MICROBIAL REDUCTIVE TRANSFORMATION OF PENTACHLORONITROBENZENE

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by

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PENTACHLORONITROBENZENE

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Dedicated to my parents and my husband

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SUMMARY

Pentachloronitrobenzene (PCNB) is an organochlorine fungicide used either as seed dressing or for soil treatment. Research was conducted to investigate the microbial reductive transformation of PCNB with cultures developed from a contaminated estuarine sediment. The biotransformation of PCNB to pentachloroaniline (PCA) occurred under all electron accepting conditions tested. Sequential dechlorination of PCA to di- and in some cases to mono-chlorinated anilines occurred under fermentative/methanogenic conditions. Based on the use of inhibitors, methanogens were not involved in the sequential dechlorination of PCA. Based on 16S rRNA gene analysis, among five known dechlorinating bacterial groups tested, only *Dehalococcoides* was detected in the mixed culture. The sequential dechlorination of PCA was simulated using a branched-chain Michaelis-Menten kinetic model. The dechlorination rate (*k'*) of the chlorinated anilines ranged from 0.25 to 1.19 μM/day and the half-saturation coefficient (K_C) ranged from 0.11 to 1.72 μM at an incubation temperature of 22°C and pH 6.9±0.1. Incubation at different temperature and pH values resulted in significant differences in the biotransformation rate and extent of PCNB in the fermentative/methanogenic enrichment culture. Incubation at 35°C resulted in significantly different product distribution. The effect of temperature on the PCA dechlorination rate was modeled using an Arrhenius relationship. Dechlorination of PCA and methanogenesis were not observed in cultures amended with completely bioavailable iron sources until all Fe^{3+} was reduced to Fe^{2+}. In contrast, PCA dechlorination took place at the same time with iron reduction in the same

mixed, methanogenic culture amended with a less bioavailable iron source (FeOOH). PCA was sequentially dechlorinated to dichloroanilines in cultures amended with low nitrate concentrations, whereas partial dechlorination of PCA to tetrachloroanilines was observed in cultures amended with high initial nitrate concentrations due to the accumulation of reduced nitrogen species (e.g., NO, N_2O). A semi-empirical molecular model (MOPAC/AM1) was used to estimate the thermodynamic and electronic properties of all chlorinated aniline congeners. These values were used to predict the sequential PCA dechlorination pathway and compare to experimentally observed dechlorination reactions. The results of this study have significant environmental implications relative to the fate and transport of PCNB, PCA and its dechlorination products in subsurface systems.

CHAPTER 1

INTRODUCTION

Anthropogenic hydrophobic organic compounds are widely distributed, environmental contaminants which persist in the environment and pose a chronic threat to the health and safety of humans and wildlife. These compounds were introduced into the environment through agricultural uses, industrial discharges or improper waste disposal practices. A large number of these hydrophobic organic compounds are toxic and suspected carcinogens. Polyhalogenated organic compounds tend to be resistant to biodegradation in aerobic environments. These compounds are strongly bound to the solid phase (soils or sediments) as a result of their low aqueous solubility and high hydrophobicity. Such compounds are more oxidized than their nonhalogenated counterparts due to the presence of highly electronegative halogen substituents, which provide molecular stability. Studies on the biodegradation of these compounds showed that anaerobic microorganisms can sequentially remove halogens by reductive dehalogenation rendering such compounds more accessible for aerobic degradation or mineralization. To date, research has focused on either the biodegradation of halogenated (Mohn and Tiedje, 1992; Holliger *et al.*, 1999) or nitroaromatic compounds (Spain, 1995; Spain *et al.*, 2000). Despite the fact that chloronitroaromatic compounds are extensively used in industry and agriculture (e.g., pesticides, fungicides, pharmaceuticals, dyes) and found worldwide in surface and subsurface soils, as well as streambed sediments, studies on their fate and transformation have been rare, especially for polychloronitro-substituted

compounds. Therefore, there is a need for the investigation of the biotransformation of chlorinated nitroaromatic compounds. To address and explore this paucity of information, the focus of the research was to elucidate the reductive biotransformation of pentachloronitrobenzene (PCNB) and chloroanilines (CAs) resulting from the reductive (bio)transformation of PCNB.

