (1 mL) were

taken daily for optical density (OD) measurement using an Evolution 60S UV-Visible Spectrophotometer (Thermo Scientific, USA). Finally, growth rates were estimated according to (Widdel, 2007) at the steepest point of the exponential phase of the growth curve.

$$\mu = \left(2.303(\log OD2 - \log OD1)\right) \div (t2 - t1)$$

Where: μ is growth rate, OD is optical density, and t is time.

3.2.6 Molecular methods

Pure isolates were plated from -80ºC stocks and incubated at 28ºC, cells were collected, and genomic DNA extraction was accomplished using the DNeasy UltraClean Microbial Kit (QIAGEN, Germany). The near-complete of the 16S rRNA gene was amplified by using 27F (AGAGTTTGATCMTGGCTCAG) and 1525R (AAGGAGGTGATCCARCCGCA) primers with the following PCR protocol: denature at 95ºC, 30 sec; anneal at 55ºC, 30 sec; extension at 72ºC, 1.5 min; and final extension 72ºC, 3 min (T100^{TM}, BIORAD). PCR products were purified using ExoSapi (Affymetrix Inc. Santa Clara, CA, USA) and sent for sequencing (**GENEWIZ,** South Plainfield, NJ). For functional gene PCR, the angular dioxygenase primers DBFA1A2F 5'-ACCCGATGACCAGCATT-3' and DBFA1A2R 5'-AAGAAGATGGCACG-3' (Habe et al., 2004; Kasuga et al., 2001; Kasuga et al., 2013) were used with the following PCR protocol: denature 94ºC, 30 sec; anneal 50ºC, 30 sec; extension 68ºC, 2 min; and final extension 68ºC, 5 min.

Phylogenetic trees were constructed using MEGA (Molecular Evolution Genetics Analysis) software version MEGAX64. Fasta files for all related strains were downloaded

from NCBI, alignment was performed using MUSCLE algorithm and, phylogeny was determined by the maximum likelihood tree method.

3.2.7 CDD and DF transformation

Resting cells were prepared with cells grown on DF as the only carbon source. The ability of the cells to transform DD, 2MCDD, and 2,7DCDD was determined as described in the three sections below, using a combination of several previously described methods with modifications (Bedard et al., 2006; Habe et al., 2002a; Iwai et al., 2005)

3.2.7.1 Preparation of resting cells Twenty mL of each isolate that grew on DF was used to inoculate 500 mL of fresh minimal medium amended with DF crystals (500 mg) as the sole carbon source and was incubated with shaking at 28ºC until mid-log phase was reached. In total, 2 L of liquid culture was prepared for each isolate. DF residual crystals were removed by filtration using a sterile Whatman Class micro-filter GF/D of 47 mm diameter (GE Health Care Buckinghamshire, UK), that allowed cells to pass but not DF crystals, as described previously. Cell harvesting was done by centrifugation at 6000 *g* for 10 min. Finally, cells were washed three times in minimal medium and re-suspended in the same medium to an OD_{600} of 2.

3.2.7.2 Transformation of 2MCDD, 2,7DCDD and DD 250 mg of floated silica powder as a carrier was placed in a 30 mL serum bottle, sealed by Teflon-lined-butyl rubber stoppers (Wheaton, part number, city), crimped and then autoclaved. CDDs, DD and DF were prepared from (1000 mg/L) stock solutions and diluted by hexane. Congeners were added to triplicate serum bottles to achieve a final concentration of 5 ppm by saturating the silica (not the glass walls of the bottles), then left open under the hood until hexane evaporated completely. Bottles were re-sealed by Teflon-lined-butyl rubber stoppers,

crimped, and then autoclaved for 40 min. Next, 5 mL of resting cell suspension was added to each serum bottle under a sterile hood, and bottles were re-sealed. Triplicate bottles with cell suspension were autoclaved and used as abiotic controls. Bottles were incubated at 28ºC and 180 rpm. One milliliter of cell suspension was removed from each microcosm for several time points over 0 to 24 hr for analysis of DD, DF and CDDs. Samples were stored at -20 ºC until extraction.

3.2.7.3 Sample Extraction p-terphenyl-d14 (5 ppm) was added to each sample from a hexane solvent stock solution (80 mg/L) as an internal standard to correct for extraction efficiency. Each sample was extracted twice by adding 1:1 volume:volume (vol:vol) ethyl acetate, vortexing vigorously for 2 min, and then shaking for 3 hr at 28ºC and 180 rpm. The organic phase was removed to a clean vial after each extraction. Extracted samples were concentrated via nitrogen evaporation (model N-EVAP 111, Organomation Associates, MA) to 2 mL and transferred to a clean vial for analysis.

3.2.8 Gas Chromatography - Mass Spectroscopy (GC-MS) Analysis

Congener concentrations were analyzed on an Agilent 6890 gas chromatograph (GC) equipped with Mass Spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA) using an HP-5MS (5% diphenyl dimethyl polysiloxane) capillary column (60m × 0.25mm i.d. × 0.25μm film thickness) capillary column (Agilent Technologies, Santa Clara, CA, USA). The initial temperature was 80ºC for 3 min, then the temperature was increased to 250ºC by 30 ºC/min and held at 250ºC for 10 min. Standards containing the congeners at 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 ppm with p-terphenyl-d14 at a constant concentration of 5 ppm was used as an internal standard. the M/Z were 168, 184, 218, 252 and 244, for DF, DD, 2MCDD, 2,7DCDD and p-terphenyl-d14, respectively. Sample concentrations were

calculated by using standard curves and internal standard correction and reported as averages of triplicates +/- one standard deviation.

3.3 Results and discussion:

3.3.1 Study site characterization and sample collection: Surficial grab samples were obtained from the LPR (Figure 3.1). During sampling the temperature of the water column above the sediment bed was 23.1ºC, the pH was 7.74, and the salinity was 6.55 ppt. The sediments were black with a fine-grained texture. The CDD/F content of the site sediment was not determined for this study. Previous work, however, indicates that surficial sediment of the LPR contains 10^4 to 10^5 ng 2,3,7,8-TCDD/kg organic carbon (OC) (Israelsson et al., 2014). In contrast, 2,3,7,8-TCDD concentrations increased with depth in consolidated sediments, reaching a maximum at 1 to 2 m depth, associated with the 1950's time horizon (Chant et al., 2010) of 10^6 to 10^7 ng/kg OC. The surficial sediments are proposed to form a "mobile pool" of 2,3,7,8-TCDD that is transported upriver by tidal activity and then downriver to Newark Bay during higher river flows (Israelsson et al., 2014). A high concentration of CDD/Fs throughout the site sediments make it suitable for isolation of microorganisms that are active on a variety of congeners because the frequency of isolation from highly contaminated sediments is higher (Futamata et al., 2004). Additionally, the unconsolidated surficial sediments are likely more exposed to oxygen than deeper sediments and thus could be favorable for isolation of aerobic DF, DD and CDD degraders.

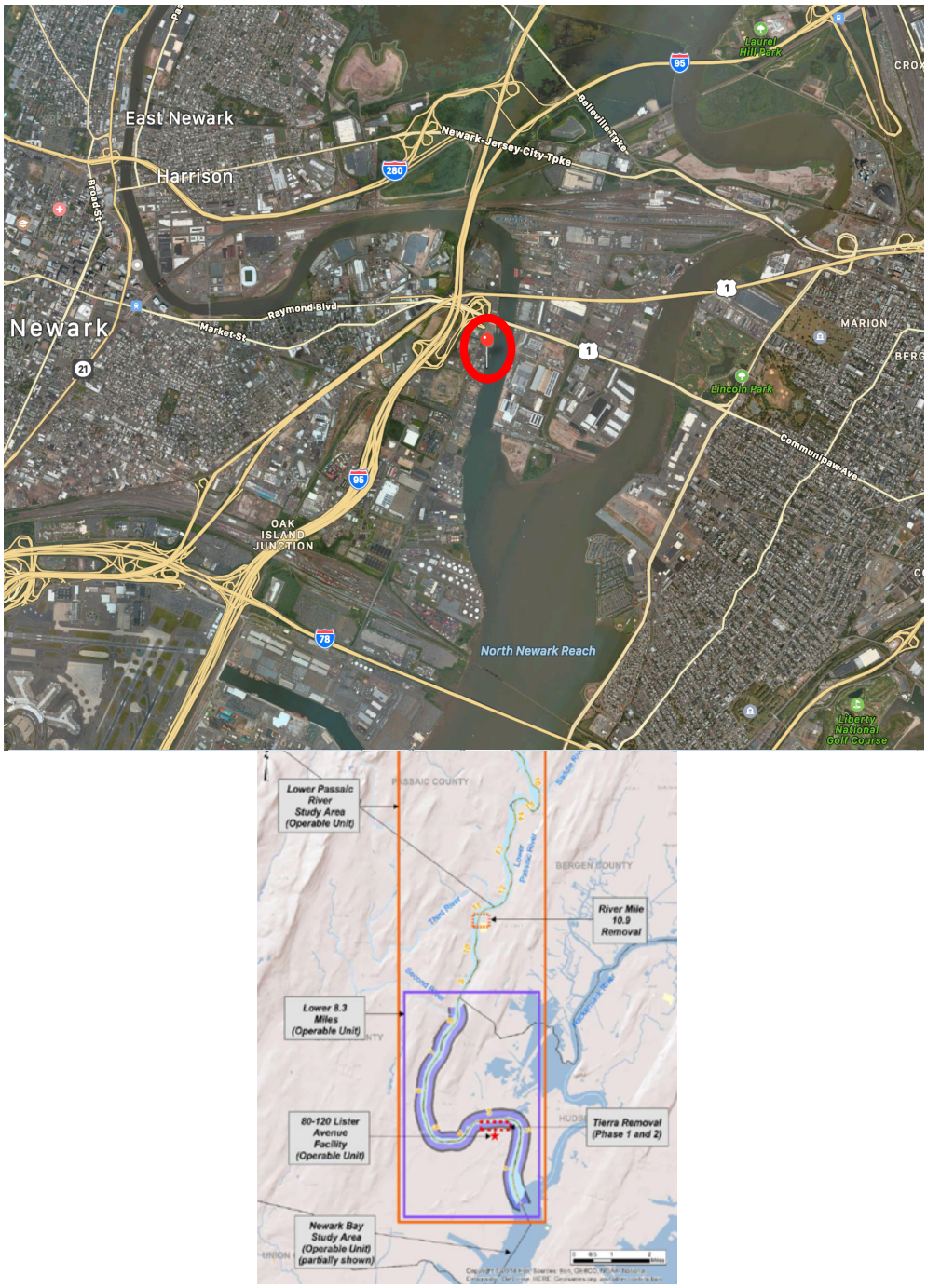


Figure 3.1 Site of study and sample collection area, it is located within the last 8.3 miles of the river, which a superfund site (Dimond Alkali) according to the EPA (Apple map June 24,2020) https://www.eenews.net/stories/1060055900.

3.3.2 Dibenzofuran enrichments and strain isolation

Passaic River sediments were enriched and incubated aerobically for three weeks with 1 g/L of DF crystals as the sole carbon source. During the second week of incubation a slight yellow color developed and by the end of the incubation period (21 days) the yellow color was obvious in live enrichments compared to the killed, abiotic and biotic controls. Enrichments were transferred twice to fresh minimal medium (MM) with DF crystals, then plated on minimal agar media with DF. The yellow color was evident in the second or third transfers. The third transfer was used as inoculum to isolate DF degraders. The yellow color in the enrichments indicates the presence of 2-hydroxy-4-[3'-oxo-3'H-benzofuran-2'-yliden] but-2-enoic acid (HOBB), an intermediate in the DF degradation pathway. HOBB is formed as a product from trihydroxylated biphenyl through the action of the catabolic pathway meta cleavage enzyme (Fortnagel et al., 1990; Kubota et al., 2005).

Three pure strains that can use DF as a sole carbon and energy source were obtained by plating serial dilutions of the active enrichments on minimal media plates with DF crystals on the lid of the plates, providing a vapor phase of DF. Several colonies developed after incubation of one week, and these were classified according to their size, color, and texture. Three pure strains that can use DF as a sole carbon and energy source were obtained. Pure strains were obtained by transferring single colonies of each colony type three times on LB agar plates. No colonies developed from plated inoculum from the killed, abiotic, and biotic controls with no DF. During their growth on minimal media plates with DF, a yellow color also formed, especially around the colonies. The 16S rRNA primer set 27F and 1522R were used to amplify the majority of the 16S rRNA gene of each isolate. The sequences of

the PCR products showed that the three isolates, denoted PR1, PR2, and PR3 are closely related to *Janibacter terrae* (99.4%, figure 3.2), *Agromyces mediolanus* (99.2% Figure. 3.3), and *Arthrobacter oryzae* (99.5%, Figure 3.4), respectively. Several strains of *Janibacter* are known as DF degraders and some of them also transform 1- and 2-MCDD (Iwai et al., 2005; Jin et al., 2006). Strain NSA3-4 belonging to the genus *Arthrobacter* also degrades DF, but without producing the yellow intermediate (Futamata et al., 2004). To our knowledge, however, this is a first report of isolates related to *Agromyces* and *Arthrobacter oryzae* being identified as DF degraders.

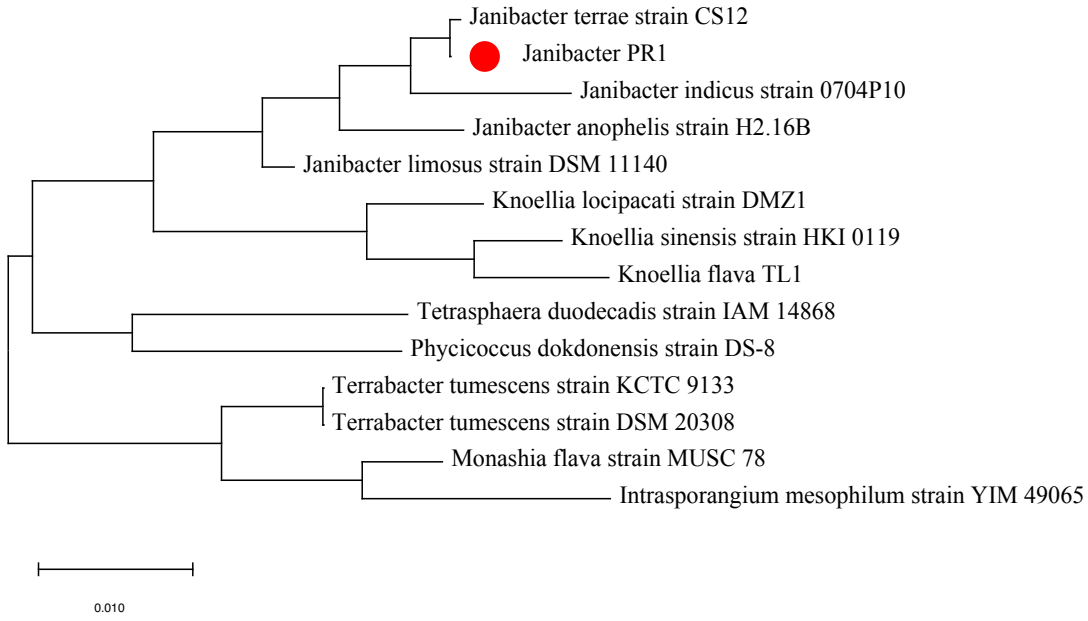


Figure 3.2 Phylogenetic tree prepared for *Janibacter* sp. PR1 using MEGA software. Red dot refers to the isolated strain

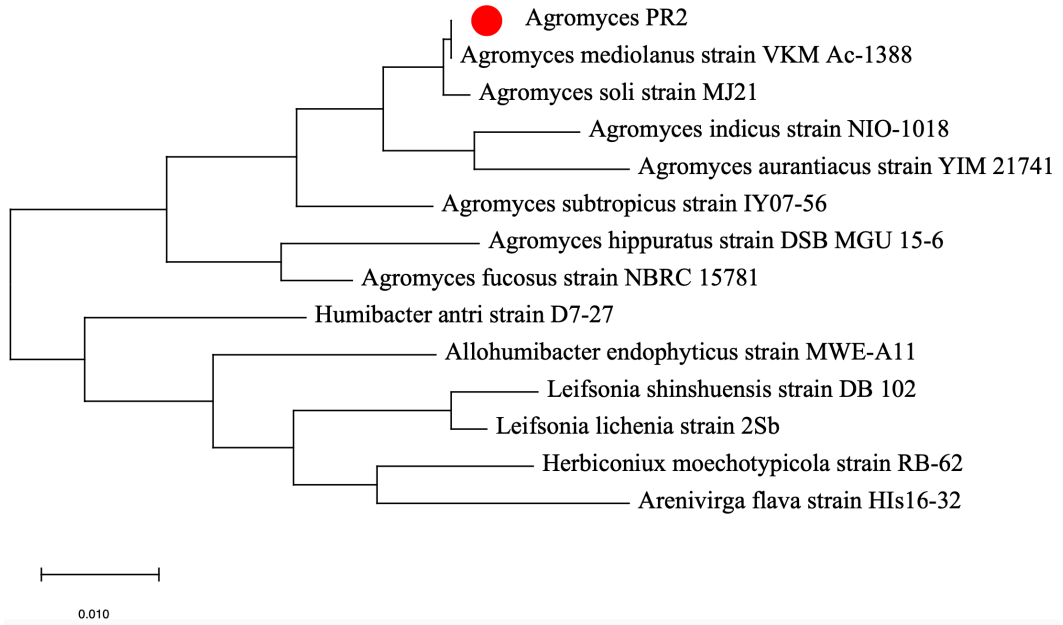


Figure 3.3 Phylogenetic tree prepared for *Agromyces* sp. PR2 using MEGA software. Red dot refers to the isolated strain.

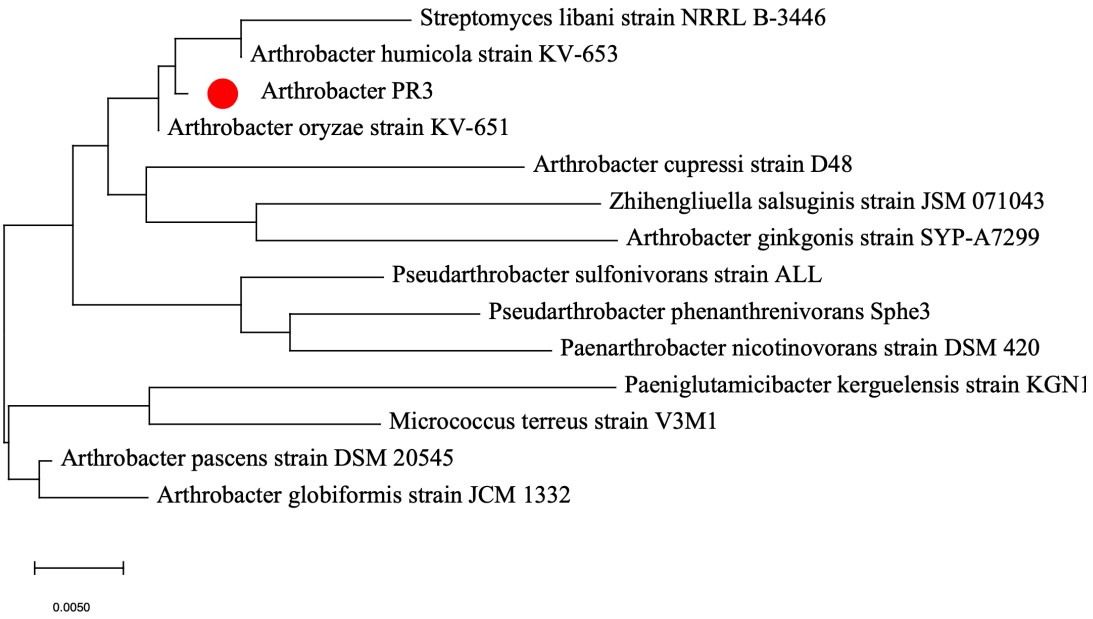


Figure 3.4 Phylogenetic tree prepared for *Arthrobacter* sp. PR3 using MEGA software, Red dot refers to the isolated strain.

3.3.3 Growth on dibenzofuran: The growth of the three isolates on DF is shown in (Figure 3.5). Slightly different rates of growth were observed for the isolates, although all reached similar ODs at the end of the incubation period, OD of 1.2 ± 0.1, at day 7 or 8, depending on the organism. *Janibacter* sp. PR1 had the highest rate of growth (0.68 d^{-1}), followed by *Agromyces* sp. PR2 (0.47 d^{-1}), then *Arthrobacter* sp. PR3 (0.34 d^{-1}) (Figure 3.5). Appearance of the yellow color was noticed at day 3 of incubation for all isolates, with slightly different color densities.

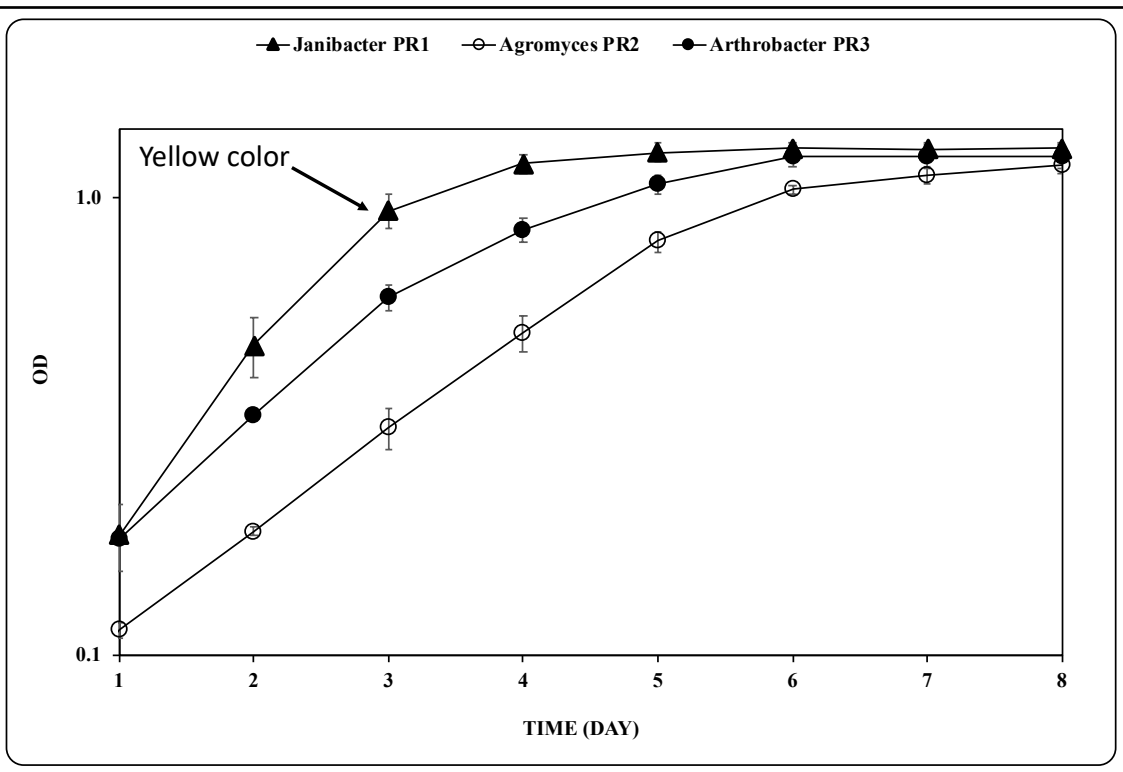


Figure 3.5 Growth curves of *Janibacter* sp. PR1, *Agromyces* sp. PR2, and *Arthrobacter* sp. PR3 on minimal medium with dibenzofuran as the sole carbon source. Each isolate was started with 0.05 OD inoculum. Symbols are averages of three replicates and error bars are one standard deviation.

3.3.4 Transformation of DD, 2-MCDD, and 2,7DCDD

Resting cells of *Agromyces* sp. PR2 and *Arthrobacter* sp. PR3*,* grown to stationary phase on DF, were examined for the ability to transform DD, 2-MCDD, and 2,7DCDD. We did not examine PR1 because very closely related strains have been tested in other studies (Iwai et al., 2005; Jin et al., 2006; Lang et al., 2003). DF was used as positive or reference reaction because it is already known that both strains could utilize and grow on it. Results showed that *Agromyces* sp. PR2 and *Arthrobacter* sp. PR3 could transform DF rapidly, as all DF was utilized by both isolates within 3 hrs. Furthermore, DF loss was detected even at the first time point at ~ 10 min (the time required to mix the vials and remove the first set of samples) (Figures 3.6 and 3.7.). Overall the rate of transformation of the compounds by the isolates was DF>DD>2MCDD, with no transformation of 2,7DCDD observed. The transformation of DD by *Agromyces* sp. PR2 was more rapid than that of *Arthrobacter* sp. PR3. It required between 6 to 12 hrs for DD to be depleted in the case of *Agromyces* sp. PR2 while some DD was still detected at 12 hrs in the case of *Arthrobacter* sp. PR3. Further, there were lower congener concentrations at 3 hrs in the case of *Agromyces* sp. PR2 (0.35 ppm DD) than for *Arthrobacter* sp. PR3 (0.92 ppm DD). No substantial loss was observed in the killed controls (Figures 3.6 and 3.7).

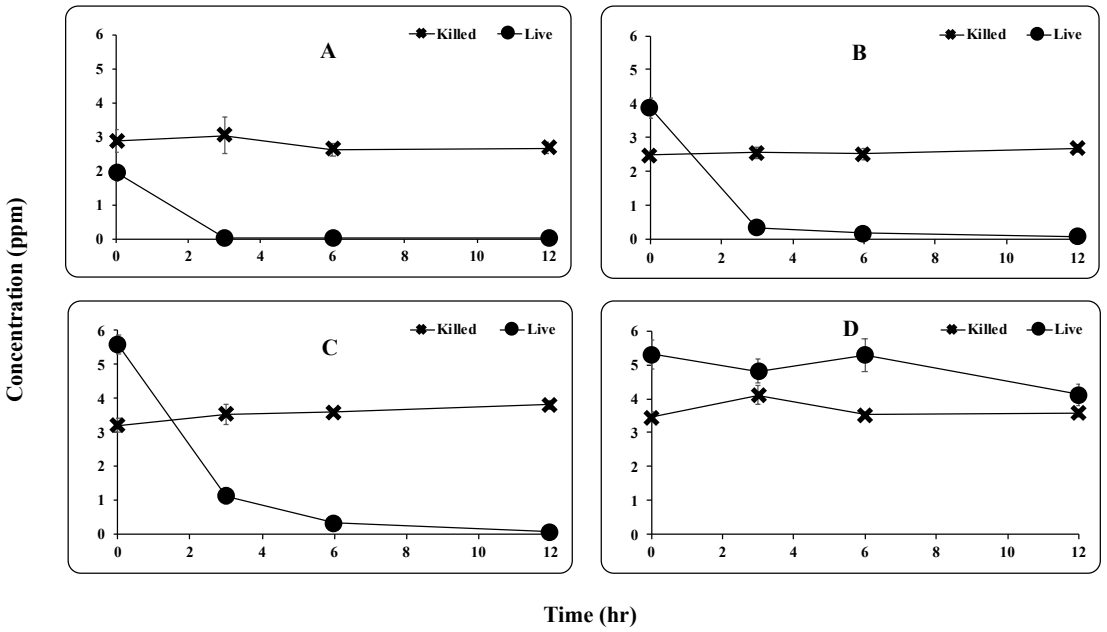


Figure 3.6 Transformation of dibenzofuran (A), dibenzo-*p*-dioxin (B), 2-monochlorodibenzo-*p*-dioxin (C), and 2,7-dichlorodibenzo-*p*-dioxin (D) by resting cells of *Agromyces* sp. PR2. Symbols are averages of three replicates and error bars are one standard deviation.

Bacterial strains isolated on DF as a sole carbon and energy source most likely also transform DD (Hong et al., 2004; Keim et al., 1999; Nam et al., 2006) due to the similarity in the chemical structures and the enzymatic system that is responsible for biodegradation possesses of both compounds (Chang, 2008; Damborsky et al., 1998; Wittich, 1998). Bioavailability and toxicity are governed by the compounds' structures (Fiedler, 2003), in addition to other parameters. Accordingly, DF and DD may be differentially available to microbes. DD has a higher molecular mass, is more hydrophobic and presumably less

bioavailable than DF (Bunge and Lechner, 2009; Urbaniak, 2013). Both isolates transformed DD, but DF transformation was faster than DD. Faster transformation of DF was also expected because the resting cells were grown with DF before conducting the transformation experiments (Parsons et al., 1998; Wilkes et al., 1996).

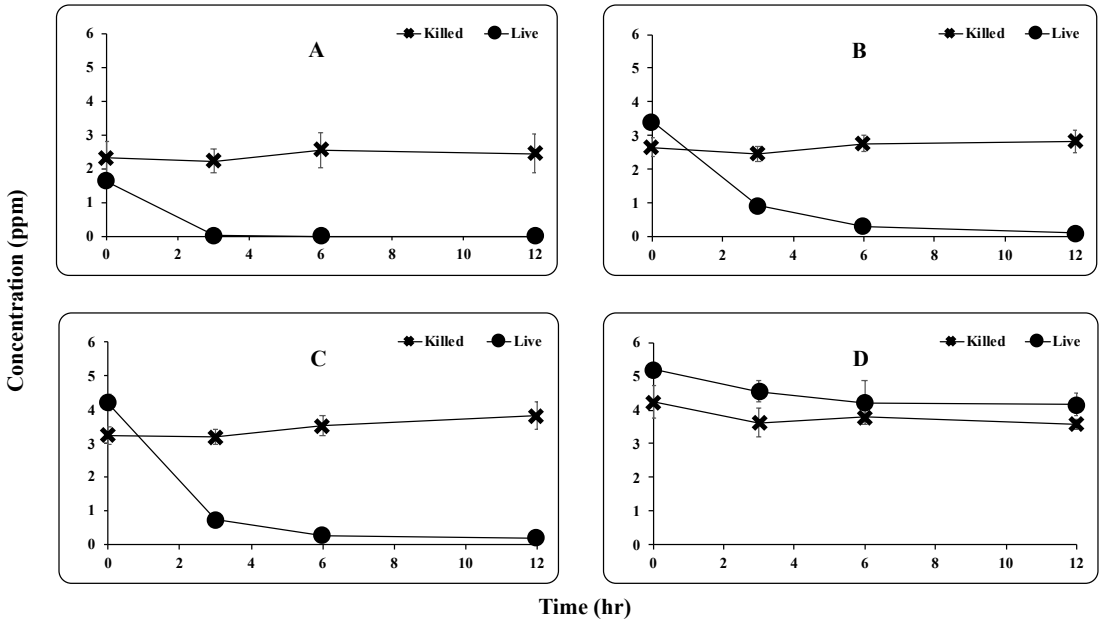


Figure 3.7 Transformation of dibenzofuran (A), dibenzo-*p*-dioxin (B), 2-monochlorodibenzo-*p*-dioxin (C), and 2,7-dichlorodibenzo-*p*-dioxin (D) by resting cells of *Arthrobacter* sp. PR3. Symbols are averages of three replicates and error bars are one standard deviation.