PCNB is an organochlorine fungicide and is extensively used as a fungicide for the prevention and control of certain soil-borne diseases on golf courses, sod farms, home lawns and institutional areas where turf is grown. As a result of its worldwide extensive use, PCNB is widespread and found in every environmental compartment (i.e., soil, water, and air). In a report published by the USGS on the occurrence of semivolatile organic compounds in streambed sediments of twenty major river basins across the United States, PCNB was detected at a maximum concentration of 180 µg/kg (Lopes and Furlong, 2001). PCNB is included in the US EPA's Toxicity Class III chemicals list and is among the thirty chemicals included in the US EPA's list of Waste Minimization Priority Chemicals. The US EPA has classified PCNB as a possible cancer causing substance (U.S. EPA, 2003).

Despite the fact that PCNB is widely used, there is a lack of information on the biodegradation potential and environmental consequences of PCNB and its metabolites under anoxic/anaerobic conditions typically encountered in water-saturated soils and sediments. The purpose of the research presented here was to investigate the biotransformation potential of PCNB under anoxic/anaerobic conditions.

The research commenced with the development of a fermentative/methanogenic PCNB-transforming, sediment-free enrichment culture. The culture transformed PCNB

first to pentachloroaniline (PCA) which was then sequentially dechlorinated to dichlorinated anilines (DCAs) under fermentative/methanogenic conditions. Abiotic assays were also performed to assess the transformation potential of PCNB in the absence of microbial activity (Chapter 5). The sediment-free enrichment culture was physiologically characterized by using specific inhibitors as well as specific electron donors. Molecular characterization of the culture was also performed to determine the microbial communities responsible for the reductive PCNB biotransformation (Chapter 6). Pathway and kinetic analysis of the sequential PCA dechlorination was performed. The microbial reductive dechlorination kinetics of PCA and all other chlorinated anilines were investigated individually. The effect of physicochemical factors such as temperature and pH on the PCNB biotransformation kinetics and pathway was investigated (Chapter 7). The effect of alternative electron acceptors on the reductive biotransformation of PCNB was tested with different electron acceptors such as nitrate and iron. PCNB biotransformation in the presence of completely bioavailable iron, less bioavailable iron, and nitrate was investigated with the fermentative/methanogenic enrichment culture (Chapter 8 and 9). Furthermore, the biotransformation potential of PCNB under denitrifying conditions, as well as its inhibitory effect on enriched denitrifying cultures were investigated (Chapter 9). Finally, semi-empirical molecular modeling of PCA and all chlorinated aniline congeners was performed to develop a quantitative theoretical prediction of the sequential reductive dechlorination pathway of PCA (Chapter 10).

This research sheds light on the significance of biological processes on the fate and transport of chlorinated nitroaromatic compounds in soil and sediment systems. In addition to natural systems, the results of this research are applicable to industrial

wastewater treatment efforts where chlorinated nitroaromatic compounds may be of concern. The research presented here provides a better understanding of the (bio)transformation kinetics, products formed, and conditions, which enhance the (bio)transformation of PCNB and its (bio)transformation products.