Very similar transformation for both isolates was observed for 2MCDD. Overall transformation was slower for 2MCDD than for DD in both isolates. *Agromyces* sp. PR2 exhibited slighty more rapid transformation. No significant loss in concentration was noted in killed controls (Figures 3.6 and 3.7). As for 2,7DCDD, resting cells of the isolates did not transform the congener. There was no transformation or substantial loss in the 2,7DCDD concentrations in live treatments compared to the killed controls, even with several days of incubation (Figures 3.6,3.7, and 3.8).

Results for the Passaic River isolates are similar to those for other strains. For example, other bacterial strains were able to transform DD and a mono-CDD/F but either did not transform DD and DF congeners with two or more chlorines or transformed them only slowly (Hong et al., 2004; Wilkes et al., 1996). Similar to our results, some bacterial species, such as *Terrabacter* sp. strain DBF63 and *Sphingomonas* sp. strain KA1, have been reported to transform DD and MCDD, but not DCDD congeners (Habe et al., 2002a; Habe et al., 2002b) In contrast, *Sphingomonas wittichii* RW1 can transform DD, MCDD, DCDD, and trichlorodibenzo-*p*-dioxin (TriCDD) (Keim et al., 1999). Furthermore, some transformation of hexachlorodibenzo-*p*-dioxin (HxCDD) congeners was reported for *Sphingomonas wittichii* RW1 (Nam et al., 2006).

Chlorine number and position play an essential role in the biodegradability of different CDD congeners. Congeners that have more chlorines and chlorines in the lateral positions are more hydrophobic, less bioavailable, more toxic, and theoretically more difficult to biodegrade than others (Schecter et al., 2006; Toussaint et al., 1998; Urbaniak, 2013). Additional chlorines, especially in the lateral positions, slow the transformation rates significantly, which may explain why neither of the isolates transformed 2,7DCDD. The

floated silica was used to supply the congeners to the cultures and prevent them from being absorbed to the glass wall of the serum bottles; this, in addition to the continuous shaking during the incubation time, were intended to enhance their bioavailability. 2,7DCDD has two chlorines in the lateral positions that could cause steric hindrance to the oxygen on the ether bridge, which is the attachment target of the enzymes. (Damborsky et al., 1998; Wittich, 1998). Transformation of non and lightly-chlorinated dioxin congeners is important especially since Passaic River sediments may release these congeners as a result of anaerobic biodegradation (Dean et al., 2020).

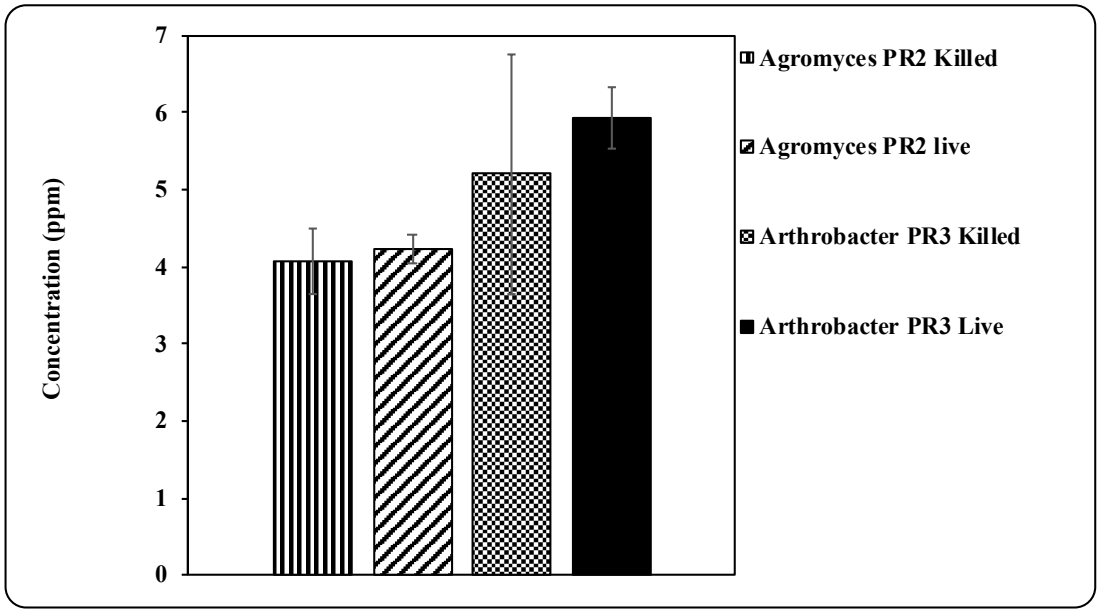


Figure 3.8 Concentration of 2,7DCDD in killed and live cultures of *Agromyces* sp. PR2 and *Arthrobacter* sp. PR3 after 5 days of incubation.

3.3.5 Characterization of angular dioxygenases

PCR amplification of the gene encoding the angular dioxygenase DbfA from the genomic DNA of *Janibacter* sp. PR1 and *Agromyces* sp. PR2 using primers DBFA1A2F and DBFA1A2 (Hong et al., 2004; Kasuga et al., 2001; Kasuga et al., 2013) yielded amplicons of the expected size (1.8 kb). The dioxygenase sequences from the two strains are 100% similar to each other. The dioxygenase gene had a high similarity (99.7%) to the angular dioxygenase genes in *Terrabacter* sp. strain DBF63 (Kasuga et al., 2001) and strain YK3 (Iida et al., 2002). This gene is also found in *Rhodococcus* sp. strain P52 (Peng et al., 2013) and strain HA01 (Aly et al., 2008); *Janibacter terrae* XJ1 (Jin et al., 2006); *Panibacillus* sp. strain YK5 (Iida et al., 2006); (Figure 3.9). No amplification occurred from the genomic DNA of *Arthrobacter* sp. PR3, and it is not known what functional gene is responsible for the observed transformation by that isolate.

The *dbfA* gene is located on plasmids (Miyauchi et al., 2008): linear plasmids (160 kb) as in *Terrabacter* sp. strain DBF63 (Nojiri et al., 2002) or circular plasmids as in *Rhodococcus* sp. strain P52 and *Terrabacter* sp. strain YK3 (Iida et al., 2002; Peng et al., 2013). Plasmids can transfer from one bacterial cell to another vertically and horizontally (Turner et al., 1998). The plasmid from *Rhodococcus* sp. strain P52 that contains the *dbfA* gene, a self-transmissible plasmid, has been transferred to *Bacillus cereus* by conjugation (Peng et al., 2013), which explains the wide distribution of the gene in different genera. It is not surprising that different isolates from the same CDD-contaminated environment would have this particular functional gene.

DbfA has a wide range of substrate activity. It can attack fluorene, phenanthrene and anthracene (Habe et al. 2004), in addition to DF, DD, and some lightly-chlorinated CDDs

(Kasuga et al., 2001). This gene has been cloned and expressed to confirm its function in DD and CDD degradation ((Kasuga et al., 2001; Kasuga et al., 2013; Kasuga et al., 1997).

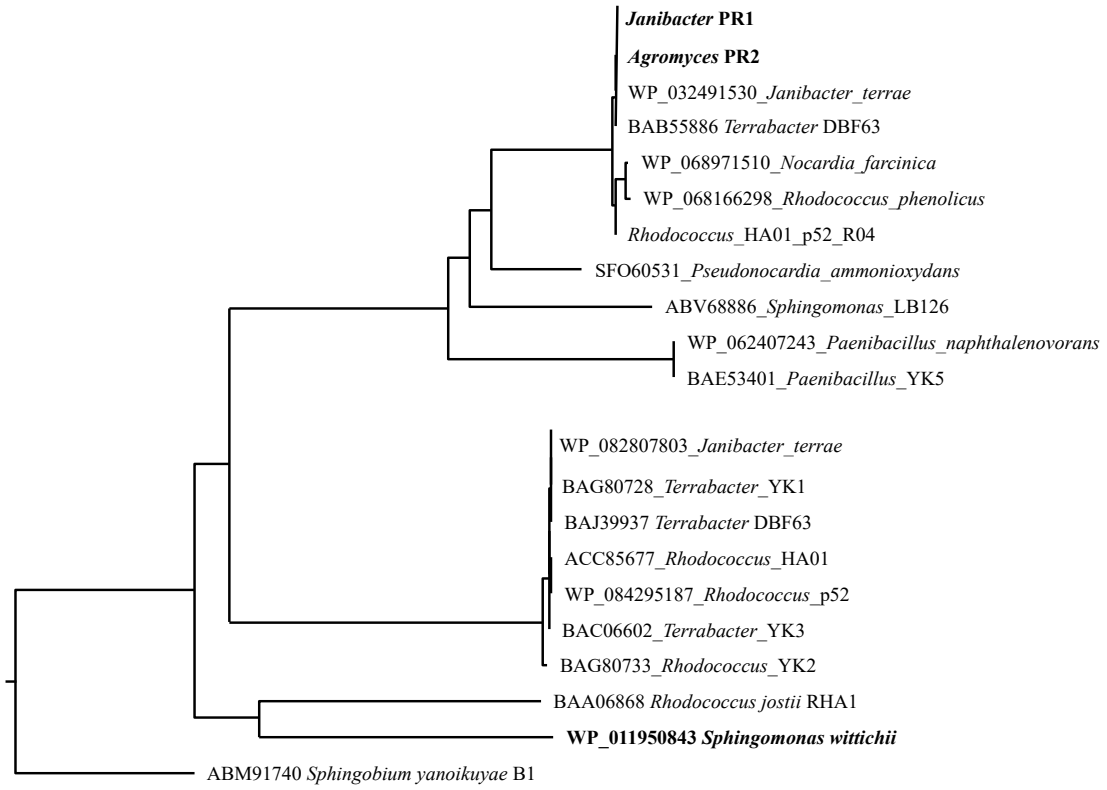


Figure 3.9 Comparison of the functional genes in the strains to selected angular dioxygenase amino acid sequences.

3.4 Conclusions

Three bacterial isolates that can use DF as a carbon and energy source were obtained from the LPR enrichments. Two of the isolates were also shown to transform non-chlorinated DD and 2MCDD, but not 2,7DCDD (the third isolate was not tested). The presence of indigenous bacteria that degrade DD and MCDD also suggests that metabolites formed from dechlorination of highly chlorinated CDDs (Barkovskii and Adriaens, 1996; Dean et al., 2020; Lohmann et al., 2000) could also be aerobically degraded in the LPR sediments. Sediment enrichments or pure cultures that could degrade these compounds aerobically do so at higher rates than the anaerobic dechlorination processes that transform highly chlorinated congeners to less chlorinated congeners. Thus, if DD or DF were formed in LPR sediments they may be rapidly depleted (Narihiro et al., 2010; Yoshida et al., 2005). Further work is needed to confirm that these organisms are active *in situ* in the river sediments. If they are, it seems that non- and mono-chlorinated DF and DD congeners could be aerobically transformed as they are formed. Further investigation could involve collection of multiple samples from different areas, including the location used in this study, of the LPR to assess how common this activity is. Isolated strains could also be used in a bioaugmentation study by providing environmental conditions that mimic the Passaic River conditions and determine potential for *in situ* activity. Since an angular dioxygenase whose function was confirmed previously in other organisms was detected in two of the pure isolates from the Passaic River sediments, expression of this gene could be an indicator of *in situ* activity.

Chapter 4

Aniline Biodegradation in Aquifers Underlying a Chemical Manufacturing Site in Southern Jersey

Abstract

We evaluated potential for aniline and parachloroaniline (PCA) biodegradation in aquifers underlying a large, chemically and geologically complex industrial site. A 55-ft sediment core was previously recovered from the site along with groundwater from differing depths from an adjacent multi-level sampling well. The sediment core was divided into 2-in slices for detailed analyses. The ~300 sediment core slices were shared among multiple teams and were characterized for *in situ* geochemistry and microbial community via sequencing of 16S rRNA genes. We used this multifaceted data to develop hypotheses for microbial activity and tested the hypotheses through laboratory scale microcosms simulating different redox conditions. Sediment from two to three 2-in core slices from different specific elevations (~ -10, -17, -20, -34 ft) were used to establish four sets of targeted microcosms. In addition, sediments from -27 to -29 ft were mixed and used for composited microcosms. Aerobic, sulfate-, nitrate-, and iron-reducing microcosms were established and incubated statically, in the dark at room temperature. The targeted microcosms showed no substantial loss of aniline or PCA. Composited microcosms established under aerobic and sulfate-reducing conditions were active, and aniline was depleted several times after being re-amended. Bacterial community analysis via next-generation sequencing showed substantial changes in the bacterial community of active microcosms compared to the original sediments. Our results indicate differential distribution of aniline biodegradation

in the core material recovered from the site, indicating heterogenous distribution of biodegradation in the subsurface.

4.1 Introduction

Aniline and chloroanilines (CANs) are organic amines widely used as production chemicals to produce dyes, plastics, pesticides, herbicides, resins, explosives and pharmaceuticals (Poste et al., 2014; Travis, 1997; Yen et al., 2008). Aniline and CANs are contaminants of concern because of their toxicity to aquatic, terrestrial and human life (Fan et al., 2011; Khan et al., 2003; Messeguer, 2011). Aniline is a probable human carcinogen, a mutagen and teratogen (Greenwood et al., 1979; NYSD E, 1998). These compounds enter the environment from mainly anthropogenic sources such as spills and improper disposal and may spread widely owing to their relatively low hydrophobicity and high solubility (Poste et al., 2014). CANs can also be produced in the environment by microbial transformation of halogenated herbicides (Häggblom, 1992; Zeyer and Kearney, 1982).

Large aniline spills have occurred over the years. For example, a 40,000 lb release at an unidentified manufacturing site in 1979 was later the subject of characterization for biodegradation potential (Kosson and Byrne, 1995). Huge spills have occurred, for example in China, including a large release of aniline that contaminated rivers and a reservoir in Shanxi and Hebei provinces (CHINADAILY USA website) and a separate incident that resulted in contamination of a water supply in a village, also in Hebei province (High levels of aniline found in Hebei water supply (CHINADAILY.com.cn).

Because of their widespread presence as pollutants in the environment and in industrial wastewater, several chemical and physical treatment methods have been used to remove

aniline and CANs from aqueous systems including electrochemical destruction (Brillas et al., 1995), chemical oxidation using pyrite, persulfate and peroxydisulfate with copper and nickel (Liang et al., 2013; Zhang et al., 2015; Zhu et al., 2019), UV radiation (Gosetti et al., 2010; Hwang et al., 1987; Sanchez et al., 2002), and reverse osmosis (Gómez et al., 2009).

Bartha and colleagues were among the first to study biodegradation of aniline. They found that biodegradation was the most important loss process for aniline in pond water, and that addition of sewage sludge inoculum enhanced biodegradation (Lyons et al., 1984; Lyons et al., 1985). Slow biodegradation of CANs was observed to occur, likely because of their binding to humic material, resulting in less bioavailability to microbes, or via abiotic reactions that formed more complex organic compounds (Latorre et al., 1984). Natural biofilms collected from stones, biofilms grown on submerged glass slides, and also planktonic cells from a river in a contaminated area showed a good capacity for aniline uptake, with a change in the microbial community noted during aniline degradation (Araya et al., 2003). Thus, in addition to concern about toxicity of the compounds, this earlier research shows that spills of aniline and CANs may exert substantial biochemical oxygen demand in natural waters, and also in wastewater treatment systems (Gheewala and Annachhatre, 1997).

Limited studies are available on aniline biodegradation under anoxic conditions. A sulfate-reducing bacterium, *Desulfobacterium anilini* (Schnell et al., 1989) was isolated on aniline and extensively characterized. Another study investigated the potential for large scale bioprocesses of treating aniline-containing wastewater under different redox conditions, as

well as at different salt concentrations and pH. Only aerobic biodegradation was successful, with no degradation observed under sulfate-reducing, nitrate-reducing or fermentative redox conditions (O'Neill et al., 2000). Aniline, but not chlorinated aniline was biodegraded under iron (III)-reducing conditions in Hudson River sediments (Alexandra De et al., 1994; Kazumi et al., 1995). Aniline was degraded under nitrate-reducing conditions in anaerobic sewage sludge and in estuarine sediments from Newtown Creek (off the East River in New York City), but no loss was observed in either inoculum under methanogenic conditions after 31 weeks (Alexandra De et al., 1994; Kazumi et al., 1995). We previously examined aniline degradation under different electron-accepting conditions in the sediments of a freshwater canal and in the adjacent aquifer at the same large chemical manufacturing site that we examined in the current study. Aniline loss was observed in nitrate- and sulfate-amended microcosms and in microcosms established to promote methanogenic conditions with times to onset differing dependent upon the exact location within the site. In that study, a phylotype closely related to *Ignavibacterium* was identified by stable isotope probing as the dominant aniline degrader in the community, in microcosms operated under methanogenic conditions (Sun et al., 2015).

In the current study we examined aniline and PCA degradation in aquifer materials from different depths in aquifers underlying the same chemical manufacturing site, though at a different location. The project began with characterization of a 60 ft core recovered from this location. The core was characterized with respect to geochemistry and 16S rRNA gene sequencing of the microbial community. These data were used to guide selection of depths for examining aniline and PCA biodegradation processes. Microorganisms were enriched in microcosms under several reducing conditions to gain an understanding of the aniline

degradation processes occurring, and to determine which conditions were required to stimulate the process. Community analysis was performed for the most active microcosms.

4.2 Materials and methods

4.2.1 Study site and sample collection

Aquifer materials were collected from a large chemical manufacturing facility located in southern New Jersey (Figure 4.1) as described in detail previously (Sun et al. 2015). The facility produced many different types of chemical products over decades of operation. The underlying aquifers are contaminated with different pollutants such as chlorobenzenes, toluene, aniline, CANs, trichloroethane, tetrachloroethene, polyaromatic hydrocarbons (PAHs) and heavy metals (Kurt and Spain, 2013; Nelson et al., 2011; Sun et al., 2015). In brief, a 60-ft sediment core was recovered in 2 ft sections from the sample collection area (Figure 4.1), stored under anoxic conditions, re-located to an anaerobic chamber at the New Jersey Institute of Technology, and divided into 2-inch slices, as described in detail elsewhere Ground water was recovered from closely matching depths from a multi-level sampling well (D14-MLS) (Table 4.1). In total, approximately 300 sediment core slices were shared among several teams and geochemistry (e.g., pH, DO, ORP, Fe, S, total VOCs, etc.) and microbial community via sequencing 16S RNA genes was performed for each slice, as described previously. Groundwater was characterized for ORP, DO, methane, nitrate, nitrite, sulfate, contaminants, etc. Specific core slices from several depths were transferred to our laboratory to evaluate microbial activity using microcosm studies. Depending upon the aniline, PCA and electron acceptor profiles, specific depth slices were selected for “targeted” microcosms (using ~6 in of core) and another set of slices (~1 – 2

ft) were mixed and used to establish "composited" microcosms. Targeted microcosms were presumed to include a more limited microbial community reflecting a limited microbial niche, while composited microcosms were presumed to encompass a broader community that included more ecological niches.

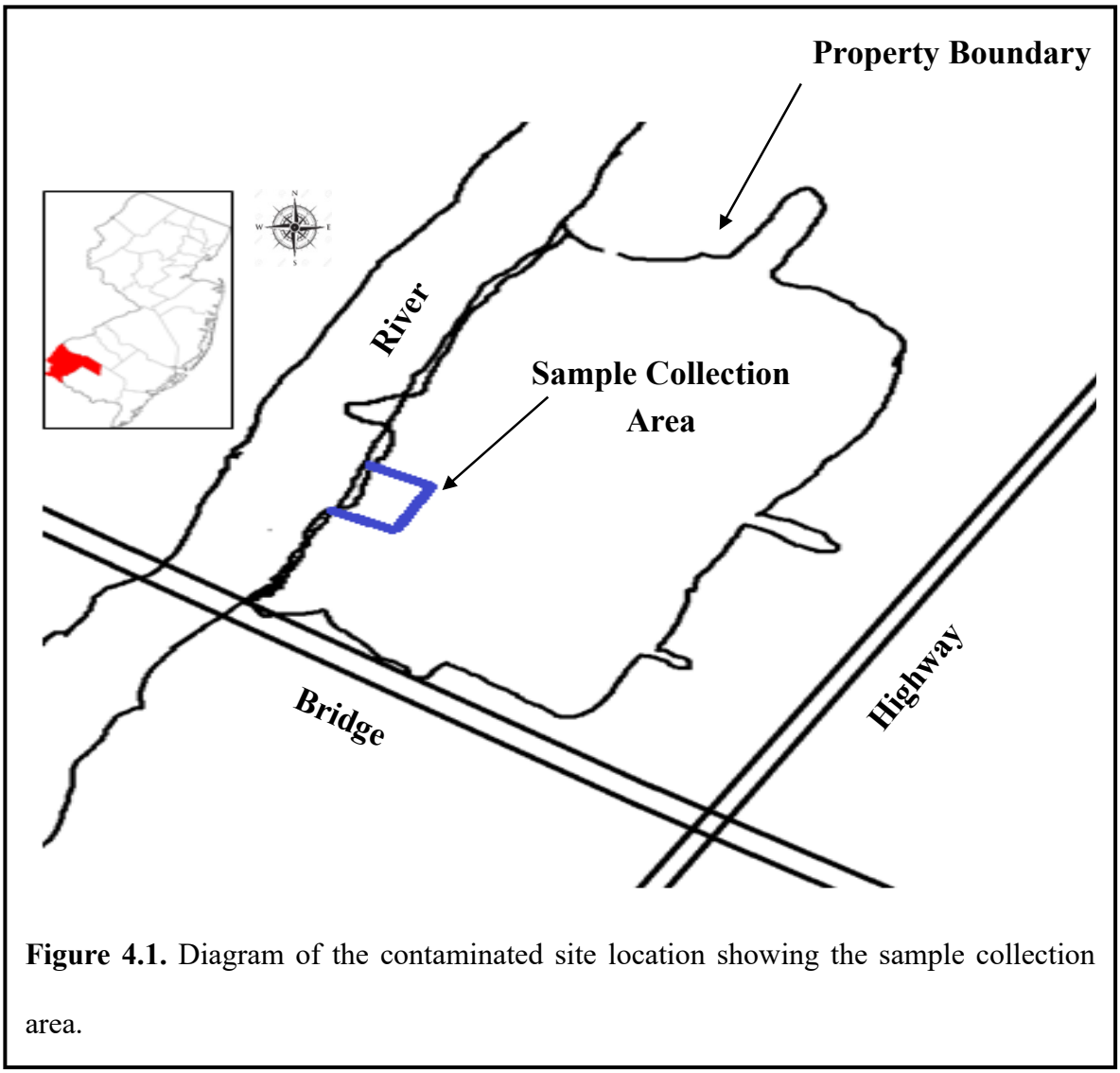


Figure 4.1. Diagram of the contaminated site location showing the sample collection area.

4.2.2 Selection of Depths and Conditions of Microcosm Studies

An extensive data set was available describing the conditions through the depth of the 60 ft core and the groundwater recovered from the multilevel sampling well. We selected depth locations for microcosms based on prevailing conditions measured in the cores and groundwater. Slices and depths were selected for example, depending upon the aniline and PCA concentration and electron acceptor profiles in groundwater (Table 4.2), along with ORP and microbial community in the core material, as information became available. As Figure 4.2 and Table 4.2 indicate, the selected depths had higher concentrations (JZM1 and JZM2), lower (JZM4), or intermediate (JZM3) concentrations of aniline and/or PCA.

Table 4.1. Sampling port designations and locations for the multi-level sampling well (D14-MLS) in the Detailed Study Area of the chemical manufacturing facility.

Well ID	Port #	Depth (below ground surface) (ft)	Port Elevation (ft)
D14-MLS	15	5.75	-1.29
D14-MLS	14	10.25	-5.79
D14-MLS	13	13.75	-9.29
D14-MLS	12	17.25	-12.79
D14-MLS	11	20.75	-16.29
D14-MLS	10	23.25	-18.79
D14-MLS	9	26.75	-22.29
D14-MLS	8	29.25	-24.79
D14-MLS	7	32.75	-28.29
D14-MLS	6	37.25	-32.79
D14-MLS	5	40.75	-36.29
D14-MLS	4	44.25	-39.79
D14-MLS	3	47.75	-43.29
D14-MLS	2	52.25	-47.79
D14-MLS	1	59.75	-55.29

Table 4.2. *In situ* groundwater concentrations for the multi-level sampling well elevations that match the approximate elevations for core slices used to form targeted microcosm sets.

Targeted Microcosm Set	Groundwater Elev (ft) (Port)	pH	Temp. (ºC)	Aniline (µg/L)	DO (mg/L)	PCA (µg/L)	Sulfate (mg/L)	Methane (µg/L)
JZM1	-24.79 (8)	6.39	26.1	3,700	9.46	620	200	610
JZM2	-24.79 (8)	6.39	26.1	3,700	9.46	620	200	610
JZM3	-5.79 (14)	7.3	NA	220	2	NA	45	17,000
JZM4	-36.29 (5)	6.79	NA	1	7.64	8	494	270

NA = not available

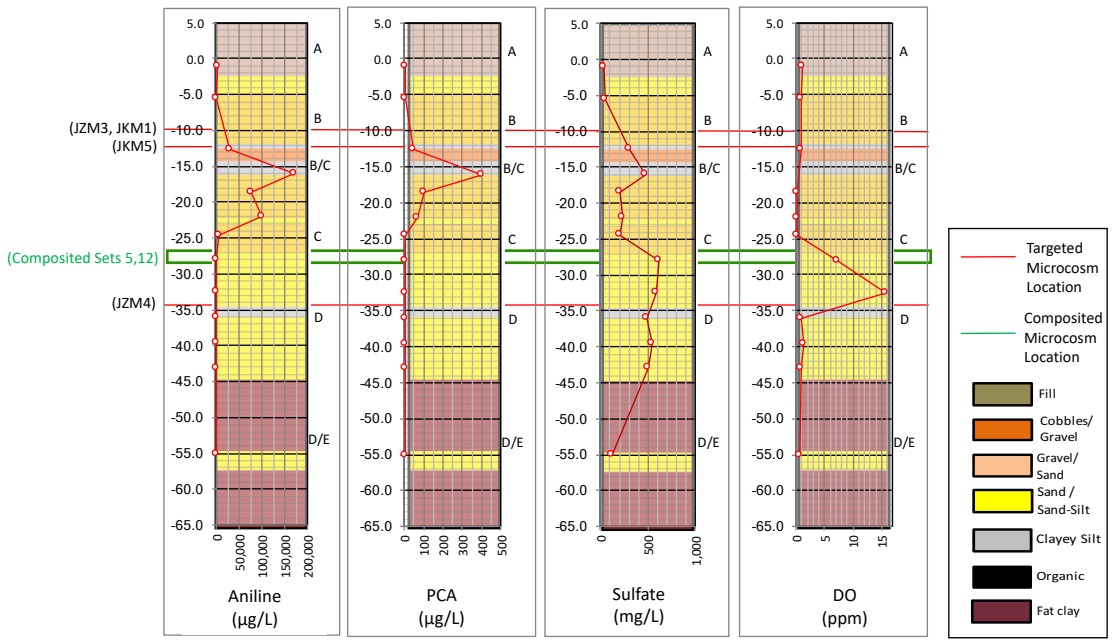


Figure 4.2. Subsurface locations of core slices used in targeted and composited microcosms in the subsurface. Left hand side Y axis is elevation (-ft) and on right hand side axis letters denote specific aquifers. Profiles of aniline, PCA, sulfate, and dissolved oxygen (DO) are show on the x axes. Profiles and graphs courtesy of Chemours and Mr. Scott Morgan of AE Com.

4.2.3 Chemicals

Aniline (99%) and parachloroaniline (PCA, 95%) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium L-lactate (≥99%) was purchased from Sigma-Aldrich (Milwaukee, WI). Acetonitrile ≥ 99.99 HPLC grade from Fisher Scientific (Fair Lawn, NJ).

4.2.4 Groundwater and Medium

Groundwater from the appropriate port of D14-MLS (Table 4.1) was purged for one hour with filter-sterilized argon to remove VOCs, filtered through a sterile 0.45 μm SUPOR membrane filter (Pall Life Sciences, Port Washington, NY) to remove cells, and stored in a sterile glass bottle at 4°C until use. To adjust the groundwater to different redox conditions, 33 mM KNO_3 and 20 mM Na_2SO_4 were added from sterile, anoxic aqueous stocks, while Fe (III) oxyhydroxide slurry was prepared and added as described below. Lactate (1 mM) was added to PCA- amended methanogenic microcosms to enhance organohalide reduction.

Medium was established with different major electron acceptors to enhance different redox processes: aerobic, nitrate-amended, Fe (III)-amended, sulfate-amended and methanogenic (i.e., no electron acceptor except CO_2). Unless otherwise noted, the medium contained the following (per liter): KCl, 1.3 g; KH_2PO_4, 0.2 g; NaCl, 1.17 g; $CaCl_2 \bullet 2H_2O$, 0.1 g; $MgCl_2 \bullet 6H_2O$, 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. Microcosms established under aerobic conditions had the headspace purged with filter-sterilized air (Airgas, Piscataway, NJ). The nitrate-amended medium contained 33 mM KNO_3 and 0.8 mM Na_2SO_4; the Fe(III)-amended media was amended with amorphous FeOOH prepared

as described below; and the sulfate-amended medium contained 20 mM Na_2SO_4. Medium was autoclaved at 121°C, cooled, then purged with sterile 30%CO_2/70%N_2 for 20 min. A $Na_2S \bullet 9H_2O$ stock solution was added to achieve 2.08 mM S^{2-} in the methanogenic, and 1.46 mM S^{2-} in the sulfate-reducing medium, as reductant. The pH was adjusted to neutrality and media was stored at room temperature until use. For more details see (Li, 2014; Sun et al., 2015).

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; $NaMoO_4 \cdot 2H_2O$, 0.1 g; and $FeCl_2 \cdot 4H_2O$, 1.49 g. Trace salts solution II contained the following (per liter): 0.03 g of $NaSeO_3$ and 0.08 g of Na_2WO_4.

Amorphous FeOOH was produced 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 to remove Na^+ (Lovley and Phillips 1986; Li, 2014). The iron floc was concentrated by centrifugation and mixed with purged site groundwater from appropriate D14-MLS ports or to anoxic medium to achieve the desired Fe (III) concentration.

4.2.5 Microcosm setup

Microcosms were generally established as described previously (Sun et al., 2015).

4.2.5.1 Targeted microcosms

Four sets of targeted microcosms were established using 15% sediment slurries (volume sediment: volume groundwater) of 40 mL in 60 mL serum bottles (Wheaton, Millville, NJ), in triplicate, and with triplicate autoclaved controls, as shown in Table 4.3 Sediments from the selected core slices were mixed under anoxic conditions in a anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). Groundwater from a closely matching depth was used to make the sediment slurry (Table 4.2). Microcosms were sealed with Teflon™ lined septa (Wheaton, Millville, NJ), and secured with crimp caps. Each microcosm was amended with 250 μM of aniline or PCA from sterile 100 mM aqueous stock solutions. Microcosms were incubated statically in the dark at room temperature.

Table 4.3. Targeted microcosms established from core material from different elevations and under different redox conditions.

Microcosm Set	Condition	Core Elev. (ft)	Core Slices	Groundwater Elev. (ft)	Amendment (**μm**)	Aniline / PCA Amendment (**μm**)
JZM1	Methanogenic-Organohalide Reducing	-17.5 to -17.8	7-1, 7-2, 7-3	-24.79	Lactate 1,000	250, PCA 250, Aniline
JZM2	Sulfate-Reducing	-20.3 to -20.7	8-7, 8-8, 8-9	-24.79	Sulfate 20,000	250, Aniline
JZM3	Aerobic	-9.8 to -10.2	3-5, 3-6, 3-7	-5.79	Sterile Air	250, Aniline
JZM4	Aerobic	-34.3 to -34.5	14B10, 14B 11	-36.29	Sterile Air	250, Aniline

4.2.5.2 Composited microcosms

Sediments from core slices encompassing the -27 to -29 ft elevation were mixed under anoxic conditions in a anaerobic chamber (Coy Laboratory Products, Grass Lake, MI), and the mixture was used to establish microcosms under aerobic, sulfate-, nitrate-, and iron-reducing conditions using minimal medium (Table 4.4). Sets of composited microcosms were established using 20% (vol:vol) sediment slurries of 35 mL in 60 mL serum bottles (Wheaton, Millville, NJ) sealed with Teflon™ lined septa (Wheaton, Millville, NJ), and secured with crimp caps. Sets of micocosms were also established in methanogenic and iron-reducing medium and autoclaved daily over three days to serve as killed controls denoted "killed" and "iron killed", respectively. Each microcosm was amended with 250 μM of aniline or PCA from sterile 100 mM aqueous stock solutions. Microcosms were monitored over approximately 600 and 500 days for aniline and PCA, respectively. Microcosms were incubated statically in the dark at room temperature.

Table 4.4 Composited microcosm study using elevation -27 to -29 ft composited core slices.

Set No.	Redox Condition	Live/Killed	Electron Donor	Electron Acceptor	Prepared In
9	Iron-Reducing	killed	aniline, PCA	Fe (III)	Medium
10	Anoxic	killed	aniline, PCA	none	Medium
11	Iron-Reducing	live	aniline, PCA	Fe (III)	Medium
12	Aerobic	live	aniline, PCA	oxygen	Medium
13	Nitrate-Reducing	live	aniline, PCA	nitrate	Medium
14	Sulfate-Reducing	live	aniline, PCA	sulfate	Medium

4.2.6 Monitoring microcosms

Microcosms were monitored periodically by removing liquid (organic acids and ions), sediment slurry (aniline and PCA) or gaseous headspace (methane) samples. Microcosms were re-amended with aniline or PCA periodically. Aerobic microcosms were re-amended or purged briefly with filter-sterilized air, periodically.

4.2.7 Sample preparation and High-Performance Liquid Chromatography (HPLC) analysis of aniline and PCA

Microcosms or cultures were mixed vigorously then 1 mL of sediment slurry or culture was sampled using a 1 mL sterile syringe and 26 G needle that had been flushed with filter-sterilized UHP N_2. Samples were either extracted directly or frozen at -20°C prior to analysis by HPLC using a modification of previously described methods (Kahng et al., 2000; Liu et al., 2015; Tanaka et al., 2009). Briefly, samples were placed in Eppendorf tubes, 1 mL of acetonitrile was added, the sample was vortexed for 3 min, and then centrifuged at 10000 *g* for 3 min. Supernatant was removed and filtered using a 0.22 μm Costar® Spin-X® centrifuge tube filter (Costar, Salt Lake City, UT). Approximately 1.5 mL extract was placed in an HPLC vial for analysis using a Beckman Coulter, (System Gold I25 Solvent Modula, System Gold I68 Detector) HPLC with diode array detector at 244 nm. The analytical column was a 5C18 120 column (250*2 mm) (Supelco Analytical, Bellefonte, PA) held at 40°C. The mobile phase was a milliQ water: acetonitrile mixture (30:70 vol:vol) and the flow rate was 0.6 mL/min. Aniline and PCA concentrations were calculated from peak areas using a five point standard curve prepared as described previously (Sun et al., 2015). All concentrations are reported as averages for triplicate bottles ± one standard deviation.

4.2.8 Bacterial Community Analysis

Community analysis using next-generation sequencing of 16S rRNA genes from original and active microcosms was performed. A sample of the original composited sediments was pelleted, water discarded and then stored at -80°C until analysis. Two of the aerobic microcosms were sampled after several amendments of aniline, and some PCA had been consumed. One mL of each sediment slurry was sampled, and DNA was extracted using a Power Soil DNA extraction kit (MoBio Laboratories Inc., Carlsbad, CA). DNA was quantified using a Qubit® ds-DNA BR Assay kit and Qubit® 2.0 fluorometer (Invitrogen, OR) and sent to MRDNA (Shallowater, TX) for sequencing. The V4 variable region of the 16S rRNA gene was amplified by MRDNA using primers 515F and 806R and the HotStarTaq Plus Master Mix Kit (Qiagen, USA) with the following PCR conditions: 94°C for 3 min, followed by 28 cycles (5 cycle used on PCR products) of 94°C for 30 sec., 53°C for 40 sec. and 72°C for 1 min, 72°C for 5 min. Ion Torrent PGM (ThermoFisher, Waltham, MA) was used to do the sequencing, then sequences were analyzed using a proprietary analysis pipeline by MRDNA: Sequences less than 150 bp and homopolymer runs exceeding 6 bp were removed. Operational taxonomic units (OTUs) were defined by clustering at 97% similarity. Finally, NCBI and RDPII websites were used to classify the OTUs.

4.3 Results and discussion

4.3.1 Chemicals profiles

In situ aniline, PCA, sulfate and dissolved oxygen concentrations in groundwater were provided to us by the on-site engineering team, while geochemical parameters for the sediment cores were provided by Professor Lisa Axe of the New Jersey Institute of Technology. The assemblages of bacteria in the core slices were provided by Dr. Frank Burns of DuPont. Based on their findings we selected the sediment core slices to establish our microcosms Figure 4.2.

4.3.2 Targeted microcosms

For targeted microcosms sediments from specific depths encompassing not more than 6 in of depth were used. Aniline or aniline plus PCA were amended to microcosms as shown in Table 4.3. After more than 400 days of monitoring the JZM1 set of microcosms showed no significant differences in either aniline or PCA concentration between the killed controls and live treatments. We noticed some abiotic loss for both compounds, that could be due to their chemical reactions with some other reactive compounds in the sediments or due to a change in pH (Minard et al., 1977). The loss is abiotic because it occurred in both the killed and the live microcosms (Figure 4.3). No activity was observed in this set of microcosms could be because a very low or absence of biomass, especially organisms could survive anaerobically and degrade aniline or PCA. Sulfate-reducing conditions (JZM2 microcosms) also showed no biological activity toward aniline (Figure 4.4). Both sets of microcosms (JZM1 and JZM2) were established using core slices that were exposed to a higher concentration of aniline and PCA in comparison to the concentrations in ground

water at other elevations (Figure 4.2), so the lack of activity could reflect the absence of active biomass in these depths (i.e., no *in situ* biodegradation). Less abiotic loss was observed in the JZM2 microcosms. In previous studies performed using soil from different sites to obtain enrichment cultures, there was also no activity in aniline biodegradation under sulfate-reducing conditions but there was activity under nitrate-reducing conditions (Kahng et al., 2000) and under aerobic conditions (Kahng et al., 2000; O'Neill et al., 2000). Aerobic sets of microcosms (JZM3 and JZM4) showed no differences in concentrations between killed controls and live treatments and abiotic loss was observed in JZM4 but not JZM3 (Figures 4.5 and 4.6).

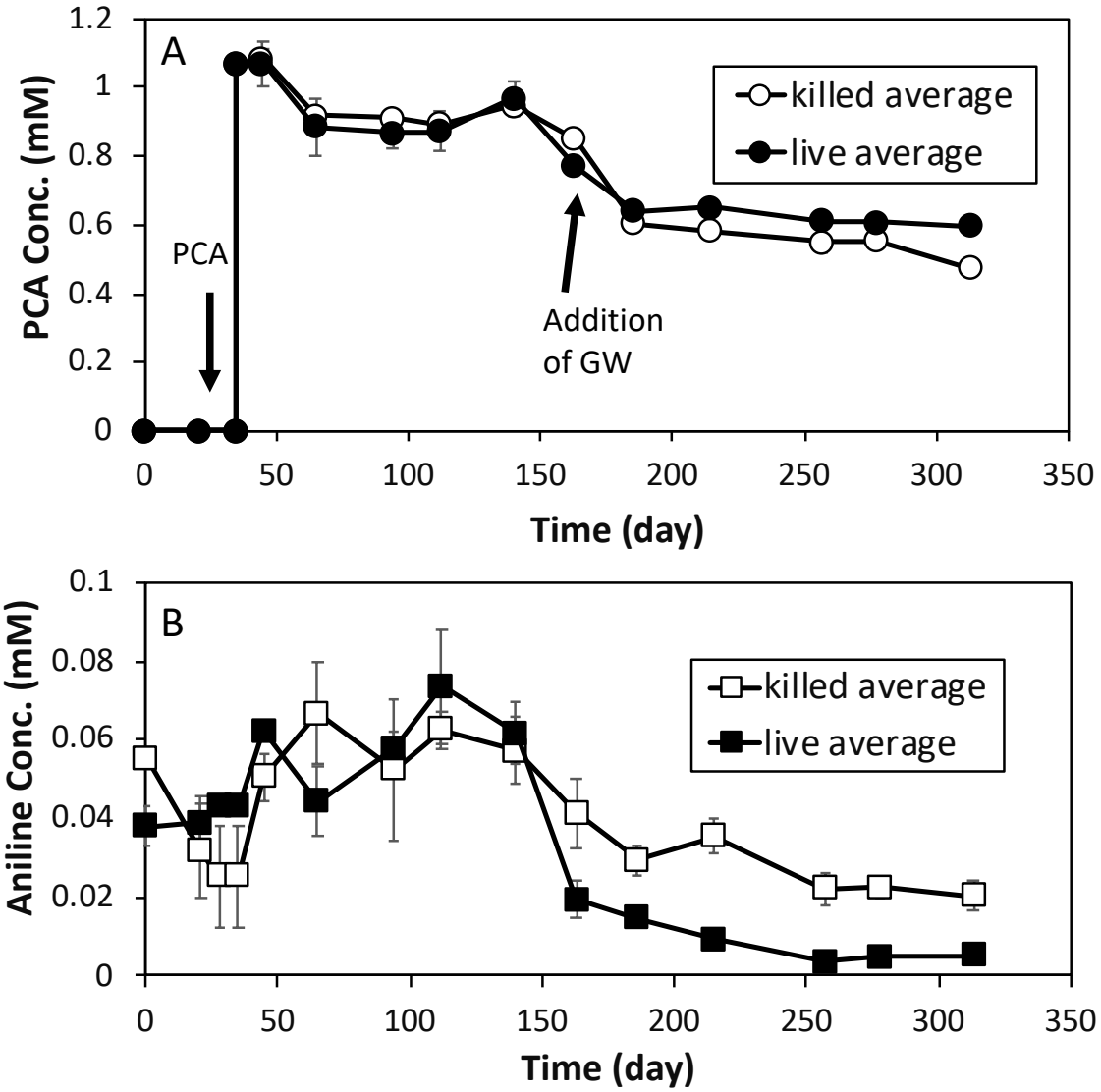


Figure 4.3 PCA (A) and aniline (B) over time in targeted microcosms JZM1 (elevation -17.5 to -17.8 ft) established under methanogenic conditions with lactate addition intended to enhance PCA dechlorination. Symbols are averages for triplicate bottles and error bars are ± one standard deviation.

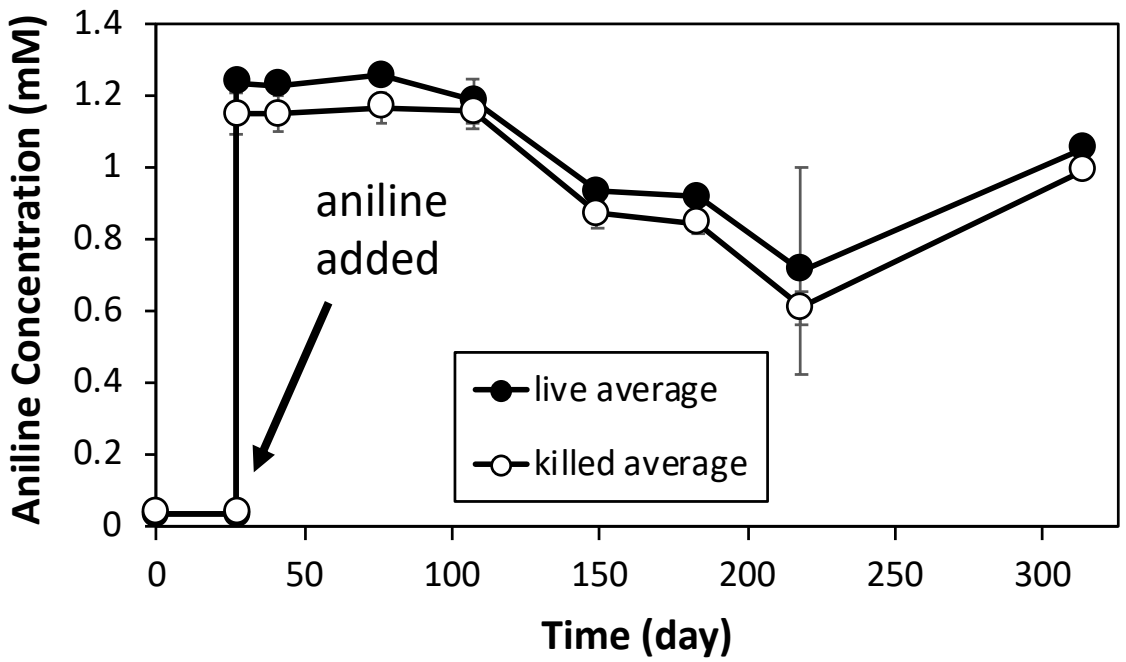


Figure 4.4 Aniline over time in targeted microcosms JZM2 (elevation -20.3 to -20.7 ft) established under sulfate-reducing conditions. ↓ indicates aniline addition. Symbols are averages for triplicate bottles and error bars are ± one standard deviation.

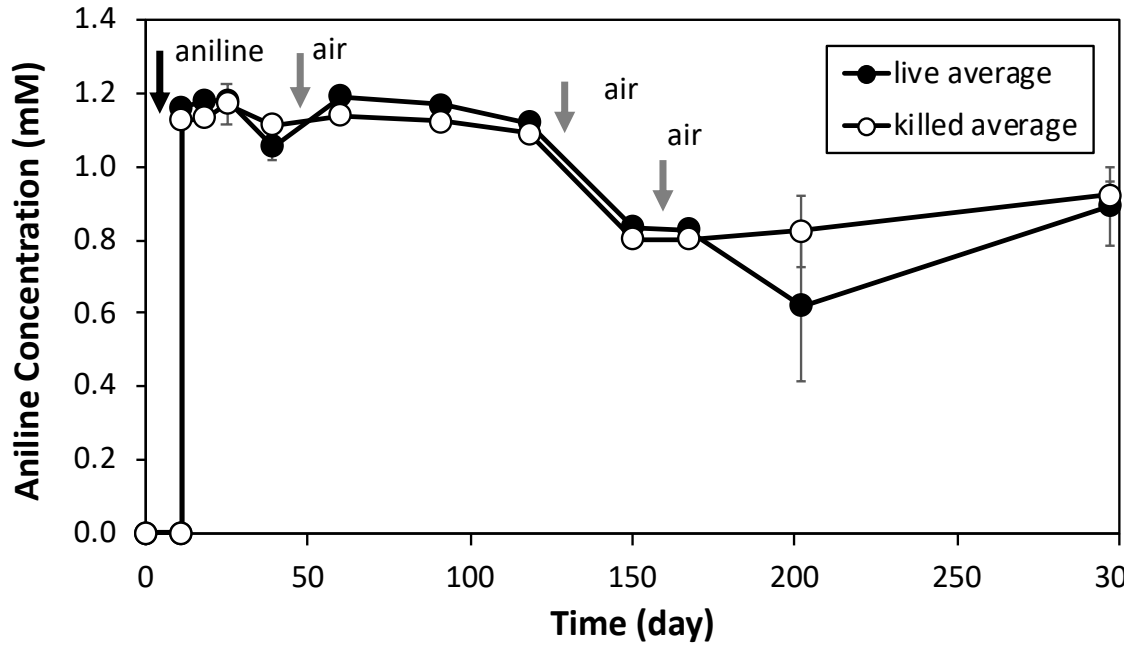


Figure 4.5 Aniline over time in targeted microcosms JZM3 established under aerobic conditions. ↓ indicated aniline addition and ↓ indicates air addition. Symbols are averages for triplicate bottles and error bars are ± one standard deviation.

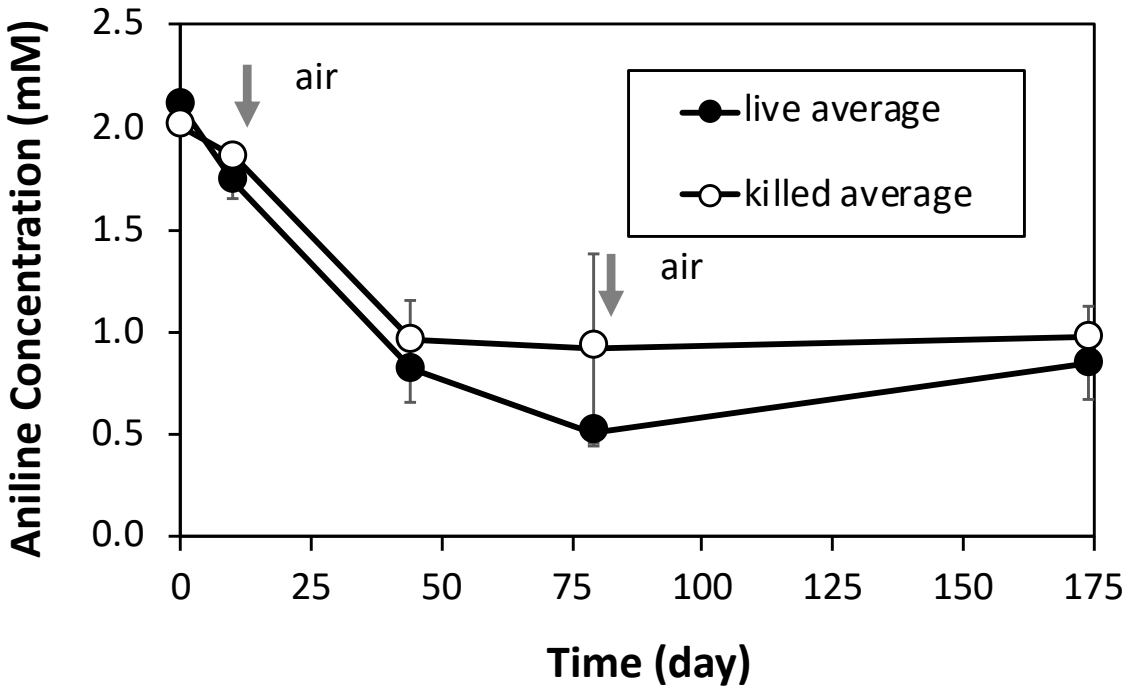


Figure 4.6 Aniline over time in targeted microcosms JZM4 (elevation -34.3 to -34.5 ft) established under aerobic conditions. ↓ indicates air addition. Symbols are averages for triplicate bottles and error bars are ± one standard deviation.

4.3.3 Composited microcosms

Because the targeted microcosms used specifically selected 2-inch slices and a corresponding redox condition, we believed they more closely mirrored the *in situ* condition (Cui et al., 2017; Ding et al., 2011; Konopka, 1993; Travkin et al., 2002). However, these treatments were not active. We therefore established composited microcosms from a larger depth interval, which should reflect a broader environment encompassing a more diverse set of conditions. Results for each set of microcosms for

both aniline and PCA are shown in Figures 4.7, 4.8, 4.10 and 4.11; while results for nitrate and sulfate concentrations are shown in Figure 4.9.

Aerobic microcosms were active, and aniline was depleted (Figure 4.7A). Depletion continued as bottles were reamended several times. Aerobically, aniline was depleted by day 69 initially, then again as aniline was reamended three times. We observed that the aniline concentration was depleted faster after the last two amendments, likely because the microbial community became adapted to use aniline and more aniline-degrading biomass grew with time due to growth and reproduction by using aniline as a carbon or carbon and nitrogen sources. Interestingly, the sediment mixture was selected from -27 to -29 ft elevations and this area had very low or no concentrations of aniline and PCA (Figure 4.2). The lack of its presence indicates that there is likely an active microbial community *in situ* at this location which led to degradation of both compounds in this area, perhaps aerobically. PCA concentrations decreased in the aerobic microcosms but were only depleted relative to the abiotic controls by day 470 of incubation (Figure 4.10A). It took longer for PCA to be depleted and that could be because PCA is more hydrophobic than aniline and could adsorb to the solid materials, and perhaps was less bioavailable to the microbes (Lide, 1991), or perhaps there were only very low numbers of organisms capable of degrading PCA present initially. Biodegradation of aniline was previously found to be faster than PCA (Zeyer and Kearney, 1982).

The aniline concentrations were not monitored as often in the microcosms established under sulfate-reducing conditions (Figure 4.7B). At day 75 the loss in live microcosms was very similar to that of the killed anoxic control. Aniline was depleted at day 316, but upon re-amendment, loss was slower than that observed in the aerobic microcosms (Figure 4.7).

This aquifer location has a substantial concentration of sulfate, which could support growth of sulfate-reducers in the area. PCA concentrations were reduced more slowly under aerobic and also under sulfate-reducing conditions, than aniline was (Figure 4.10). Limited studies have been conducted using enrichment techniques under anoxic conditions to investigate aniline biodegradation. A strain was isolated from marine sediments enriched with aniline under sulfate-reducing conditions. The organism degraded aniline completely to CO_2 and NH_3 at the same time sulfate was reduced to sulfide (Schnell et al., 1989).

There was variation in the aniline and PCA concentration in nitrate-reducing condition microcosms, especially in the first and third time points for aniline (Figure 4.8A) and PCA (Figure 4.11A). We cannot consider these signs as biological activity, since there was no substantial difference compared to the killed controls at the end of the monitoring time. In previous studies, some reported cultures that were not active under nitrate-reducing conditions (O'Neill et al., 2000), others got active cultures (Alexandra De et al., 1994). Others reported activity under both aerobic and nitrate-reducing conditions (Kahng et al., 2000). In addition, enrichment cultures that could degrade both aniline and CANs under nitrate reducing conditions have been reported (Travkin et al., 2002).

Aniline loss was observed under iron-reducing conditions (Figure 4.8B). Depletion of aniline was noted at day 75, and after re-amendment at day 200, slow, but continual loss was observed, which did not occur in the killed control. For PCA there was no significant difference between killed controls and the live treatment microcosms (Figure 4.11B). Hudson River sediments were enriched previously under iron- reducing conditions and the results showed there was biodegradation of aniline but not chloroaniline (Kazumi et al., 1995), similar to our observation. The most recent study conducted in our laboratory at a

different location within this same chemical manufacturing site showed that enrichments were active under nitrate- and sulfate-amended conditions, and methanogenic conditions. This indicates differential presence of biodegradative capacity, depending on the site locations and sampling area (Sun et al., 2015).

In this study, aerobic, iron-reducing, and sulfate-reducing conditions were the most active redox conditions for the biodegradation of aniline and PCA, when we used mixed sediments from the -27 to -29 ft elevation in the subsurface (Figures 4.7 and 4.8). It is interesting that no substantial loss of the electron acceptors sulfate and nitrate was observed for these sets of microcosms (Figure 4.9). There was an excess of sulfate and nitrate maintained in the microcosms over the incubation period, and thus lack of biodegradation of aniline or PCA was not due to limitation of the electron acceptor.

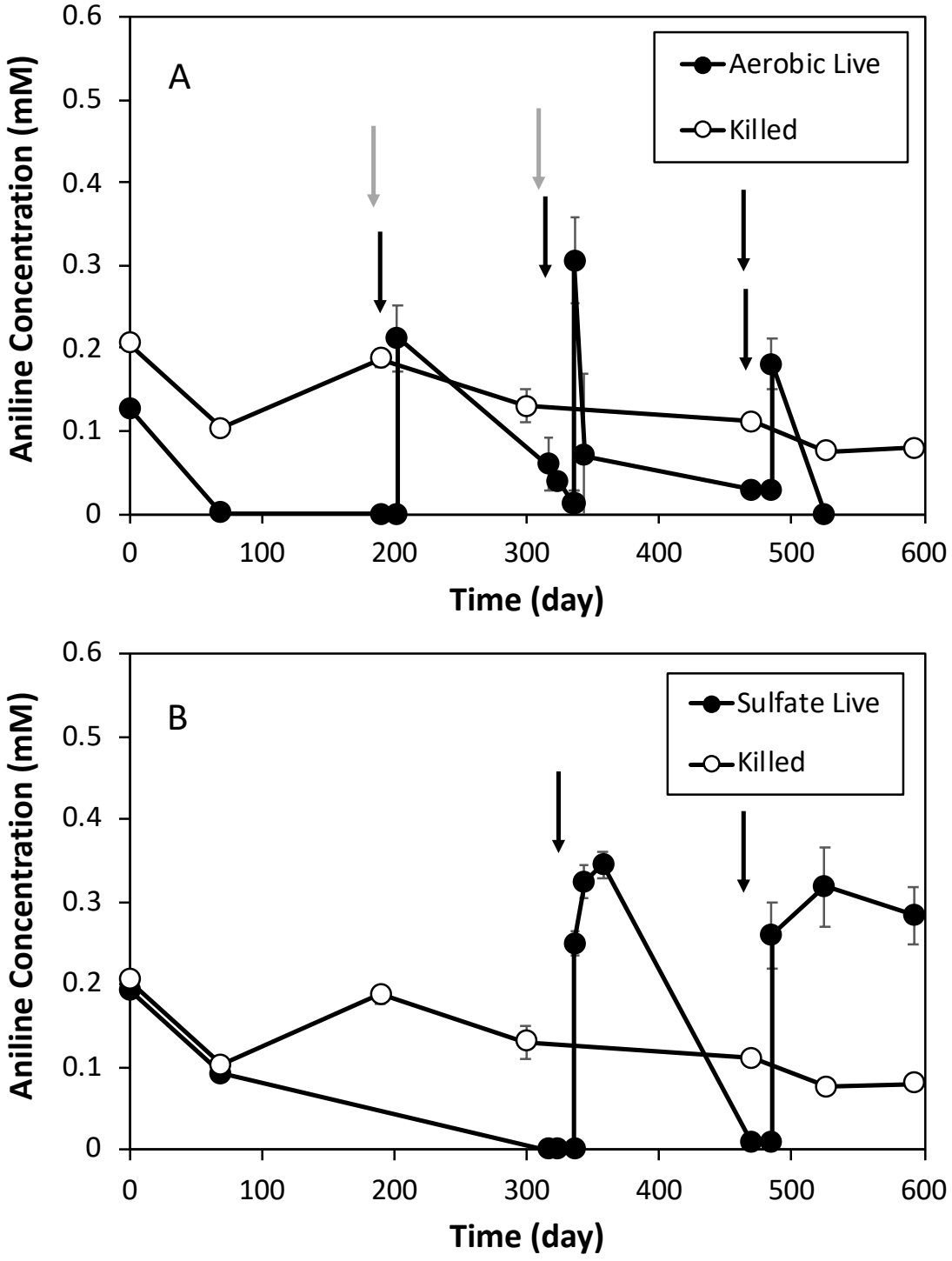


Figure 4.7 Results from composited microcosm study using cores from -27 to -29 ft elevation (composited slices D14-SCS-11-8 to 11-11; D14-SCS-12-3 to 12-9). Aniline loss in aerobic (A), sulfate amended, (B). ↓ indicates aniline re-amendment and ↓ indicates air addition. Symbols are averages for triplicate bottles and error bars are ± one standard deviation.