CHAPTER 2

BACKGROUND

2.1. Introduction

Halogenated organic compounds (HOCs) constitute one of the largest groups of environmental contaminants. The extensive use of HOCs in dyes, pesticides, fire retardants and solvents resulted in widespread environmental contamination either by extensive use (i.e., pesticides that do not degrade rapidly and persist in the environment), or by improper disposal or release. HOCs are strongly bound to the solid phase (soils or sediments) as a result of their low aqueous solubility and high hydrophobicity. Although abiotic and biotic natural attenuation processes may reduce the risks associated with HOCs, their effectiveness is highly variable and depends on contaminant chemical properties, as well as environmental conditions. Many HOCs can result in harmful effects because of their acute or chronic toxicity, or their long-term effects caused by their tendency for bioaccumulation or bioconcentration. Thus, HOCs which persist in the environment pose a chronic threat to the health and safety of humans and wildlife. Resistance to both chemical and biological degradation is one of the properties that have made HOCs useful for industrial and/or agricultural applications. However, the same properties are also the reason for many of the environmental problems related to the use of these compounds (Häggblom and Bossert, 2003).

Chloronitroaromatic compounds are an important class of HOCs. Despite the fact that chloronitroaromatic compounds are extensively used in industry and agriculture and

found worldwide in surface and subsurface soils, as well as streambed sediments, studies on their fate and transformation, especially for polychloronitro-substituted compounds, have been rare as compared to either chlorinated (Mohn and Tiedje, 1992) or nitroaromatic compounds (Spain, 1995; Spain *et al.*, 2000). Pesticides which are used in agriculture, home, or industries to regulate and control various kinds of pests and weeds are one of the major sources of chlorinated nitroaromatic compounds.

Pesticides, (i.e., herbicides, insecticides, fungicides, molluscicides, rodenticides, acaricides, nematicides) are a major source of environmental, nonpoint pollution. Agriculture accounts for 75% of total pesticide use in the U.S. (Larson *et al.*, 1997). A very large amount of pesticides is produced all over the world every year. For instance, in the U.S., in 1992, over a billion pounds of pesticides were sold (Kamrin, 1997). The fractions of the different types of pesticides used are as follows: 58.7% (647 million pounds) herbicides, 23.2% (255 million pounds) insecticides, 10.9% (120 million pounds) fungicides, and 7.2% (80 million pounds) other pesticides. The pesticide usage rate is expected to increase with a rate of about 1 to 2% per year (Kamrin, 1997). After their release into the environment, pesticides may follow different routes according to their properties, and how and when they are released. From the millions of tons of pesticides applied annually on various crops, a large fraction is deposited on the soil and nontarget organisms, as well as moving into the atmosphere and water (Pimentel and Levitan, 1986).