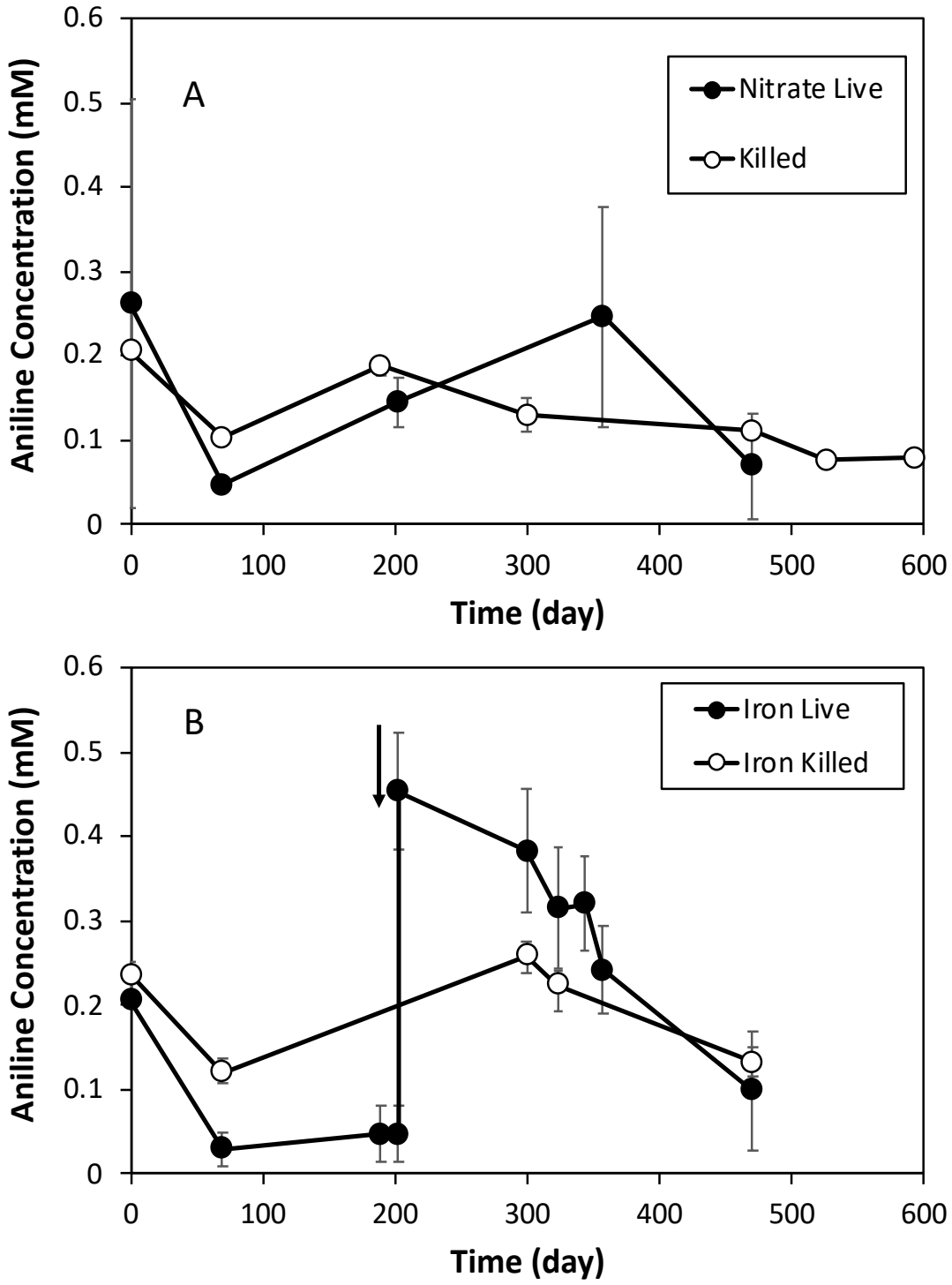


Figure 4.8 Results from composited microcosm study using cores from -27 to -29 ft elevation (composited slices D14-SCS-11-8 to 11-11; D14-SCS-12-3 to 12-9). Aniline loss in nitrate (A), iron amended, (B). ↓ indicates aniline re-amendment. Symbols are averages for triplicate bottles and error bars are ± one standard deviation.

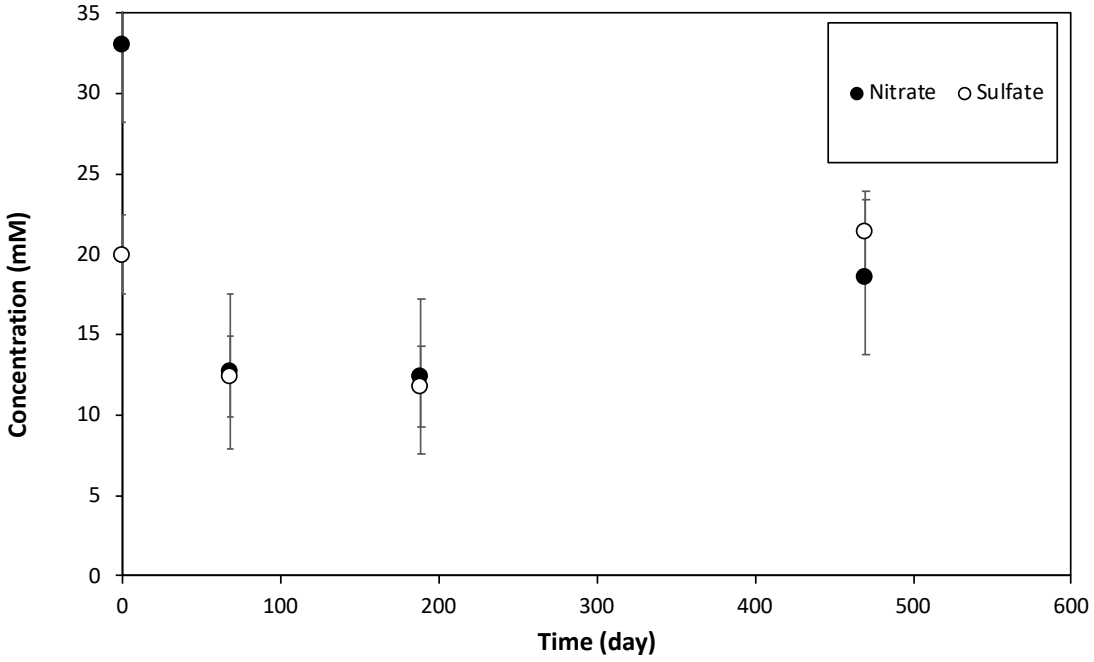


Figure 4.9 Concentration of sulfate and nitrate in the sulfate and nitrate sets of microcosms. Symbols are averages for triplicate bottles and error bars are ± one standard deviation.

In case of PCA there was some loss of PCA concentration in the aerobic and sulfate microcosms in comparison to the killed controls but, there was no loss in case of the iron and nitrate reducing conditions (Figures 4.10, 4.11).

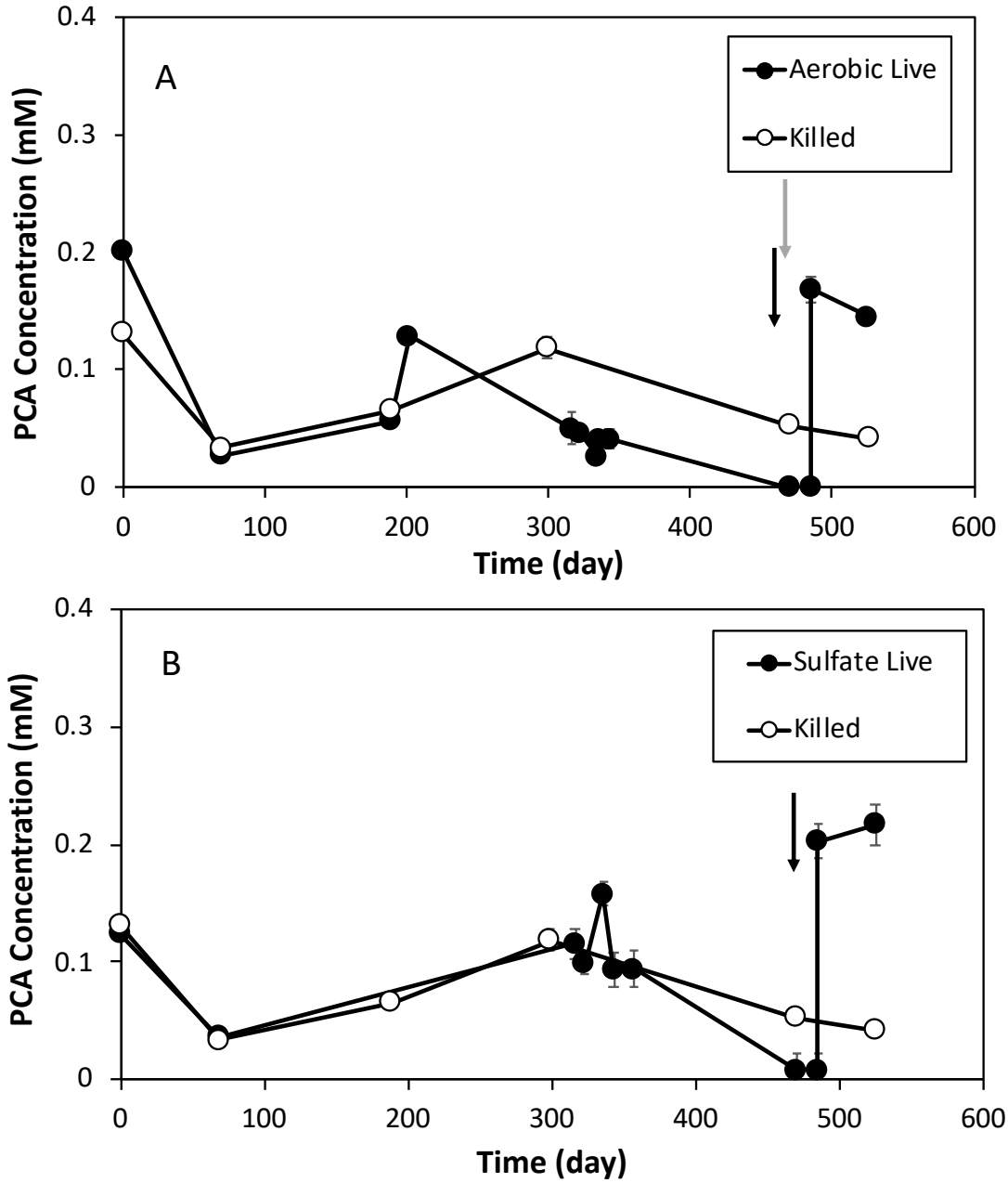


Figure 4.10 PCA over time in composited microcosms (elevation -27 to -29 ft) operated under elevation -27 to -29 ft. Aerobic (A); Sulfate-reducing (B. Killed controls included autoclaved microcosms in anoxic medium. ↓ indicates PCA re-amendment and ↓ indicates air addition. Symbols are averages for triplicate bottles and error bars are ± one standard deviation.

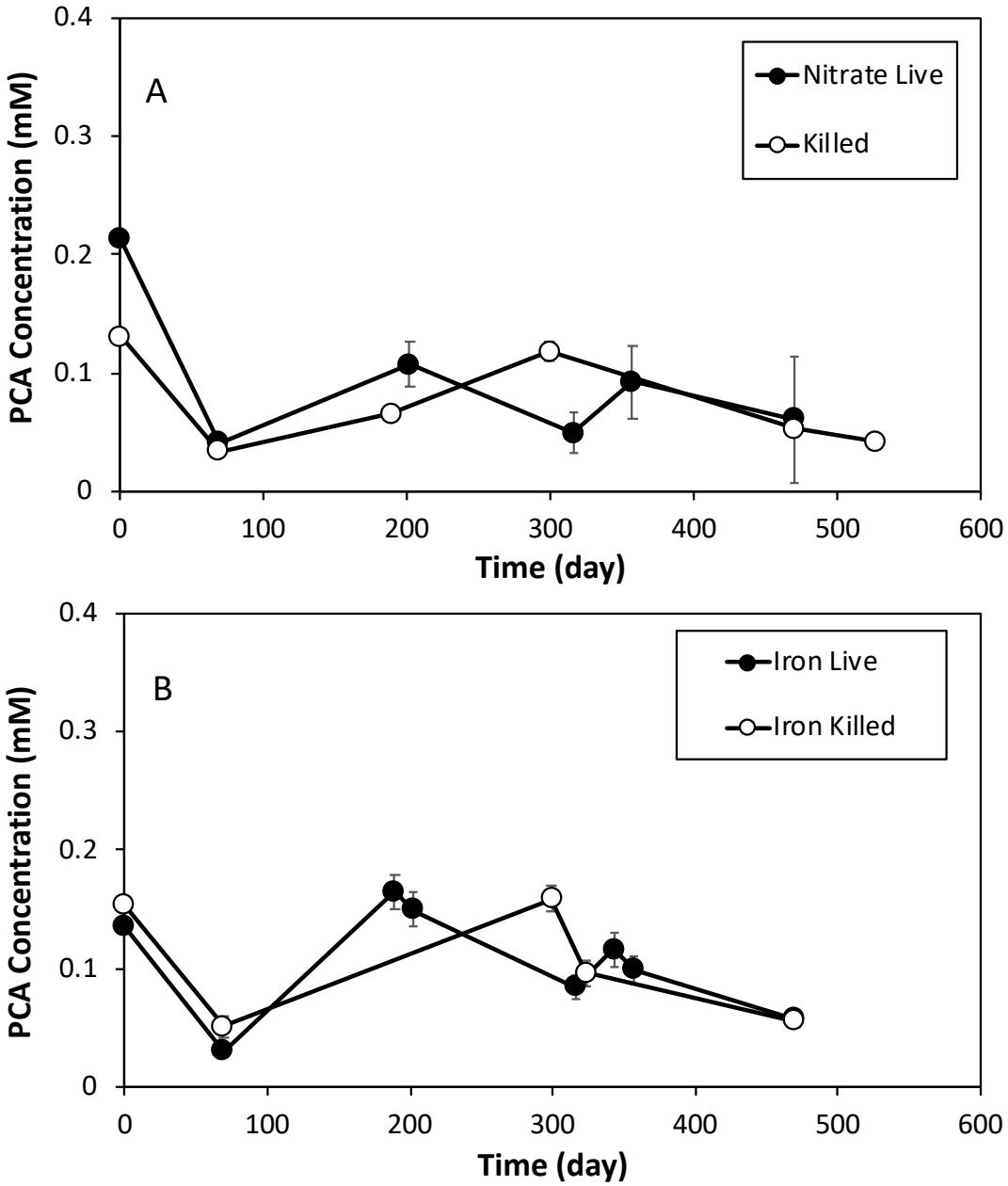


Figure 4.11 PCA over time in composited microcosms (elevation -27 to -29 ft) operated under elevation -27 to -29 ft. Nitrate (A); iron-reducing (B); iron-reducing. Killed controls included autoclaved microcosms in anoxic medium and in iron-reducing medium. Symbols are averages for triplicate bottles and error bars are ± one standard deviation.

4.3.4 Bacterial community analysis

The composited aerobic microcosms were the most active among redox conditions. Next generation sequencing of 16S rRNA genes was performed for the original mixed sediments (frozen at the time of mixing) and the active aerobic microcosm sediment slurry recovered from duplicate bottles. A change in the microbial community was expected after several amendments of aniline and some PCA had been depleted. The results were analyzed to examine the changes at the genus level (Figure 4.12). The results showed that there was a substantial change in the bacterial community between the original sediment community and the aerobic microcosms. In general, the bacterial community changes on genus level could be divided into three groups. Some genera were detected in the original sediments, but not detected in the aerobic enrichments (group 1). Some genera were not detected in the original sediments but were detected in the aerobic enrichments (group 2). While genera in group 3 were detected in both, although in differing percentages. During the biodegradation process the percentage of the first group, detected in original sediments, but not in the microcosms, included *Mezorihzobium, Streptococcus* and *Escherichia.* Thus, most likely these organisms have no role in aniline and PCA biodegradation, or other conditions of the microcosm study (e.g. the concentrations of the compounds) may have inhibited their growth. The second group, which is the largest group, increased in presence and were detected at high percentages in some cases. This included the genera *Knoellia* and *Brevundimonas.* These genera could be those mainly responsible for aniline and PCA biodegradation. The percent of the third group, including *Stenotrophomonas,* decreased in prevalence during biodegradation, and we believe these may have a lesser role in the biodegradation processes. The large shift in the bacterial community was the change of the

dominant genera. In the original sediments, the dominant genera were *Pseudomonas* and *Stenotrophomonas* and they were more than 55% of the entire community, while in the aerobic enrichments *Knoellia*, *Brevundimonas,* or *Pseudomonas* were dominant in aerobic 2 and aerobic 3 microcosm, respectively. These genera formed almost 60% of the OTUs detected in the community. *Pseudomonas* behaved differently, it was much less prevalent in aerobic 2 microcosm, but was still present at a high percentage in aerobic 3 microcosm, in comparison to the original sediments. This shift is important because it helps establish the genera that may have an essential role in the aerobic degradation processes at this site (Figure 4.12). The literature is very limited on microbial community associated with *in situ* aniline degradation, but in one previous study reported that the natural biofilm was changed during aniline degradation (Araya et al., 2003). Recent study indicates there is also a big shift in the microbial community of and several new genera appeared after aniline biodegradation (Hou et al., 2018).

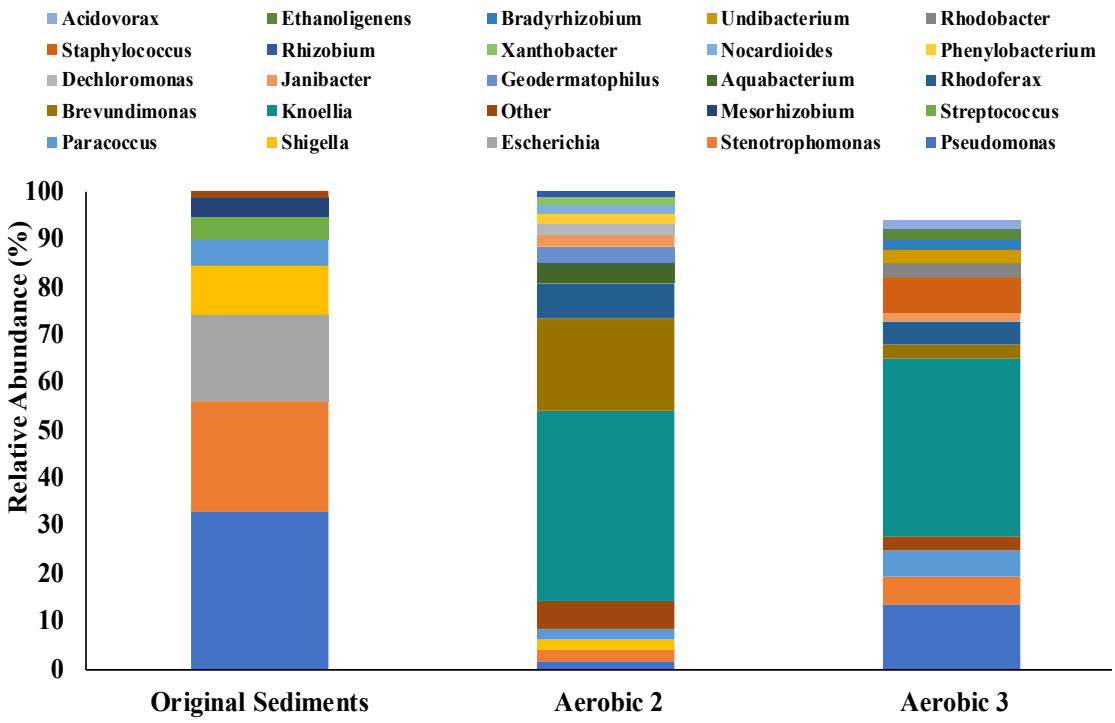


Figure 4.12 Bacterial Community analysis sequencing of 16S rRNA genes, presented at the genus level for the original mixed sediments and the active aerobic duplicate microcosms enriched on aniline and PCA.

4.4 Conclusions

A site evaluation using different microcosm sets to test biodegradation of aniline and PCA using aquifer sediment core slices established from different depths was performed. Targeted microcosms were not active in terms of aniline and PCA biodegradation, while composited microcosms showed activity. In composited microcosms, aniline was depleted several times, especially in the aerobic set of microcosms. Slower loss of aniline was noted under sulfate-amended and iron (III)-amended conditions. No loss was noticed in the nitrate-amended sets. Bacterial community analysis showed a shift between the original sediments and the aerobic microcosms.

Chapter 5

Identification of Aniline-Degrading Bacteria at an Industrial Site by Isolation and Stable Isotope Probing (SIP)

Abstract

Aniline degradation occurred under aerobic conditions only at specific depths in the heterogenous subsurface of a chemical manufacturing site. Here we characterized active aniline-degrading microbes at this depth by direct isolation and by stable isotope probing (SIP) of active microcosms. Sediment slurry from the active aerobic microcosms representing aquifer material from -27 to -29 ft elevation were plated on agar media containing aniline as the sole carbon source. A pure isolate designated *Rhodococcus* HA1, was obtained. The strain is closely related to *Rhodococcus erythropolis* (98%). HA1used aniline as a carbon and nitrogen source, but it did not degrade a closely related contaminant of concern, parachloroaniline (PCA). Active microcosms were also used to form enrichments that were amended with ^{12}C or ^{13}C labelled aniline for SIP. DNA was recovered after 96 % of the aniline was degraded and processed by fractionation. SIP fractions were subject to qPCR of 16S rRNA gene and light and heavy fractions containing the highest gene copy numbers were identified. Bacterial community analysis of 16S rRNA of the SIP fractions showed several genera are responsible for aniline degradation dominated by *Knoellia* sp. in addition to *Aquabacterium* sp. and *Brevundimonas* sp. *Rhodococcus* was also detected in the community analysis, although it was not dominant. The pure isolate, HA1, was mixed with another aniline degrader Janibacter sp. PR1, an aniline degrader isolated from a different site, to develop a bioaugmentation culture. After

bioaugmentation, non-active microcosms at other depths in the aquifer became active and aniline was depleted within two weeks.

These findings are essential for understanding biodegradation at the site and could be considered for guidance of future bioremediation processes.

5.1 Introduction

Aniline is a chemical compound used widely in industry. It is a precursor in manufacturing of dyes, pesticides, and pharmaceutical compounds, and because of its heavy use, it has emerged as a pollutant in different environments (Travis, 1997). Due to the toxicity of aniline to aquatic, terrestrial and human life, it is considered a contaminant of concern (Fan et al., 2011; Khan et al., 2003; Messeguer, 2011). Aniline is a potential human carcinogen, a mutagen and a teratogen (Greenwood et al., 1979; Wang et al., 2016). Amines in general, including aniline, enter the environment mainly from industrial wastewater and could also be released in minor quantities from natural sources (Poste et al., 2014). Several chemical and physical methods have been used in aniline treatment, but they are either not effective or are very expensive (Gómez et al., 2009; Sanchez et al., 2002; Zhu et al., 2019). Biological approaches are promising techniques to degrade aniline in a more sustainable and environmentally friendly way.

Pseudomonas putida has been characterized for aniline degradation in detail with the functional genes and degradation pathway identified (Anson and Mackinnon, 1984; Fukumori and Saint, 1997; Konopka, 1993; Nitisakulkan et al., 2014; Vangnai and Petchkroh, 2007). Several *Delftia* sp. were reported that could degrade aniline and closely related compounds, the chlorinated anilines (CANs). For example, strain AN3 could degrade aniline in high concentrations—it could use aniline at a concentration at of ≥ 53

mM of aniline (Liu et al., 2002). *Delftia tsuruhatensis* strain H1 could degrade 2-,3-, and 4-chloroaniline in mixtures, or separately (Zhang et al., 2010). *Delftia* sp. XYJ6 could degrade aniline when added at 2000 mg/L (21 mM) and a degradation pathway was proposed (Chengbin et al., 2009; Liu et al., 2002; Zhang et al., 2010). *Delftia acidovorans* strain HY99 was reported to degrade aniline both aerobically and anaerobically, using nitrate as the electron acceptor (Kahng et al., 2000). Furthermore, aniline biodegradation by *Delftia tsuruhatensis* strain 14S was studied not only in batch culture, but also as a continuous process for aniline removal (Sheludchenko et al., 2005). *Rhodococcus, Staphylococcus, Acinetobacter, and Rhizobium* sp. and many more were reported as aniline degraders (Ahmed et al., 2010; Aoki et al., 1983; Hongsawat and Vangnai, 2011; Zhang et al., 2011a). Pure cultures have been found to utilize aniline as a carbon and energy source, but also as a nitrogen source (Chengbin et al., 2009; Sheludchenko et al., 2005; Zhang et al., 2010).

Bacterial mixed communities were used in aniline degradation in industrial wastewater treatment plants. Aerobic mixed culture granules were used and showed good degradation potential in a stable sequencing airlift bioreactor. In that system the degradation rate of aniline was higher than that for CANs (Zhu et al., 2012). A mixed culture was developed and showed higher degradation rate of aniline than several pure cultures and mixed culture could tolerate higher aniline concentrations (Cui et al., 2017). Rapid aniline biodegradation was observed when aerobic granular sludge from wastewater treatment plants was used. The activated sludge formed using aniline as a carbon source could degrade aniline at a rate of 17.8 mg/L-hr under optimal conditions. (Jiang et al., 2017; Wang et al., 2011).

Bioaugmentation is a bioremediation approach used in the case of absence of active microbes in a contaminated environment by adding active pre-adapted microbes in the form of pure cultures or consortia (Tyagi et al., 2011). Environmental conditions and the nature of the pollutants should be considered before applying this type of remediation technology (Tyagi et al., 2011). Bioaugmentation was effective in treating a PCA-contaminated soil, where the bioaugmented organisms became the dominant microbial community over time after amendment to the soil (Tongarun et al., 2008).

Several known microbes could degrade aniline and can be cultivated in the laboratory successfully. This limits our knowledge of pollutant degradation since it is limited by the specific cultivated microbes. Active microbes could be unculturable, especially since it is generally known that most individual species in environmental bacterial communities are unculturable (Head et al., 1998; Hugenholtz et al., 1998). Stable isotope probing (SIP) is a molecular technique that links the microbial identity and function in the environment without enrichment or isolation. Microbes responsible for degradation of a specific substrate could be identified if they incorporate an element from an isotopically labeled version of the targeted substrate into newly produced cells. ^{13}C- and ^{15}N-labeled substances have been used for this purpose (Neufeld et al., 2007; Radajewski et al., 2000). Environmental samples or microcosms need to be incubated with the labeled substrate, for example an organic compound made with ^{13}C. During biodegradation studies the active microbes degrading the organic compound incorporate ^{13}C in newly synthesized DNA. Thus, in addition to the ^{12}C DNA normally found in the environment, active organisms will have produced ^{13}C DNA. The two types of DNA could be separated by density gradient ultracentrifugation and collected for analysis using different approaches (Figure 5.1)

(Friedrich, 2006). In this study we used SIP combined with fractionation, qPCR, and next generation sequencing to identify active microbes.

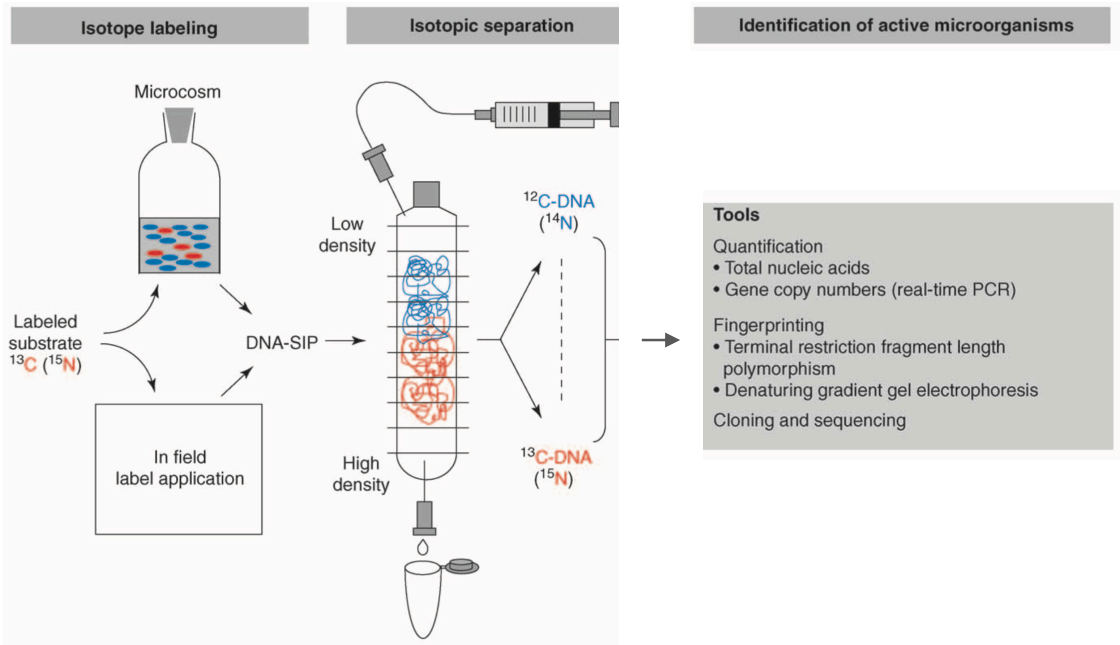


Figure 5.1 Diagram illustrating the Stable Isotope Probing (SIP) steps (Friedrich, 2006) with modification.

In addition to SIP applications using DNA, it could be done for RNA as well (Lueders et al., 2004; Manefield et al., 2002). The SIP approach not only provides useful information about the active microbes, but functional genes could also be targeted and analyzed (Friedrich, 2006).

The first reports of use of SIP was applied to degradation and uptake of one carbon molecules such as methane, methanol, and CO_2 (McDonald et al., 2005). For example, it has been used to analyze CH_4 production contributions to the atmosphere and showed that

anoxic microorganisms in rice soils contribute 10-25% of methane production globally (Lu and Conrad, 2005). Active nitrogen fixing bacteria were identified using ^{15}N labeled nitrogen and detection of *nifH* gene (Buckley et al., 2007). The technique was also used to identify active methanotrophs in soil (Morris et al., 2002), and acetate and methanol assimilating bacteria under nitrate-reducing conditions (Osaka et al., 2006).

In biodegradation, SIP has been used in many studies to detect active degraders of organic aliphatic or aromatic pollutants. For example, SIP was used to detect the benzoate-utilizing community under denitrifying conditions (Gallagher et al., 2005). Toluene degraders were also identified using SIP under sulfate- and nitrate-reducing conditions, and several genera including *Thauera* and *Desulfosporosinus* were found to be responsible for toluene degradation (Sun and Cupples, 2012). Other aromatic hydrocarbon-degraders were identified using SIP with labeled compounds such as anthracene (Zhang et al., 2011b) and salicylate, naphthalene, and phenanthrene (Friedrich, 2006). Very limited studies are available related to aniline degradation in general, and one of them was done in our laboratory. Our group coupled with SIP under anaerobic conditions to detect active aniline-degraders in a contaminated site. Phylotypes closely related to *Ignavibacterium album* and *Acidovorax spp*. were found to be the dominant aniline-degraders under methanogenic and sulfate-reducing conditions (Sun et al., 2015). Using SIP helps identify active bacteria in a specific environment and could help provide information about the optimal conditions to encourage *in situ* biodegradation processes.

This chapter describes work to identify and characterize aniline degrading bacteria at a contaminated industrial site. A pure isolate which degrades aniline was obtained and tested for biodegradation of aniline and PCA. We also used bioaugmentation to stimulate activity

in inactive microcosms through bioaugmentation. Active aniline degraders were also identified using DNA-SIP.

5.2 Materials and methods

5.2.1 Chemicals

Aniline (99%) and 4-chloroaniline (95%) were purchased from Sigma-Aldrich (St. Louis, MO).

5.2.2 Microcosms

Microcosms were established using aquifer core material and groundwater or minimal medium recovered from a large chemical manufacturing facility located in southern New Jersey. The recovery and characterization of site material and operation of microcosms used in this study is described in detail in Chapter 4 and shown in Tables 5.1 and 5.2. Specific slices of recovered aquifer core from several depths were selected for targeted microcosms (using ~6 in of core) (5.1) and another set of slices (~1 – 2 ft) were mixed and used to establish composited microcosms (Table 5.2).

Table 5.1 Targeted microcosms established under different redox conditions.

Microcosm Set	Condition	Core Elev. (ft)	Core Slices	Groundwater Elev. (ft)	Amendment (**μm**)	Aniline/PCA Amendment (**μm**)
JZM1	Methanogenic-Organohalide Reducing	-17.5 to -17.8	7-1, 7-2, 7-3	-24.79	Lactate 1000	250, PCA 250, Aniline
JZM2	Sulfate-Reducing	-20.3 to -20.7	8-7, 8-8, 8-9	-24.79	Sulfate 20,000	250, Aniline
JZM3	Aerobic	-9.8 to -10.2	3-5, 3-6, 3-7	-5.79	Sterile Air	250, Aniline
JZM4	Aerobic	-34.3 to -34.5	14B10,14B11	-36.29	Sterile Air	250, Aniline

Table 5.2 Composited microcosm study using elevation -27 to -29 ft composited slices.

Microcosm Set No.	Redox Condition	Live/Killed	Electron Donor	Electron Acceptor	Prepared In
9	Iron-Reducing	killed	aniline, PCA	Fe (III)	Medium
10	Anoxic	killed	aniline, PCA	none	Medium
11	Iron-Reducing	live	aniline, PCA	Fe (III)	Medium
12	Aerobic	live	aniline, PCA	oxygen	Medium
13	Nitrate-Reducing	live	aniline, PCA	nitrate	Medium
14	Sulfate-Reducing	live	aniline, PCA	sulfate	Medium

5.2.3 Aerobic medium

Two types of aerobic media were prepared: carbon free minimal medium (CFMM) and carbon and nitrogen free minimal medium (CNFMM). Aerobic minimal medium was prepared as described previously (Fukuda et al., 2002) with modifications. To prevent precipitation, four solutions were prepared in 250 mL of milli-Q water: solution 1 (6.4 g $Na_2HPO_4.7H_2O$ and 1.5 g KH_2PO_4); solution 2 (0.3 g NaCl and 0.5 g of NH_4Cl); solution 3 (0.5 g $(NH_4)_2SO_4$ and 0.5 g $MgSO_4•7H_2O$,); and solution 4 (0.02 g $CaCl_2$ and 0.0018 g $FeSO_4•7H_2O$). The pH of each solution was adjusted to 7. Medium solutions were autoclaved for 15 min at 121°C then cooled to 50°C before mixing under sterile conditions. Medium was stored at 4°C until use. The final medium contained (per L): 6.4 g $Na_2HPO_4•7H_2O$, 1.5 g KH_2PO_4, 0.3 g NaCl, 0.5 g NH_4Cl, 0.5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4•7H_2O$, 0.02 g $CaCl_2$, and 0.0018 g $FeSO_4•7H_2O$). In the case of CNFMM, NH_4Cl and $(NH_4)_2SO_4$ were excluded from solutions 2 and 3, respectively.

5.2.4 Dilution medium

The following components were added to 1 L of milli-Q water: 1.3 g KCl, 0.2 g KH_2PO_4, 11.5 g NaCl, 0.5 g NH_4Cl, 0.1 g $CaCl_2•2H_2O$, 3 g $MgCl_2•6H_2O$ and 2.5 g $NaHCO_3$. One ml of 1 g/L resazurin, 5 mL of vitamin solution (d-biotin, 0.02 g; folic acid, 0.02 g; pyridoxine hydrochloride, 0.1 g; thiamine hydrochloride, 0.05 g; riboflavin, 0.05 g; nicotinic acid, 0.05 g; DL-calcium pantothenate, 0.05 g; vitamin B12, 0.01 g, p-aminobenzoic acid, 0.05 g; lipoic acid (thiotic acid), 0.05 g; 1,4-naphthaquinone, 0.04 g; nicotinamide, 0.1 g; and hemin, 0.01 g per 1 L), 1 mL of trace salt I (containing per L: $MnCl_2•6H_2O$, 5 g; H_3BO_3, 0.5 g; $ZnCl_2$, 0.5 g; $CoCl_2•6H_2O$, 0.5 g; $NiCl_2•6H_2O$, 0.46 g;

$CuCl_2$•$2H_2O$, 0.3 g; $NaMoO_4$•$2H_2O$, 0.1 g; and $FeCl_2$•$4H_2O$, 1.49 g) and 0.1 mL of trace salt II solution (containing per L: 0.03 g of $NaSeO_3$ and 0.08 g of Na_2WO_4) were added. Medium was autoclaved then purged with sterile 70%N_2:30%CO_2 for 20 min. The pH was adjusted to neutrality (Li, 2014).

5.2.5 Isolation, purification and identification

Aerobic carbon free minimal media agar (CFMM) was prepared using 15 g/L noble agar. Aniline or PCA stock solution was added to achieve 1 mM as a final concentration as the only carbon source was added to the medium after cooling and before solidification. For isolation of single colonies, 100 µl of diluted (10^{-1}-10^{-6}) culture was prepared from active composited aerobic microcosms (Set 12, Table 5.2) and spread on agar plates. One plate was left without aniline and another one left without inoculum, as controls. Plates were incubated at 28°C until colonies developed after seven to nine days. Finally, different single colonies were streaked onto fresh LB agar plates to ensure pure colonies. The pure strain was stored at -80°C in 30% glycerol as described previously (Huang et al., 2018; Kahng et al., 2000).

The pure strain was plated on Luria-Bertani (LB) plate from -80°C stocks and incubated at 28°C. Cells were collected, and genomic DNA extraction was done using the DNeasy UltraClean Microbial Kit (QIAGEN, Germany). A 1500 bp fragment of the 16S rRNA gene was amplified using 27F (AGAGTTTGATCMTGGCTCAG) and 1525R (AAGGAGGTGATCCARCCGCA) PCR primers with the following PCR protocol: denature at 95°C for 30 sec, anneal at 55°C for 30 sec, extend at 72°C for 1.5 min, (30 cycles) and final extension 72°C for 3 min. Amplicons were purified using ExoSapi (Affymetrix Santa Clara, CA) and sent for sequencing to GENEWIZ South Plainfield, NJ.

5.2.6 Biodegradation of aniline and para-chloroaniline

Triplicate cultures and abiotic controls were established by adding 100 mL of aerobic CFMM and CNFMM to 160 mL sterile serum bottles under sterile conditions. Aniline and PCA stock solutions were added to the bottles to achieve 0.5 mM as a final concentration for each compound. One mL of cell suspension (optical density at 600 nm (OD_{600}) = 1) of each isolate was added to all bottles except the abiotic controls. Bottles were incubated at 28°C with 160 rpm shaking. Bioaugmentation tests were also performed by adding pure cultures (OD=1) to duplicate inactive JZM3 microcosms (non-active aerobic microcosms -9.8 to -10.2 ft elevation, Table 5.1). Bottles were sampled (1 mL) for aniline and PCA at each time point.

5.2.7 Sample preparation and high-performance liquid chromatography (HPLC) analysis of aniline and PCA

Bottles were mixed vigorously then 1 mL of culture was sampled using a 1 mL sterile syringe with 26 G needle that had been flushed with filter-sterilized UHP N_2. Samples were either extracted directly or frozen at -20°C prior to analysis by HPLC, using a modification of previously described methods (Kahng et al., 2000; Liu et al., 2015; Tanaka et al., 2009). Briefly, samples were placed in an Eppendorf tube, 1 mL of acetonitrile was added, the sample was vortexed for 3 min, and then centrifuged at 10000 *g* for 3 min. Supernatant was removed and filtered using a 0.22 μm Spin-X centrifuge tube filter (Costar®, Salt Lake City, UT). Approximately 1.5 mL extract was placed in an HPLC vial for analysis using a Beckman Coulter (System Gold I25, Solvent Module, System Gold I68 Detector) HPLC with diode array detector operated at 244 nm. The analytical column was a 5C18 120 column (250*2 mm) (Supelco Analytical, Bellefonte, PA) held at 40°C.

The mobile phase was a milliQ water: acetonitrile mixture 30:70 vol:vol and the flow rate was 0.6 mL/min. Aniline and PCA concentrations were calculated from peak areas using a five point standard curves prepared as described previously (Sun et al., 2015).

5.2.8 Stable isotope probing

5.2.8.1 Incubation with ^{13}C Aniline

Duplicates aerobic active microcosms were diluted (30/70% slurry/medium) using the dilution minimal medium to produce a larger enrichment volume for the SIP experiment. Duplicate sterile abiotic controls were established that contained medium only. Aniline was amended and monitored frequently for 118 days, at day 119 aniline was re-amended and the concentration was monitored daily for nine days. Thereafter, triplicate enrichments were created from a mixture of these diluted microcosms and incubated with either ^{12}C (regular) or ^{13}C aniline. To 75 mL serum bottles, 20 mL of the mixed aerobic diluted microcosms was added along with 20 mL of the medium, and ^{12}C- and ^{13}C-aniline were amended to achieve 0.5 mM as final concentrations. Three sets were prepared in triplicate: live enrichments with ^{12}C- and ^{13}C-aniline, and abiotic controls amended with ^{12}C-aniline. All sets were incubated in the dark at room temperature. The aniline concentration was monitored in all sets using HPLC (Sun et al., 2015).

5.2.8.2 DNA extraction and ultracentrifugation

After nine days of incubation, 10 mL of sediment slurry was withdrawn from each bottle of the ^{12}C- and ^{13}C-aniline amended sets and DNA was extracted using the Power Soil DNA extraction kit (MO BIO laboratories Inc., CA, USA) following the manufacture's

protocol. DNA from both sets was separated on a CsCl gradient as descried previously (Sun and Cupples, 2012).

5.2.8.3 Setting up CsCl Gradient and Ultracentrifugation

A CsCl solution in Tris-EDTA (TE) buffer, with a buoyant density of 1.7742 g/mL, as measured by an AR200 digital refractometer (Reichert Technologies, NY) 15 g of CsCl was dissolved in 10 mL TE buffer the refractive index (RI) around 1.4030. CsCl solution was place in the ultracentrifuge tubes. 500 ng of DNA was added in different volumes (21-35 µL) depend on the concentration of the replicate. Caps were placed on the centrifuge tubes and vortex to make sure it is completely mixed. RI was adjusted with water or CsCl stock solution to be 1.4020 ± 0.0005, do not continually add more volume to the tube. If needed, take some of the volume out of the ultracentrifuge tube. Losing some sample in this way, but this is needed. When the RI values adjusted each tube with the cap was weighed. The tubes need to be within 1 mg of each other, if necessary, take off some volume to adjust their weight. Tubes were span for- speed: 48,000; temperature: 28°C; Accel: 2; Decel: 9. The time was set for 72 hours, it is required to spin for 2 days (48 hours), but we want to make sure we have leeway in case of any problems.

5.2.8.4 Gradient Fractionation and DNA Precipitation

After centrifugation, samples were fractionated using a fraction recovery system (Beckman Coulter Inc., Brea, CA) and 24 fractions of 200 µL were collected and the buoyant density was measured for each fraction using an AR200 digital refractometer (Reichert Technologies, NY).

All tubing connections were flushed with water before connecting them all together. 24 microfuge tubes were labeled to collect the fractions of each replicate. The tubing part of

the apparatus was connected to a needle and water was pumped through the line 5 mL/min to clean out the line, the bottom line was flushed with some water as well with a syringe by hand. Each span tube was placed in the fractionation apparatus and its bottom was pierced, it should be fill and has no air bubbles. Water was pumped through the apparatus 0.2 mL/min to collect 200 µL each min for 24 minutes in 24 labeled tubes. RI was measured for each fraction, then 300 µL water, 5 µL glycogen, and 800 µL proof ethanol were added and the tubes were vortexed then incubated overnight in the refrigerator. Tubes were span at maximum speed at 4°C for 30 minutes, the supernatant was discarded without disturbing the pellet and the tubes centrifuge again for 10 min. The tubes were placed in the laminar flow hood and let the pellet dry for about 20 minutes (or until dry). DNA was resuspended in 20 µL water and the fraction stored at -20°C until use.

5.2.8.5 Quantitative PCR

Twelve fractions, for each replicate of ^{12}C and ^{13}C sets, were selected for *16S rRNA* gene quantification according to their buoyant density values. Selected fraction buoyant densities were between ~1.71 g/mL (light) and ~1.77 g/mL (heavy) (Buckley et al., 2007; Cai et al., 2016; Dumont et al., 2011; Sun and Cupples, 2012; Zhang et al., 2011b). Each reaction for qPCR was performed in technical triplicates using a QuantStudio3 (Applied Biosystems by Thermo Fisher Scientific, MA. US) in a 10 µL mixture containing 5 µL of PowerUp™ SYBR™ green Master Mix, (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA), 0.3 µL of 10 µM each primer, 3.4 µL of nuclease free water, and 1 µL of DNA as a template. The *16S rRNA* primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3') were used (Klindworth et al., 2013; Thijs

et al., 2017). Standards were produced using a TOPO TA Cloning Kit (Invitrogen, Life Technology Carlsbad, CA) for ligation. The amplicon was ligated into the pCR2.4 TOPO vector following the manufacture's protocol and a *16S rRNA* gene segment was inserted into TOP10 *E. coli* made competent following protocols described previously (Inoue et al., 1990). Cells containing the insert were grown on LB agar with ampicillin overnight at 37°C with 200 rpm shaking. Plasmid DNA was extracted using Zippy Plasmid Miniprep Kit (Zymo Research, Irvine, CA) following the manufacturer's protocol. qPCR was performed using the following protocol: 95°C for 5 min, 95°C for 30 sec, 46°C for 1 min, 72°C for 1 min, (35 cycles) then 72°C for 10 min (Klindworth et al., 2013), to confirm the insertion process. The standards were prepared by 10-fold dilution (10^{-1}-10^{-7}) of the plasmids containing the *16S rRNA* gene fragment to use in constructing a standard curve for each run. The R^2 values for standard curves were 0.993, 0.974, and 0.974, while the efficiency percentages were 79.808, 85.249, and 116.753 for replicates 1, 2, and 3, respectively. The following qPCR method was used: 50°C for 2 min and 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 1 min, and 72°C for 1 min. A melt curve analysis was done with the following protocol 95°C for 15 sec, 60°C for 1 min, and 95°C for 10 sec according to (Thijs et al., 2017) with modification. Fractions containing the light DNA of the ^{12}C-aniline amended set and the light and heavy DNA of the ^{13}C-aniline amended set was sent to MR DNA (Shallowater, TX) for sequencing. The primer set 515F/806R was used to amplify the V4 variable region of the *16S rRNA* gene in a single step using the HotStarTaq Plus master mix kit (Qiagen, USA) with the following PCR conditions: 94°C for 3 min, followed by 28 cycles (5 cycle used on PCR products) of 94°C for 30 sec, 53°C for 40 sec and 72°C for 1 min, and 72°C for 5 min. The Ion Torrent PGM (ThermoFisher,

Waltham, MA) platform was used for sequencing and sequences were analyzed by MR DNA using a proprietary analysis pipeline. Sequences less than 150 bp and homopolymer runs exceeding 6 bp were removed. Operational taxonomic units (OTUs) were defined by clustering at 97% similarity. Finally, NCBI and RDPII websites were used to classify the OTUs. (MR DNA, Shallowater, TX).

5.3 Results and discussion

5.3.1 Isolation of pure culture

One strain was isolated from active aerobic microcosms from the -27 to -29 ft elevation. According to 16S rRNA gene sequencing over 1500 bp, it is closely related to *Rhodococcus erythropolis* (98%). The strain was designated *Rhodococcus* HA1 (Figure 5.2).

The ability of the strain to degrade aniline and PCA was tested. Two types of minimal media were used, without carbon source (CFMM) and without nitrogen and carbon sources (CNFMM). The results showed that *Rhodococcus* HA1 could use aniline as both a carbon and nitrogen source with aniline almost completely depleted in 72 hrs, while PCA was not degraded in either medium type (Figures 5.3 and 5.4). Hydrophobicity and toxicity of PCA is higher than aniline so, the pure isolate could be sensitive to PCA, even in low concentration. On the other hand, PCA might be not be available for the isolate because it adsorbed to the glass walls of the microcosms (Lide, 1991) or because the strain does not have the required genes responsible for PCA degradation. It has been reported previously that aniline was degraded, but not PCA under same experimental conditions (Kazumi et al., 1995), which is similar to what we found in this study.

Eight strains of *R. erythropolis* have been reported (Aoki et al., 1983). *Rhodococcus* HA1 could use aniline as carbon and nitrogen sources and that is similar to another *R. erythropolis* strain, AN13, which could degrade catechol and also aniline through the catechol pathway (Aoki et al., 1983). The functional gene, catechol 1,2 dioxygenase was isolated and purified from the same strain (Aoki et al., 1984a). Aniline degradation by *Rhodococcus* HA1 is faster than the degradation by *R. erythropolis* AN13, requiring 72 and 96 hrs, respectively, to consume aniline completely, although with different initial concentrations. The OD_{600} increased during the degradation process and reached the highest values in 140 hrs for both media (Figure 5.4). Several strains belonging to the *Rhodococcus* genus have been reported as aniline degraders (Kaminski et al., 1983). Many pure strains have been isolated in previous studies, some of them could degrade aniline 10-fold faster than *R. erythropolis* AN13 (Aoki et al., 1984b), while others could degrade both aniline and PCA, as in *Pseudomonas sp.* (Zeyer and Kearney, 1982). Some pure strains were isolated as CANs degraders and they could also degrade several types of CANs (Hongsawat and Vangnai, 2011; Loidl et al., 1990; Zhang et al., 2010).

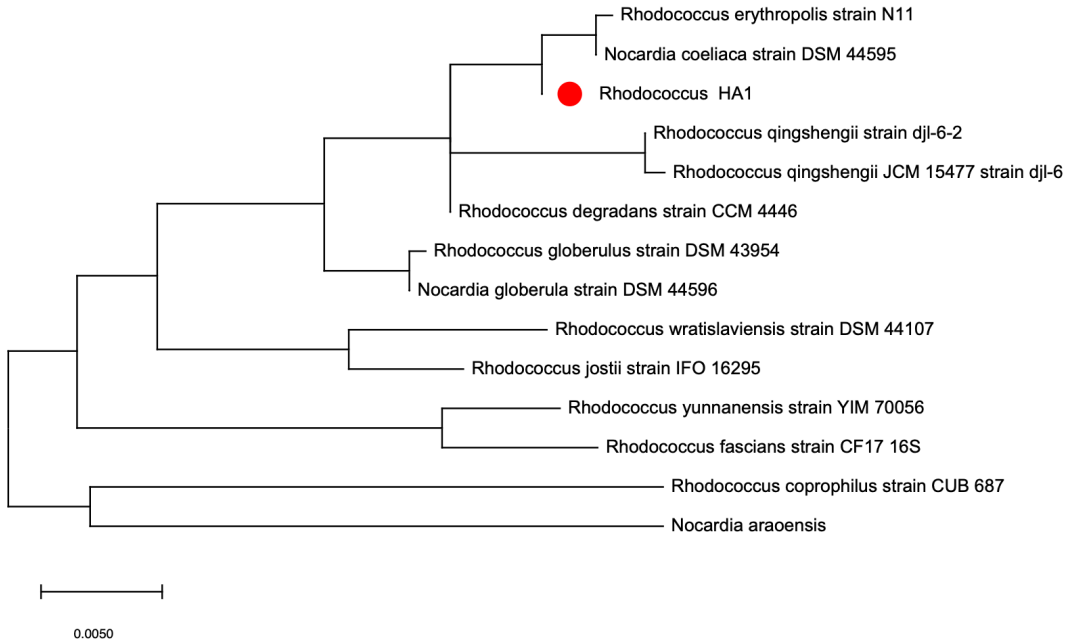


Figure 5.2 Phylogenetic tree showing *Rhodococcus* HA1. The tree was constructed using MEGA (Molecular Evolution Genetics Analysis) software.

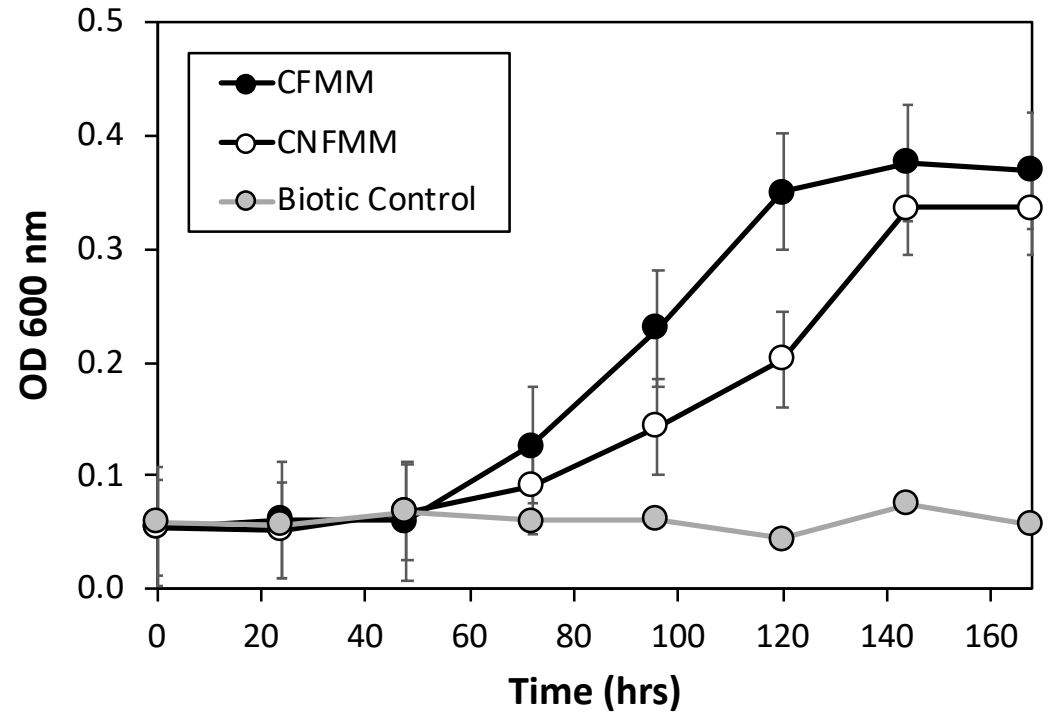


Figure 5.3 Increase in OD_{600} in pure culture *Rhodococcus* HA1 during growth on aniline in carbon free (CFMM) and carbon and nitrogen free minimal medium (CNFMM). Symbols are averages of triplicates and error bars are one standard deviation.

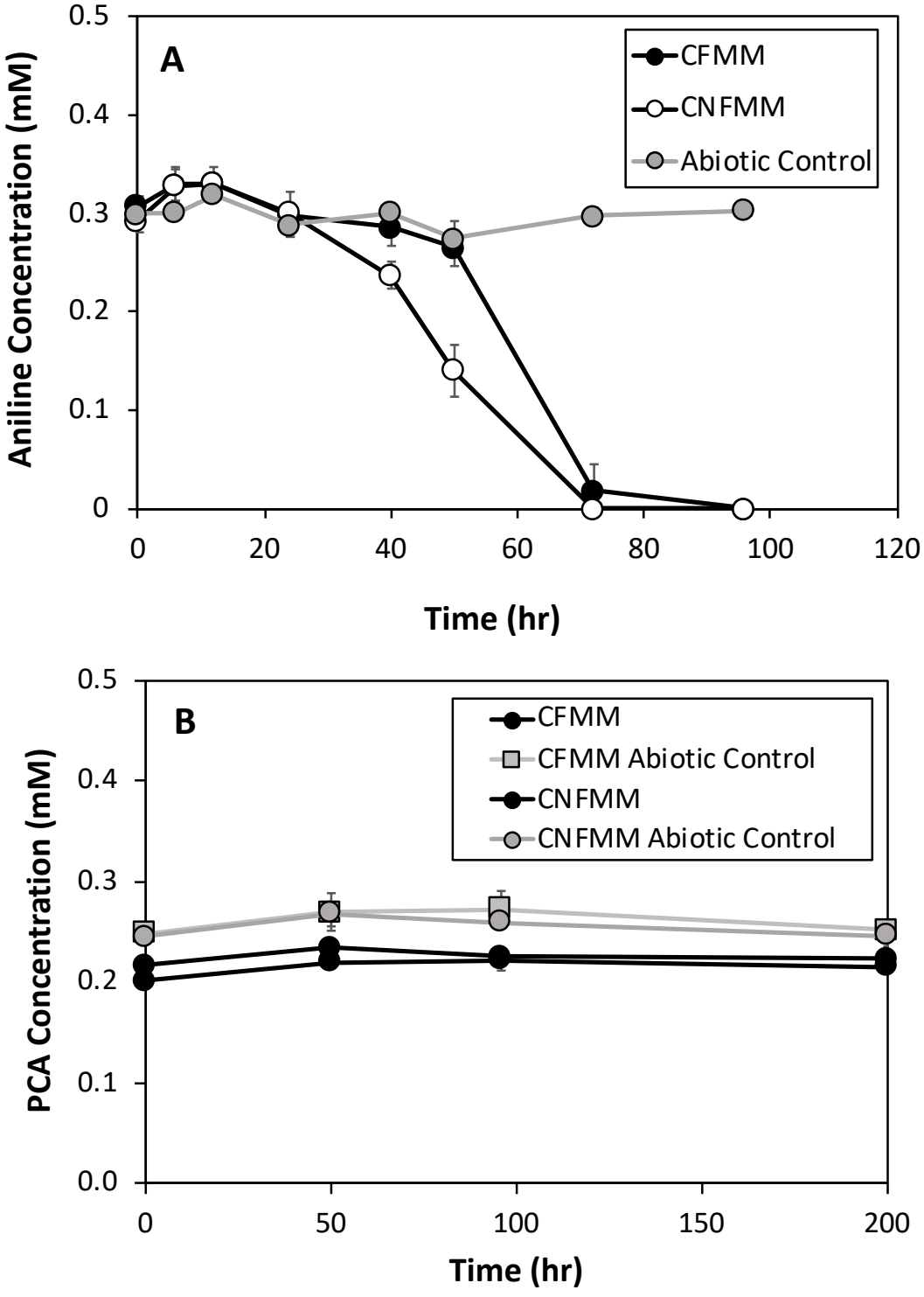


Figure 5.4 Aniline degradation (A) and lack of PCA degradation (B) by *Rhodococcus* HA1 in carbon free (CFMM) and carbon and nitrogen free minimal medium (CNFMM). Symbols are averages of triplicates and error bars are one standard deviation.

5.3.2 Bioaugmentation of non-active targeted microcosms

No targeted microcosms (Table 5.1) were active in the original microcosm study. Aerobic microcosms established from sediments of -9.8 to -10.2 elevation (JZM3) showed minimal abiotic loss of aniline. We examined addition of aniline degrading strains to these inactive microcosms in a bioaugmentation experiment. After 703 days of incubation, JZM3 microcosms were inactive. JZM3 microcosms were re-amended with sterile air and aniline. After additional sampling at day 820 there was still no activity. At day 820, a mixed cell suspension of *Rhodoccocus* HA1 and strain *Janibacter* sp. PR1, enriched in our laboratory as a dibenzofuran degrader, and also able to use aniline as a carbon source only (Almnehlawi et al., in preparation), was used in the bioaugmentation experiment. Using more than one bacterial strain ensured that metabolic diversity could be provided (Alisi et al., 2009; Rahman et al., 2002). Pure strains were mixed to achieve OD_{600} = 1 then 0.5 mL was added to duplicates of JZM3, while the third microcosm was left without cells as a non-bioaugmented control. Sterile air was added to all JZM3 microcosms. In the first week after bioaugmentation 70% of the aniline in the bioaugmented duplicates was depleted, and by the second week aniline was degraded completely (Figure 5.5). Results indicate clearly that active biomass was missing in the targeted JZM3 microcosms. As soon as the active pure isolates were added, aniline was degraded rapidly. There are limited literature reports of bioaugmentation using aniline-degrading strains, but one study found that bioaugmentation was the most effective way to remove PCA from soil samples (Tongarun et al., 2008). Many factors have to be considered to apply bioaugmentation strategies in sites such as environmental conditions including oxygen, temperature, and salinity, as well as the chemical composition and physical state of the contaminant, and the microbial

community. Sites with low or inactive microbial communities are candidates for bioaugmentation as a bioremediation technology (Tyagi et al., 2011). The inactive zones of the site under study may be a good subject for a bioaugmentation strategy because the lack of biomass, and other factors could be interfering with the biodegradation process.

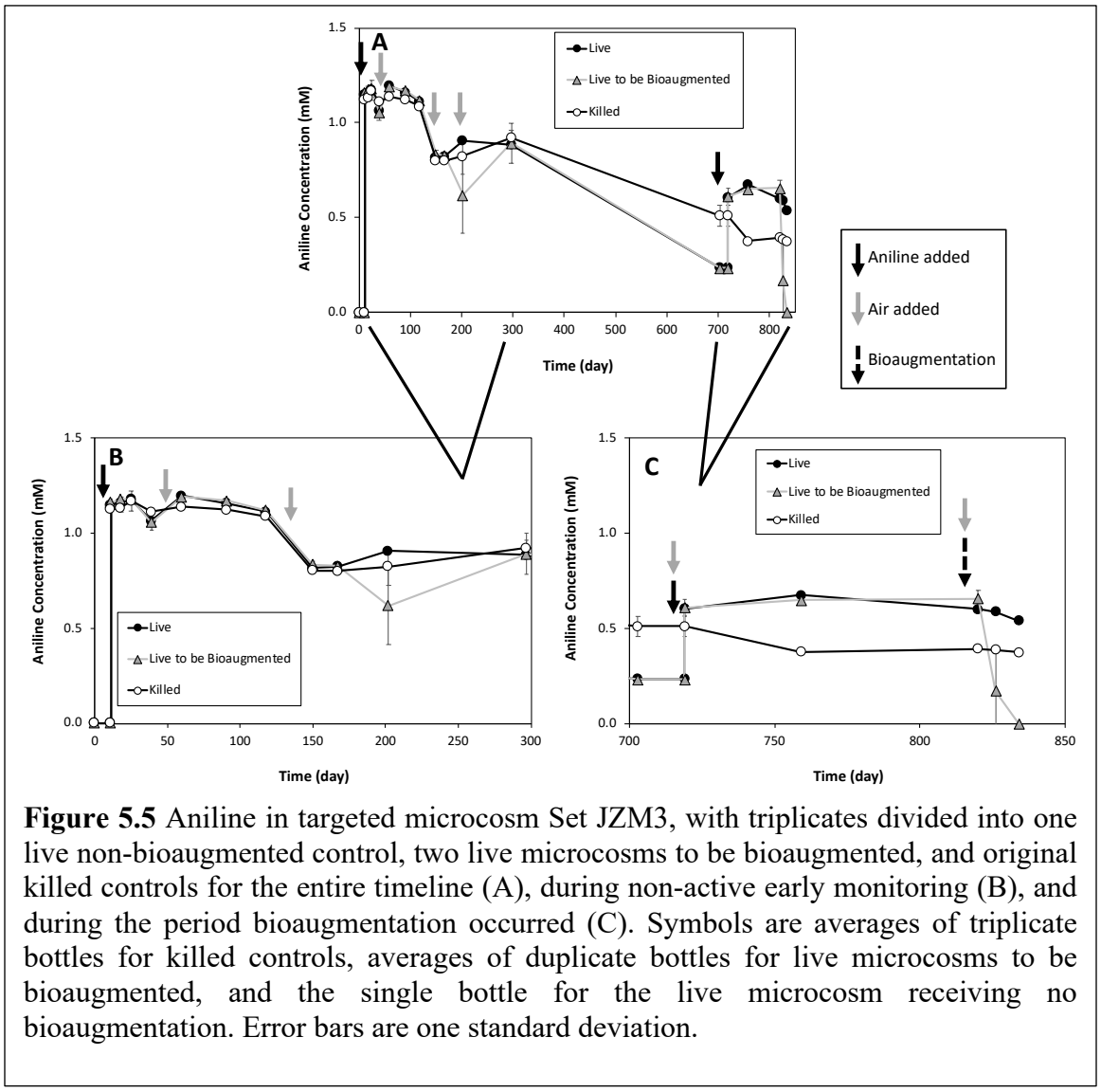


Figure 5.5 Aniline in targeted microcosm Set JZM3, with triplicates divided into one live non-bioaugmented control, two live microcosms to be bioaugmented, and original killed controls for the entire timeline (A), during non-active early monitoring (B), and during the period bioaugmentation occurred (C). Symbols are averages of triplicate bottles for killed controls, averages of duplicate bottles for live microcosms to be bioaugmented, and the single bottle for the live microcosm receiving no bioaugmentation. Error bars are one standard deviation.