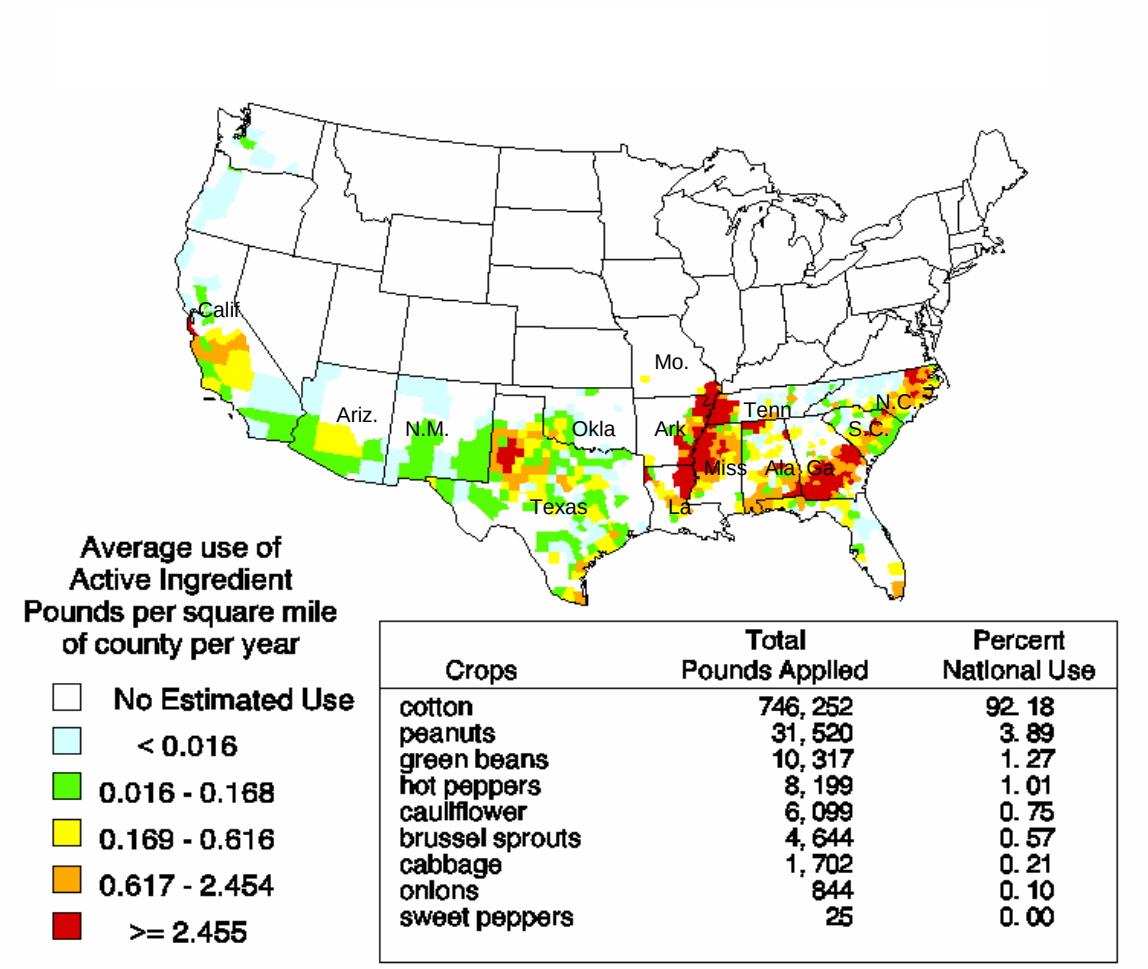
Pentachloronitrobenzene (PCNB) is an organochlorine fungicide used either as a seed dressing or for soil treatment to control a wide range of phytopathogenic fungi in crops such as potatoes, wheat, onions, lettuce, tomatoes, tulips, garlic and others, as well

as on grass, lawn flowers, ornamental shrubs and gardens (U.S. EPA, 2003a; Howard, 1991). PCNB is used to control various diseases caused by *Rhizoctonia solani,* as well as other fungal pathogens (e.g., *Botrytis* spp., *Rhizopus* spp., *Aspergillus* spp., *Pellicularia* spp., *Fusarium* spp.). PCNB is used as the key chemical in controlling karnal bunt (caused by *Tilletia caries*) in wheat (Sutton *et al*., 2004). In addition, PCNB is extensively used to prevent slime formation in industrial waters (Lièvremont *et al.*, 1996a). PCNB is mainly used as a protector that coats the surface of the plant by contact and protects against pathogen entry, as well as it inhibits production of compounds that have $-NH_2$ and -SH groups. PCNB controls fungal pathogens primarily by stopping spore formation and growth of mycelia, the vegetative part of a fungus. PCNB inhibits fungal cell division and can act as a competitive inhibitor of inositol, a required growth substance of many fungi (GCSAA, 2000). The first laboratory synthesis of PCNB was reported in 1868 (Berkowitz *et al.*, 1976). PCNB was first introduced in Germany as a soil fungicide in the 1930s to replace organomercurial seed dressings (Sinha *et al.*, 1988). PCNB is produced in the USA since 1962 (IARC, 1974). The common name for PCNB is Quintozene. Trade names for PCNB or commercial products containing it are as follows: Avicol, Botrilex, Brassicol, Earthcide, Fartox, Folosan, Fomac 2, Fungiclor, Kobu, Kobutol, KP 2, Olpisan, Pentagen, Terraclor, Terrafum, Tilcarex, Tritisan (IRPTC, 1983).