5.3.3 SIP with ^{13}C Aniline

Active aerobic microcosms (Set 12, Table 5.2) diluted to increase volume and repeatedly re-amended with aniline over 118 days. The diluted microcosms were active, and aniline was depleted several times (Figure 5.6). After aniline amendment on day 119, daily monitoring indicated nearly complete loss of aniline over 9 days (Figure 5.6).

The SIP experiment commenced by mixing then aliquoting the enriched/diluted microcosms to new triplicate treatments showed aniline loss in ^{12}C-aniline live treatments and abiotic controls and ^{13}C-aniline live treatment sets over nine days of incubation (Figure 5.7).

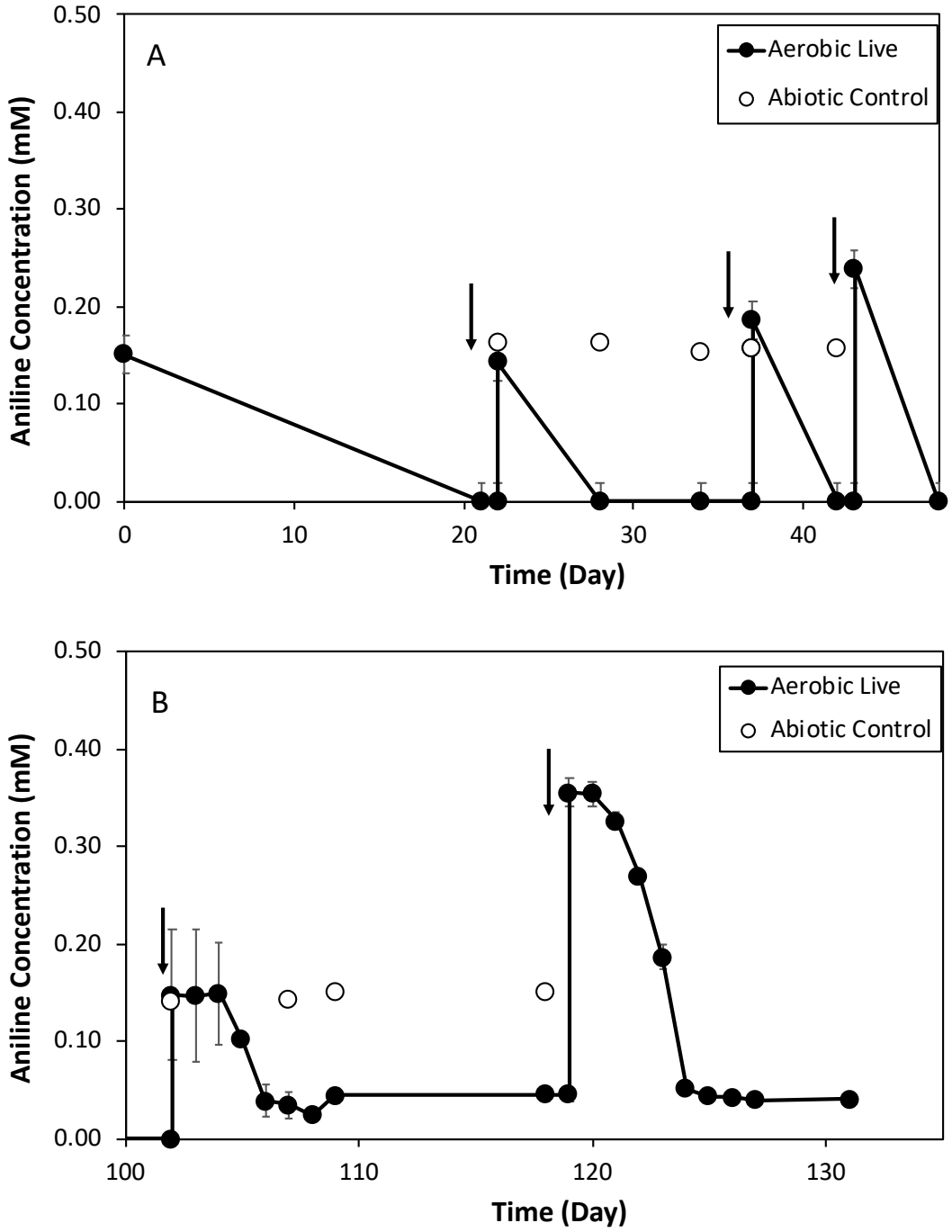


Figure 5.6 Aniline loss in active Set 12 aerobic microcosms after dilution to increase volume (A) and just prior to the SIP experiment when aniline concentration was monitored daily (B). Symbols are averages of duplicate live and triplicate abiotic controls, and error bars are one standard deviation. ↓ indicates amendment of aniline and filter-sterilized air.

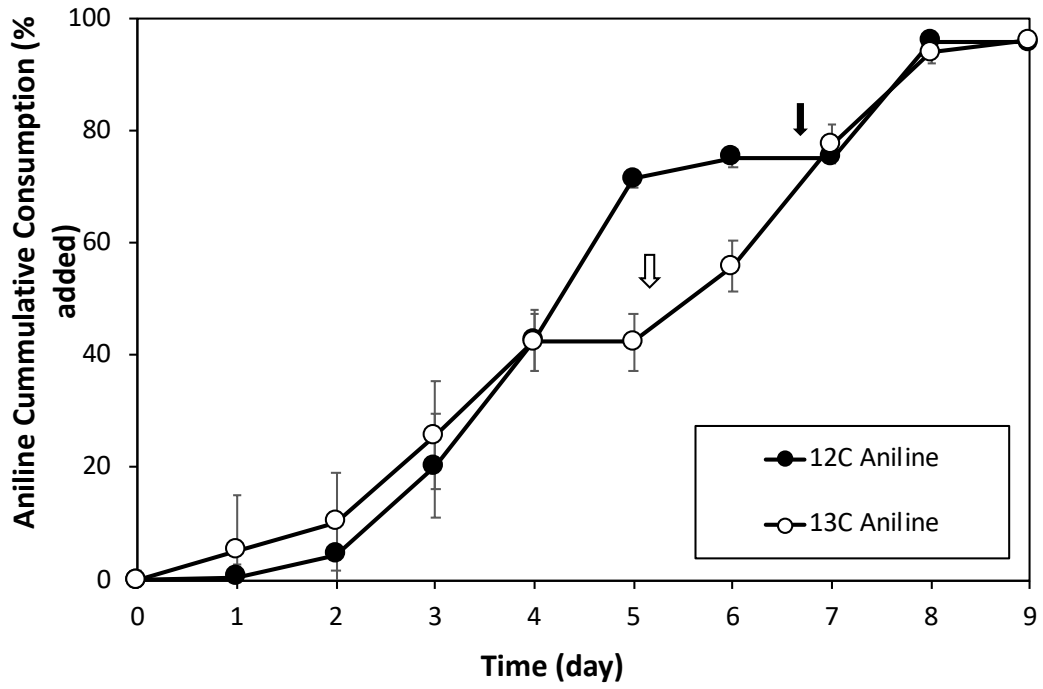


Figure 5.7 Cumulative aniline consumption during the SIP experiment by ^{12}C- and ^{13}C-aniline amended enrichments. Symbols are averages of triplicates and error bars are one standard deviation. ↓ indicates amendment of aniline and filter-sterilized air.

5.3.4 Community analysis of light and heavy SIP fractions

Genomic DNA recovered from ^{12}C- and ^{13}C-aniline amended enrichments, subjected to separation on a CsCl gradient, resulted in fractions with buoyant densities between ~1.8 g/mL to ~1.3 g/mL (for fractions 1 to 24, respectively, see Appendix). According to previous studies, we expected our targeted fractions to be ~1.71 g/mL (light, ^{12}C DNA) and ~1.77 g/mL (heavy, ^{13}C DNA) (Buckley et al., 2007; Cai et al., 2016; Dumont et al.,

2011; Sun and Cupples, 2012; Zhang et al., 2011b). We therefore performed qPCR for the 12 fractions with buoyant densities constrained by these values.

The highest gene copy numbers were detected in the light fractions of the ^{12}C microcosms, while they were detected in both the light and heavy fractions of ^{13}C set. The highest gene copy numbers were detected in fractions having buoyant density values of 1.76 g/mL (fraction 8) and 1.73 g/mL (fraction 14) for the ^{13}C set; and 1.72 g/mL (fraction 16) for the ^{12}C set for replicate 1, with similar results for replicates 2 and 3 (Figure 5.8 and Appendix ?) Results indicate there was ^{13}C incorporated in the active community DNA due to the consumption of ^{13}C labeled aniline in all replicates. There were some higher copy numbers detected in unexpected fractions of replicate 2, which could have been due to carryover of combined ^{12}C and ^{13}C together (Figure 5.8). Similar observations were reported in other studies (Sun and Cupples, 2012; Zhang et al., 2011b).

Bacterial community analysis of 16S rRNA genes of the SIP fractions showed different aniline degrading bacteria incorporated ^{13}C aniline in their DNA, but with different relative abundances. The results indicate that several bacterial genera may be responsible for aniline degradation. The community was dominant by a phylotype closely related to *Knoellia*, representing 77.3 %, 10.3%, and 82.8% of the ^{13}C DNA community (OTUs) in replicates 1, 2 and 3, respectively (Figures 5.8, 5.9 and 5.10). Phylotypes closely related to *Brevundimonas* may also be an aniline degrader, and was detected with lower relative abundances of 6.98%, 1.33%, and 2.68% of the OTUs for replicates 1, 2 and 3, respectively. A phylotype related to *Aquabacterium* showed high abundance in replicate 2, only, representing 73.41% of the OTUs. Furthermore, phylotypes related to other genera were detected in lower abundance, including *Janibacter* and *Rhizobium* (Figures 5.8, 5.9

and 5.10). Interestingly, phylotypes related to the genus *Rhodococcus*, which the isolated strain HA1 belongs to, also showed low abundance in replicate 1 and 3, 0.04% and 0.02% of the OTUs, respectively. Note that all genera representing less than 1% of the bacterial community have been summed and presented under "other" except for *Rhodococcus*, which is listed separately since it was isolated on aniline from the same original microcosm set. Overall, *Knoellia* appears to be one of the dominant aniline degraders in this bacterial community. To our knowledge, this is a first report linking it with aniline degradation. In replicate 2, a phylotype related to *Aquabacterium* was also dominant in the active community. *Aquabacterium* was isolated from the drinking water system in Berlin (Kalmbach et al., 1999). It has been also reported as an alkane degrader (Masuda et al., 2014) and linked with the aerobic biodegradation of vinyl chloride (Wilson et al., 2016). *Aquabacterium olei* sp. could be grown on diesel, gasoline and kerosene as sole carbon and energy sources (Jeong and Kim, 2015)

In Chapter 4 in this dissertation, we found that *Knoellia* was the dominant genus in the aerobic active microcosm community in comparison to the original sediment. A large shift in the bacterial community from the original sediment was apparent due to its appearance in very high abundance, followed by *Brevundimonas* (Figure 4.12 chapter 4). Here we are confirming here that *Knoellia* is the main aniline degrader in the active aerobic microcosms followed by *Brevundimonas* in term of percentage of abundancy, as determined by SIP using ^{13}C from aniline. In terms of isolation, we recovered a *Rhodococcus* strain in a pure culture from the same microcosms. *Knoellia* could be difficult to isolate in a pure culture (Hugenholtz et al., 1998), while it is notable that *Rhodococcus* strains have been isolated

in pure cultures as aniline degraders from a variety of environments (Aoki et al., 1983; Kaminski et al., 1983).

Brevundimonas is known as a CAN degrader (Arora, 2015; Surovtseva et al., 1986), and that could be the reason behind some ^{13}C incorporation in the DNA. This may also explain why there was some PCA degradation in the active aerobic microcosms in the second study of the dissertation (Figure 4.10, Table 5.2, Set 12).

Aniline degraders were identified previously at the site under study in anoxic microcosms using SIP. *Ignavibacterium album* and *Acidovorax spp.* were dominant under methanogenic and sulfidogenic conditions (Sun et al., 2015). Other contaminant degraders were also detected by this method such as toluene (Sun and Cupples, 2012), isoprene (Larke-Mejia et al., 2019) and PAHs (Morris et al., 2002; Zhang et al., 2011b). These findings confirm active aerobic metabolism of aniline at this site location. Knowledge of the active community members may be useful for monitoring aerobic bioremediation strategies.

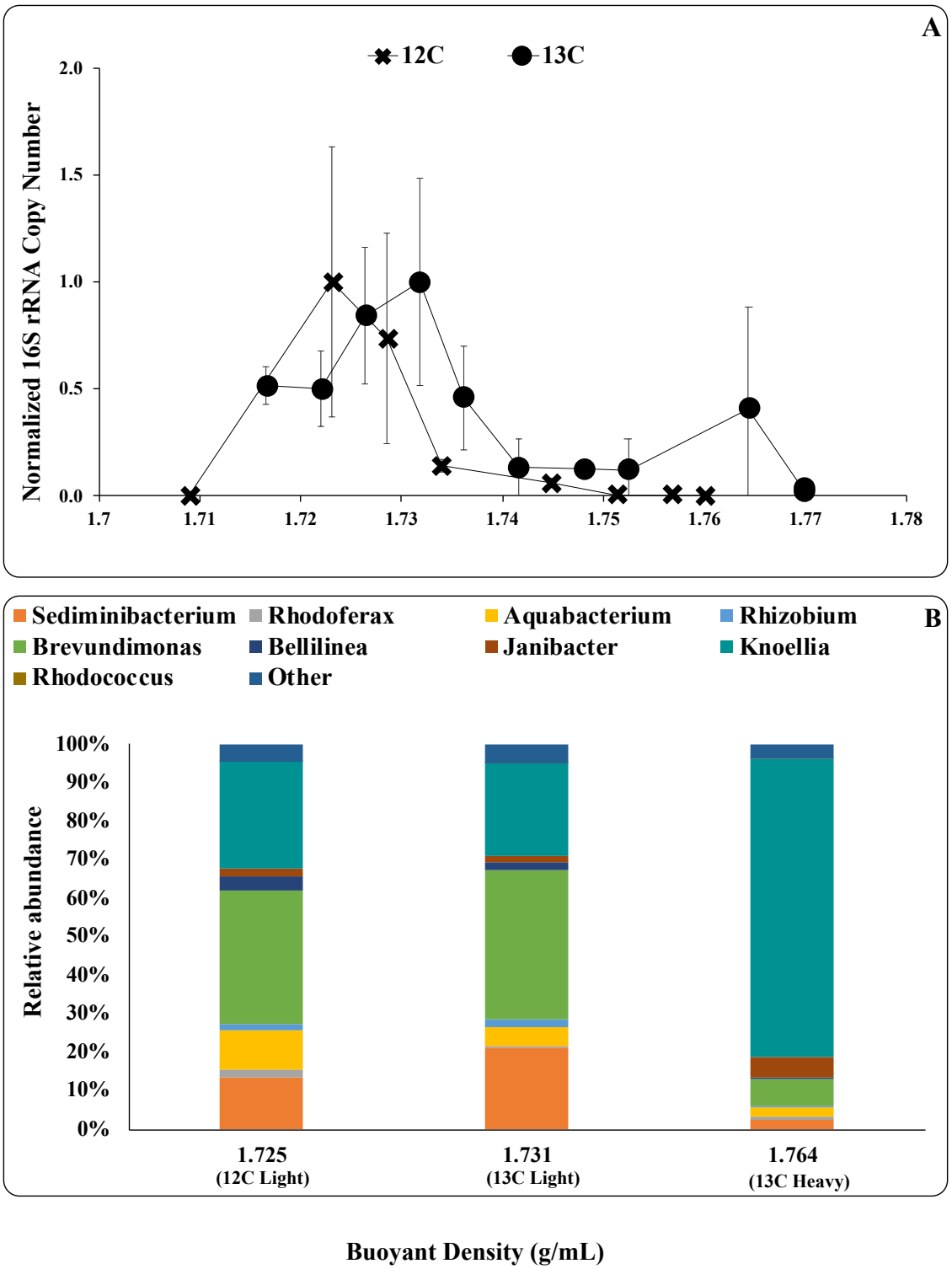


Figure 5.8 Normalized 16S rRNA gene copy number (A) and 16S rRNA gene bacterial community analysis of fractions from specific buoyant densities (B) representing ^{12}C and ^{13}C DNA, replicate 1. Symbols are averages of triplicates and error bars are one standard deviation.

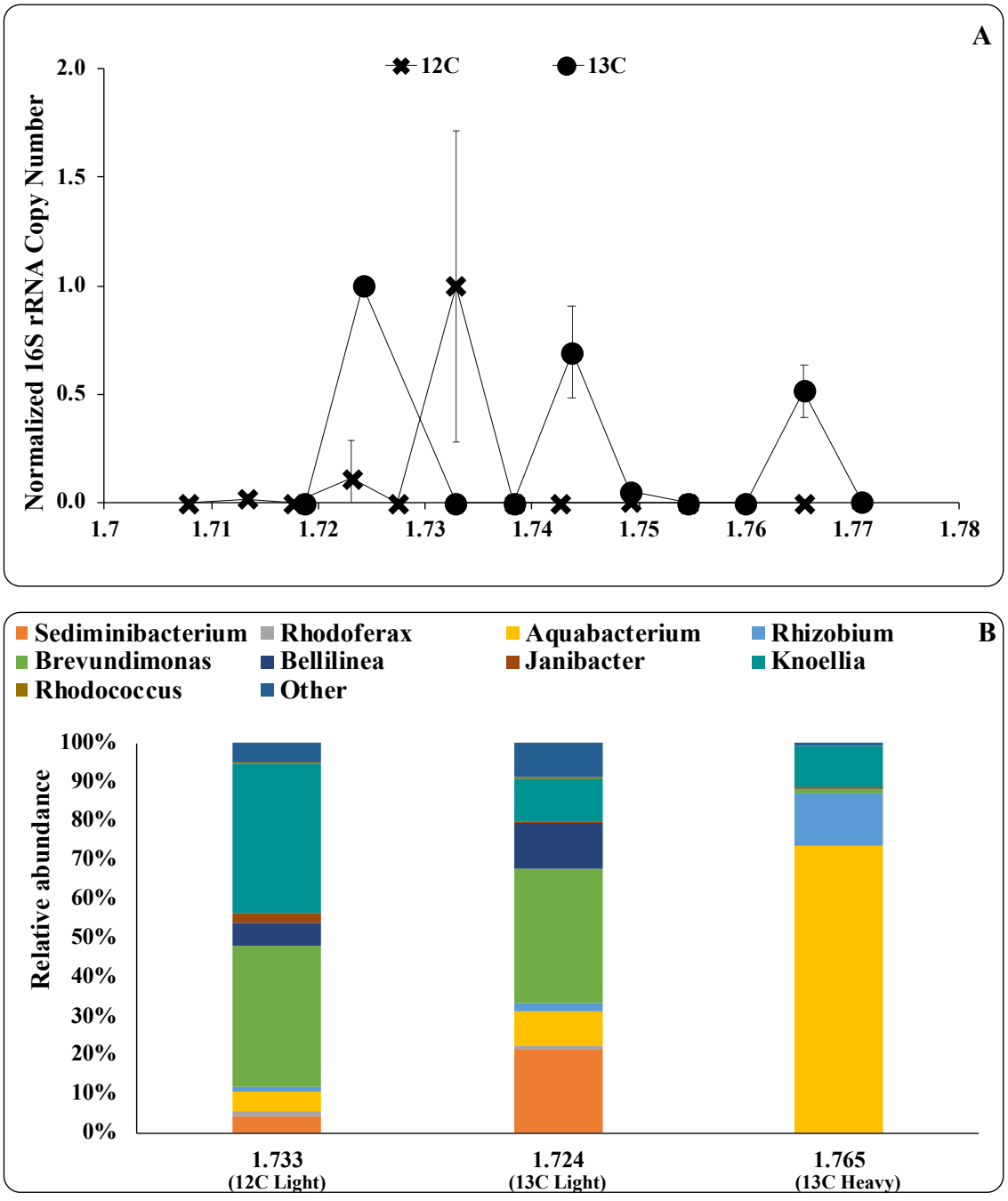


Figure 5.9 Normalized 16S rRNA gene copy number (A) and 16S rRNA gene bacterial community analysis of fractions from specific buoyant densities (B) representing ^{12}C and ^{13}C DNA, replicate 2. Symbols are averages of triplicates and error bars are one standard deviation.

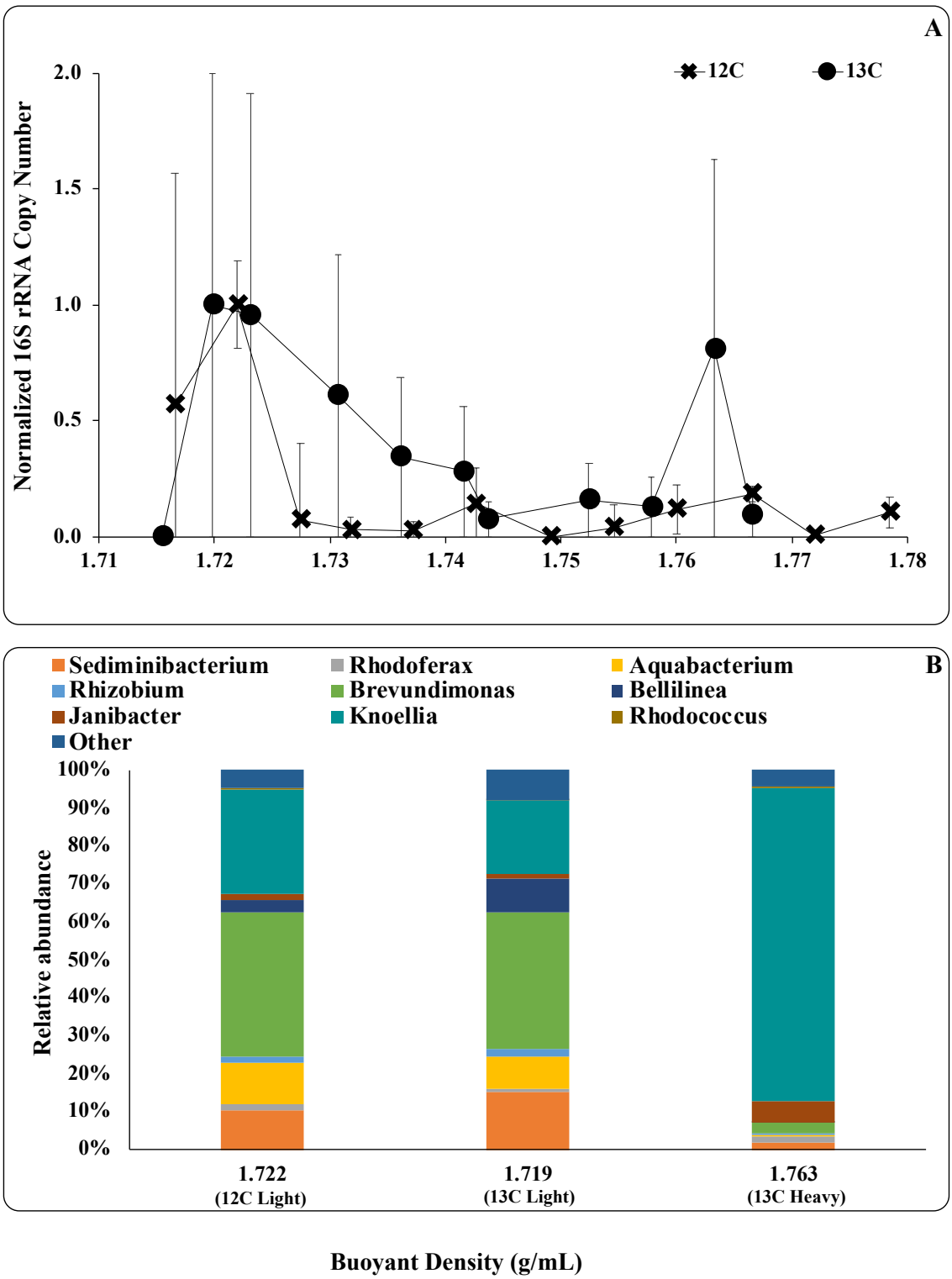


Figure 5.10 Normalized 16S rRNA gene copy number (A) and 16S rRNA gene bacterial community analysis of fractions from specific buoyant densities (B) representing ^{12}C and ^{13}C DNA, replicate 3. Symbols are averages of triplicates and error bars are one standard deviation.

5.4 Conclusions: *Rhodococcus* HA1 was isolated from active aniline degrading aerobic microcosms from an elevation of -27 to -29 ft in the subsurface of the chemical manufacturing site under study. HA1 uses aniline as a carbon and nitrogen source but did not use PCA. Bioaugmentation was a successful strategy for initiating aniline degradation in the non-active targeted microcosms from an elevation of -9 to -10.2 ft. Aniline, which showed no substantial loss over hundreds of days, was depleted in two weeks following bioaugmentation, indicating that active biomass was missing from this particular depth in the subsurface.

Assessment by SIP showed there is incorporation of ^{13}C from labeled aniline into the DNA of the active microbial community due to the consumption of aniline. Bacterial community analysis of 16S rRNA genes indicate that several genera are likely responsible for aniline degradation. Phylotypes related to *Knoellia* in addition to *Aquabacterium* and *Brevundimonas* incorporated ^{13}C from aniline into their DNA. This study provided an essential information that should be considered in case of establishing a bioremediation technology for the site.

Chapter 6 Summary and Implications

6.1 Summary

This dissertation research addressed biodegradation of two groups of aromatic contaminants at two contaminated sites in New Jersey: dibenzo-*p*-dioxin (DD) and dibenzofuran (DF) in sediments of the Passaic River located in the north; and aniline and parachloroaniline in aquifers underlying a chemical manufacturing site in the south.

Surficial sediment collected from the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8TCDD) contaminated Lower Passaic River was enriched with DF as the only carbon and energy source. DF was used in initial enrichment because it has similar structure to DD, is less expensive and less toxic, and shares a biodegradation pathway with DD.

The microorganisms in the sediments were able to degrade DF, and a known yellow intermediate, presumed to be 2-hydroxy-4-[3'-oxo-3'H-benzofuran-2'-yliden] but-2-enoic acid (HOBB), an intermediate in the DF degradation pathway, was evident in the enrichments.

Three pure strains which could degrade DF were obtained from the river sediments and according to sequencing of the 16S rRNA gene were denoted: *Janibacter* sp. PR1, *Agromyces* sp. PR2, and *Arthrobacter* sp. PR3. Two strains, *Agromyces* sp. PR2 and *Arthrobacter* sp. PR3 were able to transform DD, and 2-monochlorodibenzo-*p*-dioxin (2MCDD) but not 2,7-dichlorodibenzo-*p*-dioxin (2,7 DCDD). The key gene in the aerobic biodegradation system for DD is a dioxygenase, so it was investigated in the isolates.

Janibacter sp. PR1 and *Agromyces* sp. PR2 were found to have an angular dioxygenase gene almost identical to the angular dioxygenase gene in *Terrabacter*. The angular dioxygenase gene in *Arthrobacter* sp. PR3 was not identified.

In the second study of the dissertation, the heterogenous aquifers underlying a chemical manufacturing site in southern New Jersey were evaluated using a combination of information from the manufacturing company, and the laboratories of other collaborators, to determine whether biodegradation of pollutants is occurring. A 60-ft sediment core was previously recovered from the site along with groundwater from differing depths from an adjacent multi-level sampling well. The sediment core was divided into 2-in slices for detailed interrogation. The ~300 sediment core slices were shared among multiple teams and previously characterized for *in situ* geochemistry and microbial community via sequencing of 16S rRNA genes. Aniline and PCA were from the contaminants of concern at the site that are addressed by the research described in this dissertation. Two types microcosms from different depths were established under different redox conditions to test aniline and PCA degradation by the microbial community.

The first type of microcosms was denoted “targeted” microcosms, wherein sediment from specific depths over ~2 to 6 in was combined. The second type were denoted “composited” microcosms, wherein sediments over 2 ft depths were combined. In each case, slices from adjacent depths were combined. The composited microcosms showed more activity in comparison to the targeted ones. Aerobic and sulfate-reducing microcosms were the most active targeted microcosms while little or no activity was observed under anaerobic (methanogenic), or nitrate- and iron-reducing conditions. Bacterial community analysis was performed for the original sediments of the composited microcosms and the aerobic

microcosms, the most active condition. There was a substantial shift in the microbial community between the original sediments and the aerobic microcosms. The community was dominated by a phylotype closely related to the genus *Knoellia*.

The third study is related to the second one. The active bacteria in microbial community were identified, and active organisms were added to the inactive targeted microcosms to determine if inactivity could be overcome by bioaugmentation.

The traditional way to determine who is active was used by isolating active microbes in pure culture. Additionally, a molecular approach, stable isotope probing (SIP), was used to determined who is active without isolation, since it is generally accepted that most bacteria are difficult to isolate as pure cultures.

One pure culture was obtained from the aerobic active microcosms. It is closely related to *Rhodococcus erythropolis* and was designated *Rhodococcus* HA1. The pure isolate, along with another pure isolate (from the Passaic River site) were used in a mixture for a bioaugmentation study of selected inactive targeted microcosms. The bioaugmentation study indicated clearly that active microorganisms are missing or were present at very low levels in the targeted microcosms. Within the first week of addition of the cells most of the aniline was gone, and in the second week, aniline was depleted from the targeted microcosms.

Finally, incubation with ^{13}C-aniline showed that an active community in the composited aerobic microcosms incorporated ^{13}C in newly synthesized DNA. Bacterial community analysis and quantification using qPCR of 16S rRNA genes of the SIP fractions showed several genera are responsible for aniline degradation. The community was dominated by *Knoellia*, in addition to *Aquabacterium* and *Brevundimonas*.

6.2 Environmental Implications

Results of this dissertation have important environmental implications for bioremediation of the two contaminated sites. The Passaic River is a highly contaminated body of water in New Jersey. It is contaminated with many contaminants including primarily the polychlorinated dibenzo-*p*-dioxins and dibenzofurans (CDD/Fs), especially 2,3,7,8TCDD. The highly chlorinated congeners of CDD/Fs could be subject to anaerobic dechlorination. The results of this process would be the production of lightly or non-chlorinated congeners. Aerobic biodegradation and biotransformation of these compounds is important to prevent their accumulation in the river sediments. This is especially important after another recent study in our laboratory showed their release as a result of dechlorination of more highly chlorinated congeners (Dean et al., 2020). Findings suggested that some active aerobic microbes are present in the sediments and likely adapted to living in the presence of CDD/Fs along with PAHs, PCBs, and heavy metals. These organisms likely play a role in the natural attenuation of CDD/Fs by degrading evolved daughter products of CDD/F dechlorination. The findings may be useful in the future plan for any bioremediation of the site.

The aquifers underlying the chemical manufacturing site are highly contaminated with many chemicals including aniline and PCA. The presence of the multitude of pollutants (and dense non-aqueous phase liquid) may affect the activity of bacteria at the site. This could be the reason behind the lack of active biodegradation of aniline and PCA in the targeted microcosms. Showing activity at the site is important because it suggests that

natural attenuation is occurring and that the site could be bioremediated in the future through biostimulation of the native organisms, for example by increasing the oxygen content of the groundwater. Bioaugmentation showed that critical active biomass was missing at different depths. This should also be considered, and the results suggest that bioaugmentation or in situ re-distribution of organisms from one area of the aquifer to another, could be a suitable way to enhance bioremediation of the site. Active microbes at the site could be promoted by knowing what is missing and then adding it to the site. Further studies should be performed on the site to give better understanding of the implications for site characterization and microbial distribution on pollutant removal. Samples from different specific locations at the site could be collected and used to test biodegradation of other contaminants of concern. This could be done by establishing other microcosms under different redox conditions, or bioaugmentation could be done in a limited area of the site to see what the response would be. The studies described in this dissertation enhance the understanding of both of the sites and provide important information for application of future bioremediation technologies. Further studies are required at larger scales before application of any bioremediation technologies based on the findings described in this dissertation could be applied at the sites.

References

- Abe, T., Saito, H., Niikura, Y., Shigeoka, T., Nakano, Y., 2001. Embryonic development assay with *Daphnia magna*: application to toxicity of aniline derivatives. Chemosphere 45, 487-495.
- Abram, F.S.H., Sims, I.R., 1982. The Toxicity of Aniline to Rainbow-Trout. Water Res 16, 1309-1312.
- Adrian, L., Dudkova, V., Demnerova, K., Bedard, D.L., 2009. "*Dehalococcoides*" sp. strain CBDB1 extensively dechlorinates the commercial polychlorinated biphenyl mixture aroclor 1260. Appl Environ Microbiol 75, 4516-4524.
- Ahmed, S., Ahmed, S., Nisar, M.F., Hussain, K., Majeed, A., Ghumroo, P.B., Afghan, S., Shahzad, A., Nawaz, K., Ali, K., 2010. Isolation and characterization of a bacterial strain for aniline degradation. Afr J Biotechnol 9, 1173-1179.
- Alexandra De, M., O'Connor, O. A., & Kosson, D. S. (1994). Metabolism of aniline under different anaerobic electron-accepting and nutritional conditions. Environmental Toxicology and Chemistry: An International Journal, 13(2), 233-239.
- Alisi, C., Musella, R., Tasso, F., Ubaldi, C., Manzo, S., Cremisini, C., Sprocati, A.R., 2009. Bioremediation of diesel oil in a co-contaminated soil by bioaugmentation with a microbial formula tailored with native strains selected for heavy metals resistance. Science of the Total Environment 407, 3024-3032.
- Aly, H.A., Huu, N.B., Wray, V., Junca, H., Pieper, D.H., 2008. Two angular dioxygenases contribute to the metabolic versatility of dibenzofuran-degrading *Rhodococcus* sp. strain HA01. Appl Environ Microbiol 74, 3812-3822.
- Anson, J.G., Mackinnon, G., 1984. Novel *Pseudomonas* plasmid involved in aniline degradation. Appl Environ Microbiol 48, 868-869.
- Aoki, K., Konohana, T., Shinke, R., Nishira, H.J.A., chemistry, b., 1984a. Purification and characterization of catechol 1, 2-dioxygenase from aniline-assimilating *Rhodococcus erythropolis* AN-13. Agricultural and Biological Chemistry 48, 2087-2095.
- Aoki, K., Ohtsuka, K., Shinke, R., Nishira, H., 1983. Microbial-Metabolism of Aromatic-Amines .2. Isolation of Aniline-Assimilating Bacteria and Physiological Characterization of Aniline Biodegradation in *Rhodococcus-Erythropolis* an-13. Agricultural and Biological Chemistry 47, 2569-2575.
- Aoki, K., Ohtsuka, K., Shinke, R., Nishira, H., 1984b. Rapid Biodegradation of Aniline by *Frateuria* species ANA-18 and Its Aniline Metabolism. Agricultural and Biological Chemistry 48, 865-872.
- Araya, R., Yamaguchi, N., Tani, K., Nasu, M., 2003. Change in the bacterial community of natural river biofilm during biodegradation of aniline-derived compounds determined by denaturing gradient gel electrophoresis. J Health Sci 49, 379-385.
- Armengaud, J., Happe, B., Timmis, K.N., 1998. Genetic analysis of dioxin dioxygenase of *Sphingomonas* sp. Strain RW1: Catabolic genes dispersed on the genome. J Bacteriol 180, 3954-3966.

- Arora, P.K., 2015. Bacterial degradation of monocyclic aromatic amines. Front Microbiol 6, 820.
- Barkovskii, A.L., Adriaens, P., 1996. Microbial dechlorination of historically present and freshly spiked chlorinated dioxins and diversity of dioxin-dechlorinating populations. Appl. Environ. Microbiol. 62, 4556-4562.
- Bedard, D.L., Bailey, J.J., Reiss, B.L., Jerzak, G.V., 2006. Development and characterization of stable sediment-free anaerobic bacterial enrichment cultures that dechlorinate aroclor 1260. Appl Environ Microbiol 72, 2460-2470.
- Binh, N.D., Imsapsangworn, C., Kim Oanh, N.T., Parkpian, P., Karstensen, K., Giao, P.H., DeLaune, R.D., 2016. Sequential anaerobic-aerobic biodegradation of 2,3,7,8-TCDD contaminated soil in the presence of CMC-coated nZVI and surfactant. Environ Technol 37, 388-398.
- Bopp, R.F., Gross, M.L., Tong, H., Simpson, H.J., Monson, S.J., Deck, B.L., Moser, F.C., 1991. A Major Incident of Dioxin Contamination - Sediments of New-Jersey Estuaries. Environ Sci Technol 25, 951-956.
- Brillas, E., Bastida, R.M., Llosa, E., 1995. Electrochemical destruction of aniline and 4-chloroaniline for wastewater treatment using a carbon-PTFE O 2-fed cathode. The Electrochemical Society 142, 1733-1741.
- Buckley, D.H., Huangyutitham, V., Hsu, S.-F., Nelson, T.A., 2007. Stable isotope probing with 15N2 reveals novel noncultivated diazotrophs in soil. Appl. Environ. Microbiol. 73, 3196-3204.
- Bunge, M., Lechner, U., 2009. Anaerobic reductive dehalogenation of polychlorinated dioxins. Appl Microbiol Biotechnol 84, 429-444.
- Bünz, P.V., Cook, A.M.J.J.o.b., 1993. Dibenzofuran 4,4a-dioxygenase from *Sphingomonas* sp. strain RW1: angular dioxygenation by a three-component enzyme system. J Bacteriol 175, 6467-6475.
- Burkhardt-Holm, P., Oulmi, Y., Schroeder, A., Storch, V., Braunbeck, T., 1999. Toxicity of 4-chloroaniline in early life stages of zebrafish (Danio rerio): II. Cytopathology and regeneration of liver and gills after prolonged exposure to waterborne 4-chloroaniline. Arch Environ Contam Toxicol 37, 85-102.
- Bus, J.S., Popp, J.A., 1987. Perspectives on the mechanism of action of the splenic toxicity of aniline and structurally-related compounds. Food Chem Toxicol 25, 619-626.
- Cai, Y., Zheng, Y., Bodelier, P.L., Conrad, R., Jia, Z., 2016. Conventional methanotrophs are responsible for atmospheric methane oxidation in paddy soils. Nature communications 7, 1-10.
- Chai, B., Tsoi, T.V., Iwai, S., Liu, C., Fish, J.A., Gu, C., Johnson, T.A., Zylstra, G., Teppen, B.J., Li, H., Hashsham, S.A., Boyd, S.A., Cole, J.R., Tiedje, J.M., 2016. *Sphingomonas wittichii* Strain RW1 Genome-Wide Gene Expression Shifts in Response to Dioxins and Clay. PLoS One 11, e0157008.
- Chakraborty, J., Das, S., 2016a. Molecular perspectives and recent advances in microbial remediation of persistent organic pollutants. Environ Sci Pollut Res Int 23, 16883-16903.
- Chakraborty, J., Das, S., 2016b. Molecular perspectives and recent advances in microbial remediation of persistent organic pollutants. Environmental Science and Pollution Research 23, 16883-16903.

- Chambers, R.C., Davis, D.D., Habeck, E.A., Roy, N.K., Wirgin, I., 2012. Toxic effects of PCB126 and TCDD on shortnose sturgeon and Atlantic sturgeon. Environmental toxicology and chemistry 31, 2324-2337.
- Chang, Y.S., 2008. Recent developments in microbial biotransformation and biodegradation of dioxins. J Mol Microbiol Biotechnol 15, 152-171.
- Chant, R.J., Fugate, D., Garvey, E., 2010. The Shaping of An Estuarine Superfund Site: Roles of Evolving Dynamics and Geomorphology. Estuaries and Coasts 34, 90-105.
- Chastain Jr, J.E., Pazdernik, T.L., 1985. 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD)-induced immunotoxicity. International journal of immunopharmacology 7, 849-856.
- Chengbin, X., Jun, N., Hai, Y., Xudong, S., Jiye, H.J.C.J.o.C.E., 2009. Biodegradation of aniline by a newly isolated *Delftia* sp. XYJ6. Chinese Journal of Chemical Engineering 17, 500-505.
- Cui, D., Shen, D., Wu, C., Li, C., Leng, D., Zhao, M., 2017. Biodegradation of aniline by a novel bacterial mixed culture AC. International Biodeterioration & Biodegradation 125, 86-96.
- Damborsky, J., Lynam, M., Kuty, M., 1998. Structure-biodegradability relationships for chlorinated dibenzo-p-dioxins and dibenzofurans, Biodegradation of dioxins and furans. Springer, pp. 165-228.
- Dean, R.K., Schneider, C.R., Almnehlawi, H.S., Dawson, K.S., Fennell, D.E., 2020. 2,3,7,8-Tetrachlorodibenzo-p-dioxin Dechlorination is Differentially Enhanced by Dichlorobenzene Amendment in Passaic River, NJ Sediments. Environ Sci Technol 54, 8380-8389.
- Ding, C., Li, Z.X., Yan, J.L., 2011. Isolation, identification and degradation characterization of a p-chloroaniline degrading strain. Bull Environ Contam Toxicol 86, 454-459.
- Dumont, M.G., Pommerenke, B., Casper, P., Conrad, R., 2011. DNA-, rRNA-and mRNA-based stable isotope probing of aerobic methanotrophs in lake sediment. Environmental Microbiology 13, 1153-1167.
- Ehrlich, R., Wenning, R., Johnson, G., Su, S., Paustenbach, D., 1994. A mixing model for polychlorinated dibenzo-p-dioxins and dibenzofurans in surface sediments from Newark Bay, New Jersey using polytopic vector analysis. Archives of environmental contamination and toxicology 27, 486-500.
- Fábrega-Duque, J.R., Jafvert, C.T., Li, H., Lee, L.S. 2000. Modeling abiotic processes of aniline in water-saturated soils. Environ. Sci. Technol 34, 1687-1693.
- Fan, X., Wang, J., Soman, K.V., Ansari, G., Khan, M.F., 2011. Aniline-induced nitrosative stress in rat spleen: proteomic identification of nitrated proteins. Toxicology and applied pharmacology 255, 103-112.
- Fan, Z., Casey, F.X., Larsen, G.L., Hakk, H., 2006. Fate and transport of 1278-TCDD, 1378-TCDD, and 1478-TCDD in soil-water systems. Sci Total Environ 371, 323-333.
- Fennell, D.E., Nijenhuis, I., Wilson, S.F., Zinder, S.H., Häggblom, M.M., 2004. *Dehalococcoides ethenogenes* Strain 195 Reductively Dechlorinates Diverse Chlorinated Aromatic Pollutants. Environ Sci Technol 38, 2075-2081.

- Ferrario, J.B., Byrne, C.J., Cleverly, D.H., 2000. 2,3,7,8-dibenzo-p-dioxins in mined clay products from the United States: Evidence for possible natural origin. Environ Sci Technol 34, 4524-4532.
- Fiedler, H., 2003. Dioxins and furans (PCDD/PCDF), Persistent Organic Pollutants. Springer, pp. 123-201.
- Fiedler, H., 2007. National PCDD/PCDF release inventories under the Stockholm Convention on Persistent Organic Pollutants. Chemosphere 67, S96-108.
- Field, J., Sierra-Alvarez, R.J.S.D., 2007. Biodegradability of chlorinated aromatic compounds. Science Dossier 12.
- Field, J.A., Sierra-Alvarez, R., 2008. Microbial degradation of chlorinated dioxins. Chemosphere 71, 1005-1018.
- Fortnagel, P., Harms, H., Wittich, R.M., Krohn, S., Meyer, H., Sinnwell, V., Wilkes, H., Francke, W., 1990. Metabolism of Dibenzofuran by *Pseudomonas* sp. Strain HH69 and the Mixed Culture HH27. Appl Environ Microbiol 56, 1148-1156.
- Friedrich, M.W., 2006. Stable-isotope probing of DNA: insights into the function of uncultivated microorganisms from isotopically labeled metagenomes. Current Opinion in Biotechnology 17, 59-66.
- Fu, Q.S., Barkovskii, A.L., Adriaens, P., 2001. Dioxin cycling in aquatic sediments: the Passaic River Estuary. Chemosphere 43, 643-648.
- Fukuda, K., Nagata, S., Taniguchi, H., 2002. Isolation and characterization of dibenzofuran-degrading bacteria. FEMS Microbiol Lett 208, 179-185.
- Fukumori, F., Saint, C.P., 1997. Nucleotide sequences and regulational analysis of genes involved in conversion of aniline to catechol in *Pseudomonas putida* UCC22(pTDN1). J Bacteriol 179, 399-408.
- Futamata, H., Uchida, T., Yoshida, N., Yonemitsu, Y., Hiraishi, A.J.M., environments, 2004. Distribution of dibenzofuran-degrading bacteria in soils polluted with different levels of polychlorinated dioxins. Microbes Environ 19, 172-177.
- Gallagher, E., McGuinness, L., Phelps, C., Young, L., Kerkhof, L., 2005. 13C-carrier DNA shortens the incubation time needed to detect benzoate-utilizing denitrifying bacteria by stable-isotope probing. Appl Environ Microbiol. 71, 5192-5196.
- Gheewala, S.H., Annachhatre, A.P., 1997. Biodegradation of aniline. Water Sci Technol 36, 53-63.
- Gómez, J.L., León, G., Hidalgo, A.M., Gómez, M., Murcia, M.D., Griñán, G., 2009. Application of reverse osmosis to remove aniline from wastewater. Desalination 245, 687-693.
- Gosetti, F., Bottaro, M., Gianotti, V., Mazzucco, E., Frascarolo, P., Zampieri, D., Oliveri, C., Viarengo, A., Gennaro, M.C., 2010. Sun light degradation of 4-chloroaniline in waters and its effect on toxicity. A high performance liquid chromatography - Diode array - Tandem mass spectrometry study. Environ Pollut 158, 592-598.
- Greenwood, D.R., Kingsbury, G., Cleland, J., 1979. A handbook of key federal regulations and criteria for multimedia environmental control. US Environmental Protection Agency, Office of Research and Development.

- Habe, H., Ashikawa, Y., Saiki, Y., Yoshida, T., Nojiri, H., Omori, T., 2002a. *Sphingomonas* sp. strain KA1, carrying a carbazole dioxygenase gene homologue, degrades chlorinated dibenzo-p-dioxins in soil. FEMS Microbiol Lett 211, 43-49.
- Habe, H., Chung, J.S., Kato, H., Ayabe, Y., Kasuga, K., Yoshida, T., Nojiri, H., Yamane, H., Omori, T., 2004. Characterization of the upper pathway genes for fluorene metabolism in *Terrabacter* sp. strain DBF63. Journal of Bacteriol 186, 5938-5944.
- Habe, H., Ide, K., Yotsumoto, M., Tsuji, H., Yoshida, T., Nojiri, H., Omori, T., 2002b. Degradation characteristics of a dibenzofuran-degrader *Terrabacter* sp. strain DBF63 toward chlorinated dioxins in soil. Chemosphere 48, 201-207.
- Häggblom, M.M., 1992. Microbial breakdown of halogenated aromatic pesticides and related compounds. FEMS Microbiol Rev 9, 29-71.
- Häggblom, M.M., Bossert, I.D., 2004. Halogenated organic compounds-a global perspective, Dehalogenation. Springer, pp. 3-29.
- Halden, R.U., Halden, B.G., Dwyer, D.F., 1999. Removal of Dibenzofuran, Dibenzo-p-Dioxin, and 2-Chlorodibenzo-p-Dioxin from Soils Inoculated with *Sphingomonas* sp. Strain RW1. Appl Environ Microbiol 65, 2246-2249.
- Happe, B., Eltis, L., Poth, H., Hedderich, R., Timmis, K., 1993. Characterization of 2, 2', 3-trihydroxybiphenyl dioxygenase, an extradiol dioxygenase from the dibenzofuran-and dibenzo-p-dioxin-degrading bacterium *Sphingomonas* sp. strain RW1. J Bacteriol 175, 7313-7320.
- Head, I., Saunders, J., Pickup, R., 1998. Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. Microbial Ecology 35, 1-21.
- Hites, R. A. (1990). "Environmental behavior of chlorinated dioxins and furans." Accounts of Chemical Research 23: 194-201.
- Hites, R. A. (2011). "Dioxins: An overview and history." Environmental Science & Technology 45: 16-20.
- Hong, H.B., Chang, Y.S., Choi, S.D., Park, Y.H., 2000. Degradation of dibenzofuran by *Pseudomonas putida* PH-01. Water Res 34, 2404-2407.
- Hong, H.B., Chang, Y.S., Nam, I.H., Fortnagel, P., Schmidt, S., 2002. Biotransformation of 2,7-Dichloro- and 1,2,3,4-Tetrachlorodibenzo-p-Dioxin by *Sphingomonas wittichii* RW1. Appl Environ Microbiol 68, 2584-2588.
- Hong, H.B., Nam, I.H., Murugesan, K., Kim, Y.M., Chang, Y.S., 2004. Biodegradation of dibenzo-p-dioxin, dibenzofuran, and chlorodibenzo-p-dioxins by *Pseudomonas veronii* PH-03. Biodegradation 15, 303-313.
- Hongsawat, P., Vangnai, A.S., 2011. Biodegradation pathways of chloroanilines by *Acinetobacter baylyi* strain GFJ2. J Hazard Mater 186, 1300-1307.
- Hou, L., Wu, Q., Gu, Q., Zhou, Q., Zhang, J., 2018. Community Structure Analysis and Biodegradation Potential of Aniline-Degrading Bacteria in Biofilters. Curr Microbiol 75, 918-924.
- Huang, J., Ling, J., Kuang, C., Chen, J., Xu, Y., Li, Y., 2018. Microbial biodegradation of aniline at low concentrations by *Pigmentiphaga daeguensis* isolated from textile dyeing sludge. International Biodeterioration & Biodegradation 129, 117-122.

- Hugenholtz, P., Goebel, B.M., Pace, N.R., 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol 180, 4765-4774.
- Hwang, H.M., Hodson, R.E., Lee, R.F., 1987. Degradation of Aniline and Chloroanilines by Sunlight and Microbes in Estuarine Water. Water Res 21, 309-316.
- Iannuzzi, T., Huntley, S., Schmidt, C., Finley, B., McNutt, R., Burton, S.J.C., 1997. Combined sewer overflows (CSOs) as sources of sediment contamination in the lower Passaic River, New Jersey. I. Priority pollutants and inorganic chemicals. Pergamon 34, 213-231.
- Iannuzzi, T.J., 2002. A common tragedy: History of an urban river. Amherst Scientific Publishers.
- Iida, T., Mukouzaka, Y., Nakamura, K., Kudo, T., 2002. Plasmid-Borne Genes Code for an Angular Dioxygenase Involved in Dibenzofuran Degradation by *Terrabacter* sp. Strain YK3. Appl Environ Microbiol 68, 3716-3723.
- Iida, T., Nakamura, K., Izumi, A., Mukouzaka, Y., Kudo, T., 2006. Isolation and characterization of a gene cluster for dibenzofuran degradation in a new dibenzofuran-utilizing bacterium, *Paenibacillus* sp. strain YK5. Arch Microbiol 184, 305-315.
- Inoue, H., Nojima, H., Okayama, H., 1990. High efficiency transformation of *Escherichia coli* with plasmids. Gene 96, 23-28.
- Israelsson, P.H., Quadrini, J.D., Connolly, J.P., 2014. Fate and transport of hydrophobic organic chemicals in the lower Passaic River: Insights from 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin. Estuaries and Coasts 37, 1145-1168.
- Iwai, S., Yamazoe, A., Takahashi, R., Kurisu, F., Yagi, O., 2005. Degradation of mono-chlorinated dibenzo-p-dioxins by *Janibacter* sp. strain YA isolated from river sediment. Curr Microbiol 51, 353-358.
- Jenkins, F.P., Robinson, J.A., Gellatly, J.B., Salmond, G.W., 1972. The no-effect dose of aniline in human subjects and a comparison of aniline toxicity in man and the rat. Food Cosmet Toxicol 10, 671-679.
- Jeong, S.-W., Kim, J., 2015. Aquabacterium olei sp. nov., an oil-degrading bacterium isolated from oil-contaminated soil. International Journal of Systematic and Evolutionary Microbiology 65, 3597-3602.
- Jiang, Y., Wei, L., Yang, K., Shi, X., Wang, H., 2017. Rapid formation of aniline-degrading aerobic granular sludge and investigation of its microbial community succession. Journal of Cleaner Production 166, 1235-1243.
- Jin, S., Zhu, T., Xu, X., Xu, Y., 2006. Biodegradation of dibenzofuran by *Janibacter terrae* strain XJ-1. Curr Microbiol 53, 30-36.
- Kahng, H.Y., Kukor, J.J., Oh, K.H., 2000. Characterization of strain HY99, a novel microorganism capable of aerobic and anaerobic degradation of aniline. FEMS Microbiol Lett 190, 215-221.
- Kalmbach, S., Manz, W., Wecke, J., Szewzyk, U., 1999. *Aquabacterium* gen. nov., with description of Aquabacterium citratiphilum sp. nov., Aquabacterium parvum sp. nov. and Aquabacterium commune sp. nov., three in situ dominant bacterial species from the Berlin drinking water system. Int J Syst Bacteriol 49 Pt 2, 769-777.

- Kaminski, U., Janke, D., Prauser, H., Fritsche, W., 1983. Degradation of aniline and monochloroanilines by *Rhodococcus* sp. An 117 and a *pseudomonad*: a comparative study. Z Allg Mikrobiol 23, 235-246.
- Kao, C.M., Chen, S.C., Liu, J.K., Wu, M.J., 2001. Evaluation of TCDD biodegradability under different redox conditions. Chemosphere 44, 1447-1454.
- Kao, C.M., Wu, M.J., 2000. Enhanced TCDD degradation by Fenton's reagent preoxidation. J Hazard Mater 74, 197-211.
- Kasuga, K., Habe, H., Chung, J.-S., Yoshida, T., Nojiri, H., Yamane, H., Omori, T., 2001. Isolation and characterization of the genes encoding a novel oxygenase component of angular dioxygenase from the gram-positive dibenzofuran-degrader *Terrabacter* sp. strain DBF63. Biochemical and biophysical research communications 283, 195-204.
- Kasuga, K., Nitta, A., Kobayashi, M., Habe, H., Nojiri, H., Yamane, H., Omori, T., Kojima, I., 2013. Cloning of dfdA genes from *Terrabacter* sp. strain DBF63 encoding dibenzofuran 4,4a-dioxygenase and heterologous expression in Streptomyces lividans. Appl Microbiol Biotechnol 97, 4485-4498.
- Kasuga, K., Nojiri, H., Yamane, H., Kodama, T., Omori, T., 1997. Cloning and characterization of the genes involved in the degradation of dibenzofuran by *Terrabacter* sp. strain DBF63. J Ferment Bioeng 84, 387-399.
- Kazumi, J., Häggblom, M.M., Young, L.Y., 1995. Degradation of Monochlorinated and Nonchlorinated Aromatic Compounds under Iron-Reducing Conditions. Appl Environ Microbiol 61, 4069-4073.
- Keim, T., Francke, W., Schmidt, S., Fortnagel, P., 1999. Catabolism of 2,7-dichloro- and 2,4,8-trichlorodibenzofuran by *Sphingomonas* sp strain RW1. J Ind Microbiol Biot 23, 359-363.
- Khan, M.F., Wu, X., Kaphalia, B.S., Boor, P.J., Ansari, G., 2003. Nitrotyrosine formation in splenic toxicity of aniline. Toxicology 194, 95-102.
- Khan, M.F., Wu, X., Kaphalia, B.S., Boor, P.J., Ansari, G.A., 1997. Acute hematopoietic toxicity of aniline in rats. Toxicol Lett 92, 31-37.
- Klečka, G., Gibson, D.J.B.J., 1979. Metabolism of dibenzo [1, 4] dioxan by a *Pseudomonas* species. Biochem. J. 180, 639-645.
- Klečka, G.M., Gibson, D.T.J.A., microbiology, e., 1980. Metabolism of dibenzo-p-dioxin and chlorinated dibenzo-p-dioxins by a *Beijerinckia* species. Appl Environ Microbiol 39, 288-296.
- Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic acids research 41, e1-e1.
- Konopka, A., 1993. Isolation and Characterization of a Subsurface Bacterium That Degrades Aniline and Methylanilines. FEMS Microbiol Lett 111, 93-99.
- Konopka, A., Knight, D., Turco, R.F., 1989. Characterization of a *Pseudomonas* sp. Capable of Aniline Degradation in the Presence of Secondary Carbon Sources. Appl Environ Microbiol 55, 385-389.
- Kosson, D.S., Byrne, S.V., 1995. Interactions of aniline with soil and groundwater at an industrial spill site. Environ Health Persp 103, 71-73.

- Kubota, M., Kawahara, K., Sekiya, K., Uchida, T., Hattori, Y., Futamata, H., Hiraishi, A., 2005. *Nocardioides aromaticivorans* sp. nov., a dibenzofuran-degrading bacterium isolated from dioxin-polluted environments. Syst Appl Microbiol 28, 165-174.
- Kuhn, E.P., Suflita, J.M., 1989. Sequential Reductive Dehalogenation of Chloroanilines by Microorganisms from a Methanogenic Aquifer. Environ Sci Technol 23, 848-852.
- Kuokka, S., Rantalainen, A.L., Häggblom, M.M., 2014. Anaerobic reductive dechlorination of 1,2,3,4-tetrachlorodibenzofuran in polychlorinated dibenzo-p-dioxin- and dibenzofuran-contaminated sediments of the Kymijoki River, Finland. Chemosphere 98, 58-65.
- Kurt, Z., Spain, J.C., 2013. Biodegradation of chlorobenzene, 1,2-dichlorobenzene, and 1,4-dichlorobenzene in the vadose zone. Environ Sci Technol 47, 6846-6854.
- Lang, E., Kroppenstedt, R.M., Swiderski, J., Schumann, P., Ludwig, W., Schmid, A., Weiss, N., 2003. Emended description of *Janibacter terrae*, including ten dibenzofuran-degrading strains and *Janibacter brevis* as its later heterotypic synonym. Int J Syst Evol Microbiol 53, 1999-2005.
- Larke-Mejia, N.L., Crombie, A.T., Pratscher, J., McGenity, T.J., Murrell, J.C., 2019. Novel Isoprene-Degrading Proteobacteria From Soil and Leaves Identified by Cultivation and Metagenomics Analysis of Stable Isotope Probing Experiments. Front Microbiol 10, 2700.
- Latorre, J., Reineke, W., Knackmuss, H.J., 1984. Microbial-Metabolism of Chloroanilines - Enhanced Evolution by Natural Genetic Exchange. Archives of Microbiology 140, 159-165.
- Li, C., Zheng, M., Zhang, B., Gao, L., Liu, L., Zhou, X., Ma, X., Xiao, K., 2012. Long-term persistence of polychlorinated dibenzo-p-dioxins and dibenzofurans in air, soil and sediment around an abandoned pentachlorophenol factory in China. Environ Pollut 162, 138-143.
- Li, H., Lee, L.S.J.E.s., technology, 1999. Sorption and abiotic transformation of aniline and α-naphthylamine by surface soils. Environ. Sci. Technol. 33, 1864-1870.
- Li, Y., 2014. Biotransformation of aniline, para-chloroaniline and pentachloronitrobenzene and environmental implications, Rutgers University-Graduate School-New Brunswick.
- Liang, H.-y., Zhang, Y.-q., Huang, S.-b., Hussain, I., 2013. Oxidative degradation of p-chloroaniline by copper oxidate activated persulfate. Chemical Engineering Journal 218, 384-391.
- Lide, D.R., 1991. PhD., CRC Handbook of Chemistry and Physics. CRC Press Boca Raton Ann Arbor Boston.
- Liu, H., Park, J.W., Häggblom, M.M., 2014. Enriching for microbial reductive dechlorination of polychlorinated dibenzo-p-dioxins and dibenzofurans. Environ Pollut 184, 222-230.
- Liu, Y.B., Qu, D., Wen, Y.J., Ren, H.J., 2015. Low-temperature biodegradation of aniline by freely suspended and magnetic modified *Pseudomonas migulae* AN-1. Appl Microbiol Biotechnol 99, 5317-5326.

- Liu, Z., Yang, H., Huang, Z., Zhou, P., Liu, S.J., 2002. Degradation of aniline by newly isolated, extremely aniline-tolerant *Delftia* sp. AN3. Appl Microbiol Biotechnol 58, 679-682.
- Lohman, K., Seigneur, C., 2001. Atmospheric fate and transport of dioxins: local impacts. Chemosphere 45, 161-171.
- Lohmann, R., Nelson, E., Eisenreich, S.J., Jones, K.C., 2000. Evidence for dynamic air− water exchange of PCDD/Fs: A study in the Raritan Bay/Hudson River Estuary. Environ Sci Technol 34, 3086-3093.
- Loidl, M., Hinteregger, C., Ditzelmuller, G., Ferschl, A., Streichsbier, F., 1990. Degradation of Aniline and Monochlorinated Anilines by Soil-Born *Pseudomonas-Acidovorans* Strains. Archives of Microbiology 155, 56-61.
- Loyola-Sepúlveda, R., Salamanca, M.O., Gutiérrez-Baeza, F., Chandia, C.V., Bravo-Linares, C., Mudge, S.M., 2018. Contributions of dioxins and furans to the urban sediment signature: The role of atmospheric particles. Science of the total Environment 615, 751-760.
- Lu, Y., Conrad, R., 2005. In situ stable isotope probing of methanogenic archaea in the rice rhizosphere. Science 309, 1088-1090.
- Lueders, T., Manefield, M., Friedrich, M.W., 2004. Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. Environmental Microbiology 6, 73-78.
- Lyons, C.D., Katz, S., Bartha, R., 1984. Mechanisms and pathways of aniline elimination from aquatic environments. Appl Environ Microbiol 48, 491-496.
- Lyons, C.D., Katz, S.E., Bartha, R., 1985. Persistence and mutagenic potential of herbicide-derived aniline residues in pond water. Bull Environ Contam Toxicol 35, 696-703.
- Mackay, D., Shiu, W.-Y., Lee, S.C., 2006. Handbook of physical-chemical properties and environmental fate for organic chemicals. CRC press.
- Manefield, M., Whiteley, A.S., Griffiths, R.I., Bailey, M.J., 2002. RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. Appl Environ Microbiol. 68, 5367-5373.
- Masuda, H., Shiwa, Y., Yoshikawa, H., Zylstra, G.J., 2014. Draft Genome Sequence of the Versatile Alkane-Degrading Bacterium Aquabacterium sp. Strain NJ1. Genome Announc 2, e01271-01214.
- McDonald, I.R., Radajewski, S., Murrell, J.C., 2005. Stable isotope probing of nucleic acids in methanotrophs and methylotrophs: a review. Organic Geochemistry 36, 779-787.
- New York State Department of Environmental Conservation 1998. New York State -Human Health Fact Sheet **substrate:** Aniline CAS registry number**:** 62-53-3.
- Messeguer, A., 2011. Potential implication of aniline derivatives in the Toxic Oil Syndrome (TOS). Chemico-Biological Interactions 192, 136-141.
- Miller, T.R., Delcher, A.L., Salzberg, S.L., Saunders, E., Detter, J.C., Halden, R.U., 2010. Genome sequence of the dioxin-mineralizing bacterium *Sphingomonas wittichii* RW1. J Bacteriol 192, 6101-6102.

- Minard, R.D., Russel, S., Bollag, J.M., 1977. Chemical transformation of 4-chloroaniline to a triazene in a bacterial culture medium. J Agric Food Chem 25, 841-844.
- Miyauchi, K., Sukda, P., Nishida, T., Ito, E., Matsumoto, Y., Masai, E., Fukuda, M., 2008. Isolation of dibenzofuran-degrading bacterium, *Nocardioides* sp. DF412, and characterization of its dibenzofuran degradation genes. J Biosci Bioeng 105, 628-635.
- Monna, L., Omori, T., Kodama, T., 1993. Microbial degradation of dibenzofuran, fluorene, and dibenzo-p-dioxin by *Staphylococcus auriculans* DBF63. Appl Environ Microbiol 59, 285-289.
- Morris, S.A., Radajewski, S., Willison, T.W., Murrell, J.C., 2002. Identification of the functionally active methanotroph population in a peat soil microcosm by stable-isotope probing. Appl Environ Microbiol. 68, 1446-1453.
- Nam, I.H., Kim, Y.M., Schmidt, S., Chang, Y.S., 2006. Biotransformation of 1,2,3-tri- and 1,2,3,4,7,8-hexachlorodibenzo-p- dioxin by *Sphingomonas wittichii* strain RW1. Appl Environ Microbiol 72, 112-116.
- Narihiro, T., Kaiya, S., Futamata, H., Hiraishi, A., 2010. Removal of polychlorinated dioxins by semi-aerobic fed-batch composting with biostimulation of "*Dehalococcoides*". J Biosci Bioeng 109, 249-256.
- Nelson, J.L., Fung, J.M., Cadillo-Quiroz, H., Cheng, X., Zinder, S.H., 2011. A role for *Dehalobacter* spp. in the reductive dehalogenation of dichlorobenzenes and monochlorobenzene. Environ Sci Technol 45, 6806-6813.
- Neufeld, J.D., Vohra, J., Dumont, M.G., Lueders, T., Manefield, M., Friedrich, M.W., Murrell, J.C., 2007. DNA stable-isotope probing. Nature protocols 2, 860.
- Niittynen, M., Simanainen, U., Syrjala, P., Pohjanvirta, R., Viluksela, M., Tuomisto, J., Tuomisto, J.T., 2007. Differences in acute toxicity syndromes of 2,3,7,8-tetrachlorodibenzo-p-dioxin and 1,2,3,4,7,8-hexachlorodibenzo-p-dioxin in rats. Toxicology 235, 39-51.
- Nitisakulkan, T., Oku, S., Kudo, D., Nakashimada, Y., Tajima, T., Vangnai, A.S., Kato, J., 2014. Degradation of chloroanilines by toluene dioxygenase from *Pseudomonas putida* T57. J Biosci Bioeng 117, 292-297.
- Niu, Z., Boggs, J.E., 1984. The Structure of Aniline. J Mol Struc-Theochem 18, 381-389.
- NJDEP. 2012. "DEP Issues Reminder About Ban on Blue Claw Crabbing"; New Jersey Department of Environmental Protection. (accessed on the internet December 2019) https://www.nj.gov/dep/newsrel/2012/12_0088.htm].
- NJDEP and NJDOH. 2019. Fish Smart, Eat Smart A guide to Health Advisories for Eating Fish and Crabs Caught in New Jersey Waters. New Jersey Department of Environmental Protection and New Jersey Department of Health. (accessed on the internet December 2019) https://www.nj.gov/dep/dsr/Fish_Advisories_2019.pdf]
- Nojiri, H., Kamakura, M., Urata, M., Tanaka, T., Chung, J.S., Takemura, T., Yoshida, T., Habe, H., Omori, T., 2002. Dioxin catabolic genes are dispersed on the Terrabacter sp. DBF63 genome. Biochem Biophys Res Commun 296, 233-240.
- Nojiri, H., Omori, T., 2002. Molecular bases of aerobic bacterial degradation of dioxins: involvement of angular dioxygenation. Biosci Biotechnol Biochem 66, 2001-2016.

- O'Neill, F.J., Bromley-Challenor, K.C.A., Greenwood, R.J., Knapp, J.S., 2000. Bacterial growth on aniline: Implications for the biotreatment of industrial wastewater. Water Res 34, 4397-4409.
- Öberg, L.G., Glas, B., Swanson, S.E., Rappe, C., Paul, K.G., 1990. Peroxidase-catalyzed oxidation of chlorophenols to polychlorinated dibenzo-p-dioxins and dibenzofurans. Archives of environmental contamination and toxicology 19, 930-938.
- Osaka, T., Yoshie, S., Tsuneda, S., Hirata, A., Iwami, N., Inamori, Y., 2006. Identification of acetate-or methanol-assimilating bacteria under nitrate-reducing conditions by stable-isotope probing. Microbial Ecology 52, 253-266.
- Oulmi, Y., Braunbeck, T., 1996. Toxicity of 4-chloroaniline in early life-stages of zebrafish (Brachydanio rerio): I. cytopathology of liver and kidney after microinjection. Arch Environ Contam Toxicol 30, 390-402.
- Parette, R., Velinsky, D.J., Pearson, W.N., 2018. Reconstruction of historical 2,3,7,8-tetrachlorodibenzo-p-dioxin discharges from a former pesticide manufacturing plant to the Lower Passaic River. Chemosphere 212, 1125-1132.
- Parsons, J.R., de Bruijne, J.A., Weiland, A.R., 1998. Biodegradation pathway of 2-chlorodibenzo-p-dioxin and 2-chlorodibenzofuran in the biphenyl-utilising strain JB1. Chemosphere 37, 1915-1922.
- Patrizi, B., Cumis, M.S., Viciani, S., D'Amato, F., Foggi, P., 2014. Characteristic vibrational frequencies of toxic polychlorinated dibenzo-dioxins and -furans. J Hazard Mater 274, 98-105.
- Peng, P., Yang, H., Jia, R., Li, L., 2013. Biodegradation of dioxin by a newly isolated *Rhodococcus* sp. with the involvement of self-transmissible plasmids. Appl Microbiol Biotechnol 97, 5585-5595.
- Pohjanvirta, R., Miettinen, H., Sankari, S., Hegde, N., Linden, J., 2012. Unexpected gender difference in sensitivity to the acute toxicity of dioxin in mice. Toxicol Appl Pharmacol 262, 167-176.
- Pohjanvirta, R., Unkila, M., Tuomisto, J., 1993. Comparative acute lethality of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), 1, 2, 3, 7, 8-pentachlorodibenzo-p-dioxin and 1, 2, 3, 4, 7, 8-hexachlorodibenzo-p-dioxin in the most TCDD-susceptible and the most TCDD-resistant rat strain. Pharmacology & toxicology 73, 52-56.
- Poste, A.E., Grung, M., Wright, R.F., 2014. Amines and amine-related compounds in surface waters: a review of sources, concentrations and aquatic toxicity. Sci Total Environ 481, 274-279.
- Radajewski, S., Ineson, P., Parekh, N.R., Murrell, J.C., 2000. Stable-isotope probing as a tool in microbial ecology. Nature 403, 646-649.
- Rahman, K., Banat, I., Thahira, J., Thayumanavan, T., Lakshmanaperumalsamy, P., 2002. Bioremediation of gasoline contaminated soil by a bacterial consortium amended with poultry litter, coir pith and rhamnolipid biosurfactant. Bioresource Technology 81, 25-32.
- Sakaki, T., Munetsuna, E., 2010. Enzyme systems for biodegradation of polychlorinated dibenzo-p-dioxins. Appl Microbiol Biotechnol 88, 23-30.
- Sanchez, M., Wolfger, H., Getoff, N., 2002. Radiation-induced degradation of 4-chloroaniline in aqueous solution. Radiat Phys Chem 65, 611-620.

- Schecter, A., 2013. Dioxins and health. Springer Science & Business Media.
- Schecter, A., Birnbaum, L., Ryan, J.J., Constable, J.D., 2006. Dioxins: an overview. Environ Res 101, 419-428.
- Schnell, S., Bak, F., Pfennig, N., 1989. Anaerobic degradation of aniline and dihydroxybenzenes by newly isolated sulfate-reducing bacteria and description of *Desulfobacterium anilini*. Arch Microbiol 152, 556-563.
- Sheffield, A., 1985. Sources and releases of PCDD's and PCDF's to the Canadian environment. Chemosphere 14, 811-814.
- Sheludchenko, M.S., Kolomytseva, M.P., Travkin, V.M., Akimov, V.N., Golovleva, L.A., 2005. Degradation of aniline by *Delftia tsuruhatensis* 14S in batch and continuous processes]. Prikl Biokhim Mikrobiol 41, 530-534.
- Shen, J.Y., Zhang, C., Sun, X.Y., Li, J.S., Wang, L.J., 2011. Enhanced Biodegradation of Aniline under Anoxic Condition. Advanced Materials Research 356-360, 25-30.
- Siegrist, R.L., Crimi, M., Brown, R.A., 2011. In situ chemical oxidation: Technology description and status, In Situ Chemical Oxidation for Groundwater Remediation. Springer, pp. 1-32.
- Sorg, O., 2014. AhR signalling and dioxin toxicity. Toxicol Lett 230, 225-233.
- Sun, W., Cupples, A.M., 2012. Diversity of five anaerobic toluene-degrading microbial communities investigated using stable isotope probing. Appl Environ Microbiol. 78, 972-980.
- Sun, W., Li, Y., McGuinness, L.R., Luo, S., Huang, W., Kerkhof, L.J., Mack, E.E., Häggblom, M.M., Fennell, D.E., 2015. Identification of Anaerobic Aniline-Degrading Bacteria at a Contaminated Industrial Site. Environ Sci Technol 49, 11079-11088.
- Surovtseva, E., Ivoylov, V., Karasevich, Y., 1986. Metabolism of chlorinated anilines in *Pseudomonas diminuta*. Microbiology. 55, 591-595.
- Suzuki, H., Kido, T., Okamoto, R., Nhu, D.D., Nishijo, M., Nakagawa, H., Tawara, K., Horikawa, H., Sato, Y., Dung, P.T., Thom L.H., Hung, N.N., 2014. The relationship between dioxin congeners in the breast milk of Vietnamese women and sister chromatid exchange. Int J Mol Sci 15, 7485-7499.
- Tanaka, T., Hachiyanagi, H., Yamamoto, N., Iijima, T., Kido, Y., Uyeda, M., Takahama, K., 2009. Biodegradation of Endocrine-Disrupting Chemical Aniline by Microorganisms. J Health Sci 55, 625-630.
- Thijs, S., Op De Beeck, M., Beckers, B., Truyens, S., Stevens, V., Van Hamme, J.D., Weyens, N., Vangronsveld, J., 2017. Comparative evaluation of four bacteria-specific primer pairs for 16S rRNA gene surveys. Frontiers in Microbiology 8, 494.
- Tongarun, R., Luepromchai, E., Vangnai, A.S., 2008. Natural attenuation, biostimulation, and bioaugmentation in 4-chloroaniline-contaminated soil. Curr Microbiol 56, 182-188.
- Toussaint, M., Van Herwijnen, R., Parsons, J.R., 1998. Anaerobic bacterial dehalogenation of polyhalogenated dioxins and furans, Biodegradation of Dioxins and Furans. Springer, pp. 29-60.

- Travis, A.S., 1997. Poisoned groundwater and contaminated soil: the tribulations and trial of the first major manufacturer of aniline dyes in Basel. Environmental History 2, 343-365.
- Travkin, V., Baskunov, B.P., Golovlev, E.L., Boersma, M.G., Boeren, S., Vervoort, J., van Berkel, W.J.H., Rietjens, I.M.C.M., Golovleva, L.A., 2002. Reductive deamination as a new step in the anaerobic microbial degradation of halogenated anilines. FEMS Microbiol Lett 209, 307-312.
- Trotter, J., Whitlow, S., Zobel, T., 1966. The crystal and molecular structure of p-chloroaniline. Inorg. Phys. Theor., 353-356.
- Tu, L.-K., Wu, Y.-L., Wang, L.-C., Chang-Chien, G.-P., 2011. Distribution of polybrominated dibenzo-p-dioxins and dibenzofurans and polybrominated diphenyl ethers in a coal-fired power plant and two municipal solid waste incinerators. Aerosol and Air Quality Resarch 11, 596-615.
- Turner, P.E., Cooper, V.S., Lenski, R.E., 1998. Tradeoff between horizontal and vertical modes of transmission in bacterial plasmids. Evolution 52, 315-329.
- Tyagi, M., da Fonseca, M.M.R., de Carvalho, C.C., 2011. Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. Biodegradation 22, 231-241.
- Urata, M., Uchida, E., Nojiri, H., Omori, T., Obo, R., Miyaura, N., Ouchiyama, N., 2004. Genes involved in aniline degradation by *Delftia acidovorans* strain 7N and its distribution in the natural environment. Biosci Biotechnol Biochem 68, 2457-2465.
- Urbaniak, M., 2013. Biodegradation of PCDDs/PCDFs and PCBs, Biodegradation Engineering and Technology.
- USEPA, I., 2011. Integrated risk information system. Environmental protection agency region I. United State Environmental Protection Agency, Washington, DC.
- USEPA. 2014. Title of the report. [URL: (accessed on the internet December 2019) www.epa.gov/region2/passaicriver/pdf/prap_overview_fact_sheet_english_june_2014.pdf.
- USEPA. 2016. Record of Decision. Lower 8.3 Miles of the Lower Passaic River Part of the Diamond Alkali Superfund Site Essex and Hudson Counties, New Jersey. (accessed on the internet December 2019) https://semspub.epa.gov/work/02/396055.pdf]
- USEPA. Health and Environmental Effects Profile for Aniline. U.S. Environmental Protection Agency, Washington, D.C., EPA/600/X-85/356 (NTIS PB88174974).
- Van den Berg, M., Birnbaum, L.S., Denison, M., De Vito, M., Farland, W., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A., Haws, L., Rose, M., Safe, S., Schrenk, D., Tohyama, C., Tritscher, A., Tuomisto, J., Tysklind, M., Walker, N., Peterson, R.E., 2006. The 2005 World Health Organization reevaluation of human and Mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicol Sci 93, 223-241.
- Van den Berg, M., Denison, M.S., Birnbaum, L.S., DeVito, M.J., Fiedler, H., Falandysz, J., Rose, M., Schrenk, D., Safe, S., Tohyama, C., 2013. Polybrominated dibenzo-p-dioxins, dibenzofurans, and biphenyls: inclusion in the toxicity equivalency factor concept for dioxin-like compounds. Toxicological Sciences 133, 197-208.

- Vangnai, A.S., Petchkroh, W., 2007. Biodegradation of 4-chloroaniline by bacteria enriched from soil. FEMS Microbiol Lett 268, 209-216.
- Vargas, C., Fennell, D., Häggblom, M., 2001. Anaerobic reductive dechlorination of chlorinated dioxins in estuarine sediments. Appl Microbiol Biot 57, 786-790.
- Wagner, H.-C., Schramm, K.-W., Hutzinger, O., 1990. Biogenes polychloriertes dioxin aus trichlorphenol. Umweltwissenschaften und Schadstoff-Forschung 2, 63-65.
- Wang, D., Zheng, G., Wang, S., Zhang, D., Zhou, L., 2011. Biodegradation of aniline by Candida tropicalis AN1 isolated from aerobic granular sludge. Journal of Environmental Sciences 23, 2063-2068.
- Wang, Y., Gao, H., Na, X.L., Dong, S.Y., Dong, H.W., Yu, J., Jia, L., Wu, Y.H., 2016. Aniline Induces Oxidative Stress and Apoptosis of Primary Cultured Hepatocytes. Int J Environ Res Public Health 13.
- Widada, J., Nojiri, H., Yoshida, T., Habe, H., Omori, T., 2002. Enhanced degradation of carbazole and 2,3-dichlorodibenzo-p-dioxin in soils by *Pseudomonas resinovorans* strain CA10. Chemosphere 49, 485-491.
- Widdel, F., 2007. Theory and measurement of bacterial growth. Di dalam Grundpraktikum Mikrobiologie 4, 1-11.
- Wilkes, H., Wittich, R., Timmis, K.N., Fortnagel, P., Francke, W., 1996. Degradation of Chlorinated Dibenzofurans and Dibenzo-p-Dioxins by *Sphingomonas* sp. Strain RW1. Appl Environ Microbiol 62, 367-371.
- Wilson, F.P., Liu, X., Mattes, T.E., Cupples, A.M., 2016. *Nocardioides, Sediminibacterium, Aquabacterium, Variovorax, and Pseudomonas* linked to carbon uptake during aerobic vinyl chloride biodegradation. Environ Sci Pollut Res Int 23, 19062-19070.
- Wittich, R.-M., 1998. Aerobic degradation by bacteria of dibenzo-p-dioxins, dibenzofurans, diphenyl ethers and their halogenated derivatives, Biodegradation of Dioxins and Furans. Springer, pp. 1-28.
- Wittich, R.M., Strompl, C., Moore, E.R., Blasco, R., Timmis, K.N., 1999. Interaction of *Sphingomonas and Pseudomonas* strains in the degradation of chlorinated dibenzofurans. J Ind Microbiol Biotechnol 23, 353-358.
- Wittich, R.M., Wilkes, H., Sinnwell, V., Francke, W., Fortnagel, P., 1992. Metabolism of dibenzo-p-dioxin by *Sphingomonas* sp. strain RW1. Appl Environ Microbiol 58, 1005-1010.
- Yen, J.-H., Tsai, P.-W., Chen, W.-C., Wang, Y.-S., 2008. Fate of anilide and aniline herbicides in plant-materials-amended soils. Journal of Environmental Science and Health, Part B 43, 382-389.
- Yoshida, N., Takahashi, N., Hiraishi, A., 2005. Phylogenetic characterization of a polychlorinated-dioxin- dechlorinating microbial community by use of microcosm studies. Appl Environ Microbiol 71, 4325-4334.
- You, I.S., Bartha, R., 1982. Metabolism of 3,4-Dichloroaniline by *Pseudomonas Putida*. J Agr Food Chem 30, 274-277.
- Zanaroli, G., Negroni, A., Häggblom, M.M., Fava, F., 2015. Microbial dehalogenation of organohalides in marine and estuarine environments. Curr Opin Biotechnol 33, 287-295.

- Zeyer, J., Kearney, P.C., 1982. Microbial-Degradation of Para-Chloroaniline as Sole Carbon and Nitrogen-Source. Pestic Biochem Phys 17, 215-223.
- Zeyer, J., Wasserfallen, A., Timmis, K.N., 1985. Microbial mineralization of ring-substituted anilines through an ortho-cleavage pathway. Appl Environ Microbiol 50, 447-453.
- Zhang, G.X., Ren, S.Z., Xu, M.Y., Zeng, G.Q., Luo, H.D., Chen, J.L., Tan, Z.Y., Sun, G.P., 2011a. *Rhizobium borbori* sp. nov., aniline-degrading bacteria isolated from activated sludge. Int J Syst Evol Microbiol 61, 816-822.
- Zhang, L.L., He, D., Chen, J.M., Liu, Y., 2010. Biodegradation of 2-chloroaniline, 3-chloroaniline, and 4-chloroaniline by a novel strain *Delftia tsuruhatensis* H1. J Hazard Mater 179, 875-882.
- Zhang, S., Wan, R., Wang, Q., Xie, S., 2011b. Identification of anthracene degraders in leachate-contaminated aquifer using stable isotope probing. International Biodeterioration & Biodegradation 65, 1224-1228.
- Zhang, T., Zhang, J., Liu, S., Liu, Z., 2008. A novel and complete gene cluster involved in the degradation of aniline by *Delftia sp*. AN3. Journal of Environmental Sciences 20, 717-724.
- Zhang, Y., Tran, H.P., Hussain, I., Zhong, Y., Huang, S., 2015. Degradation of p -chloroaniline by pyrite in aqueous solutions. Chemical Engineering Journal 279, 396-401.
- Zhao, Y.Y., Tao, F.M., Zeng, E.Y., 2008. Theoretical study of the quantitative structure-activity relationships for the toxicity of dibenzo-p-dioxins. Chemosphere 73, 86-91.
- Zhu, J., Chen, C., Li, Y., Zhou, L., Lan, Y., 2019. Rapid degradation of aniline by peroxydisulfate activated with copper-nickel binary oxysulfide. Separation and Purification Technology 209, 1007-1015.
- Zhu, L., Lv, M., Dai, X., Xu, X., Qi, H., Yu, Y., 2012. Reaction kinetics of the degradation of chloroanilines and aniline by aerobic granule. Biochemical Engineering Journal 68, 215-220.

- https://www.eenews.net/stories/1060055900 (accessed on the internet February 2020)
- http://usa.chinadaily.com.cn/epaper/2013-01/11/content_16105865.htm (accessed February 24, 2020
- http://www.chinadaily.com.cn/china/////2013-04/08/content_16384273.htm (accessed February 24, 2020)

Appendices

Table 1A Fractions with refractive index and buoyant density values replicate1 ^{12}C set. Highlighted fractions were subject for qPCR analysis. Red highlighted fractions were sent for sequencing, every three adjacent fractions were combined together and sent for sequencing as one sample.

Fraction	Refractive Index	Buoyant Density (g/mL)
1	1.4092	1.80673476
2	1.4094	1.80890682
3	1.4087	1.80130461
4	1.408	1.7937024
5	1.4071	1.78392813
6	1.4068	1.78067004
7	1.4062	1.77415386
8	1.4056	1.76763768
9	1.4049	1.76003547
10	1.4046	1.75677738
11	1.4041	1.75134723
12	1.4035	1.74483105
13	1.4031	1.74048693
14	1.4025	1.73397075
15	1.402	1.7285406
16	1.4015	1.72311045
17	1.4015	1.72311045
18	1.4002	1.70899206
19	1.3989	1.69487367
20	1.3967	1.67098101
21	1.392	1.6199376
22	1.3844	1.53739932
23	1.3741	1.42553823
24	1.3632	1.30716096

Table 2A Fractions with refractive index and buoyant density values replicate2 ^{12}C set. Highlighted fractions were subject for qPCR analysis. Red highlighted fractions were sent for sequencing, every three adjacent fractions were combined together and sent for sequencing as one sample.

Fraction	Refractive Index	Buoyant Density (g/mL)
1	1.4076	1.78935828
2	1.4085	1.79913255
3	1.4082	1.79587446
4	1.4073	1.78610019
5	1.4066	1.77849798
6	1.4065	1.77741195
7	1.4054	1.76546562
8	1.4049	1.76003547
9	1.4044	1.75460532
10	1.4039	1.74917517
11	1.4033	1.74265899
12	1.4029	1.73831487
13	1.4024	1.73288472
14	1.4019	1.72745457
15	1.4015	1.72311045
16	1.401	1.7176803
17	1.4006	1.71333618
18	1.4001	1.70790603
19	1.3996	1.70247588
20	1.3994	1.70030382
21	1.3969	1.67315307
22	1.3915	1.61450745
23	1.3809	1.49938827
24	1.3635	1.31041905

Table 3A Fractions with refractive index and buoyant density values replicate 3 ^{12}C set. Highlighted fractions were subject for qPCR analysis. Red highlighted fractions were sent for sequencing, every three adjacent fractions were combined together and sent for sequencing as one sample.

Fraction	Refractive Index	Buoyant Density (g/mL)
1	1.397	1.6742391
2	1.4055	1.76655165
3	1.4071	1.78392813
4	1.4066	1.77849798
5	1.406	1.7719818
6	1.4055	1.76655165
7	1.4049	1.76003547
8	1.4044	1.75460532
9	1.4039	1.74917517
10	1.4033	1.74265899
11	1.4028	1.73722884
12	1.4023	1.73179869
13	1.4019	1.72745457
14	1.4014	1.72202442
15	1.4009	1.71659427
16	1.4003	1.71007809
17	1.3997	1.70356191
18	1.3993	1.69921779
19	1.3989	1.69487367
20	1.3982	1.68727146
21	1.3963	1.66663689
22	1.3912	1.61124936
23	1.3807	1.49721621
24	1.365	1.3267095

Table 4A Fractions with refractive index and buoyant density values replicate1 ^{13}C set. Highlighted fractions were subject for qPCR analysis. Red highlighted fractions were sent for sequencing, every three adjacent fractions were combined together and sent for sequencing as one sample.

Fraction	Refractive Index	Buoyant Density (g/mL)
1	1.4081	1.79478843
2	1.4083	1.79696049
3	1.408	1.7937024
4	1.4075	1.78827225
5	1.407	1.7828421
6	1.4058	1.76980974
7	1.4058	1.76980974
8	1.4053	1.76437959
9	1.4047	1.75786341
10	1.4042	1.75243326
11	1.4038	1.74808914
12	1.4032	1.74157296
13	1.4027	1.73614281
14	1.4023	1.73179869
15	1.4018	1.72636854
16	1.4014	1.72202442
17	1.4009	1.71659427
18	1.4003	1.71007809
19	1.3996	1.70247588
20	1.3973	1.67749719
21	1.3927	1.62753981
22	1.3851	1.54500153
23	1.3739	1.42336617
24	1.3616	1.28978448

Table 5A Fractions with refractive index and buoyant density values replicate 2 ^{13}C set. Highlighted fractions were subject for qPCR analysis. Red highlighted fractions were sent for sequencing, every three adjacent fractions were combined together and sent for sequencing as one sample.

Fraction	Refractive Index	Buoyant Density (g/mL)
1	1.4069	1.78175607
2	1.4085	1.79913255
3	1.4082	1.79587446
4	1.4078	1.79153034
5	1.4074	1.78718622
6	1.4069	1.78175607
7	1.4063	1.77523989
8	1.4059	1.77089577
9	1.4054	1.76546562
10	1.4049	1.76003547
11	1.4044	1.75460532
12	1.4039	1.74917517
13	1.4034	1.74374502
14	1.4029	1.73831487
15	1.4024	1.73288472
16	1.402	1.7285406
17	1.4016	1.72419648
18	1.4011	1.71876633
19	1.4007	1.71442221
20	1.4003	1.71007809
21	1.3997	1.70356191
22	1.398	1.6850994
23	1.3928	1.62862584
24	1.3843	1.53631329

Table 6A Fractions with refractive index and buoyant density values replicate 3 ^{13}C set. Highlighted fractions were subject for qPCR analysis. Red highlighted fractions were sent for sequencing, every three adjacent fractions were combined together and sent for sequencing as one sample.

Fraction	Refractive Index	Buoyant Density (g/mL)
1	1.4046	1.75677738
2	1.4069	1.78175607
3	1.4066	1.77849798
4	1.4062	1.77415386
5	1.4055	1.76655165
6	1.4052	1.76329356
7	1.4047	1.75786341
8	1.4042	1.75243326
9	1.4034	1.74374502
10	1.4032	1.74157296
11	1.4027	1.73614281
12	1.4022	1.73071266
13	1.4015	1.72311045
14	1.4012	1.71985236
15	1.4008	1.71550824
16	1.4003	1.71007809
17	1.3999	1.70573397
18	1.3994	1.70030382
19	1.3991	1.69704573
20	1.3985	1.69052955
21	1.3972	1.67641116
22	1.3928	1.62862584
23	1.3826	1.51785078
24	1.3679	1.35820437