Although PCNB is used throughout the U.S., most of its usage is found in the southeastern region because of its effectiveness against phytopathogenic fungi associated with crops predominantly found in this region. For example, in 2000, from the total reported PCNB usage of 543,500 lbs of active ingredient, 331,100 lbs (i.e., 61%) were

used in seven States in the region (AL, FL, GA, LA, MS, NC, SC, and TN)(Lopes and Furlong, 2001)(Figure 2.1). Between 1990 and 2000, the estimated average annual total PCNB usage in the U.S. was reported to be approximately 1,825,000 lbs. of active ingredient for 794,000 acres treated (Sutton *et al*., 2004).

Approximately 21.5 metric tons of the technical material is sold annually in the European Union (Syracuse Research Corporation, 2003). PCNB is registered in the United Kingdom, Spain, Greece and Cyprus within the European Union (EU). PCNB is banned in some European countries such as Finland (1996), Germany (1987), Italy (1973), The Netherlands (1979), Poland (1988), and Sweden (1985). As a result of its extensive use worldwide, PCNB is widespread and found in the soil, water and air (Finnegan *et al.*, 1958). PCNB is not expected to be very mobile in most soils due to its tendency to sorb to organic matter. However, on sandy soils which have low organic matter, PCNB may leach to groundwater (Sutton *et al.,* 2004). PCNB can also move to surface waters in runoff and through spray drift (particularly for foliar applications such as to turf). PCNB can also move to areas distant from its use sites via long-range atmospheric transport (Sutton *et al.,* 2004). In a recently published report by the USGS on the occurrence of semivolatile organic compounds in streambed sediments of twenty major river basins across the US, PCNB was detected at a maximum concentration of 180 µg/kg (Lopes and Furlong, 2001). Pentachloroaniline (PCA), the main (bio)transformation product of PCNB under anoxic/anaerobic conditions was detected at a range of 0.1 to 10.1 ppm in soil samples from a field previously treated with PCNB (Sutton *et al*., 2004). Samples from agricultural field soils from Belgium and Denmark were reported to contain PCA at a range of 0.85-2.5 ppm (Sutton *et al*., 2004).



Crops	Total Pounds Applied	Percent National Use
cotton	746, 252	92. 18
peanuts	31, 520	3. 89
green beans	10, 317	1. 27
hot peppers	8, 199	1. 01
cauliflower	6, 099	0. 75
brussel sprouts	4, 644	0. 57
cabbage	1, 702	0. 21
onions	844	0. 10
sweet peppers	25	0. 00

Figure 2.1. USGS national water quality assessment; annual PCNB use map in the US. The map is based on state-level estimates of pesticide use rates for individual crops, which have been compiled by the National Center for Food and Agricultural Policy (NCFAP) for 1995-1998, and on 1997 Census of agriculture county crop acreage (http://ca.water.usgs.gov/cgi-bin/pnsp/pesticide_use_maps_1997.pl?map=W5021).

2.2. Properties and toxicity of PCNB

The molecular structure of PCNB consists of a benzene ring, a nitro group and five chlorine substituents (Figure 2.2). PCNB is a colorless crystalline solid at room temperature and has a melting point of 142 -146°C. The commercial product may have a light-yellow to cream color with a musty odor. PCNB has a low aqueous solubility (0.44 mg/L at 25ºC), a high tendency to partition into natural organic matter (log K_{ow} = 4.64) and a relatively low vapor pressure (5.0 x 10^{-5} mm Hg at 20°C) (Ware, 1982). The reported PCNB solubility values in various solvents are as follows: acetonitrile, 70 g/kg; cyclohexane, 70 g/kg; ethanol, 20 g/kg; ethyl acetate, 210 g/kg; heptane, 30 g/kg; methanol, 20 g/kg; toluene, 1400 g/kg (FAO, 1995).

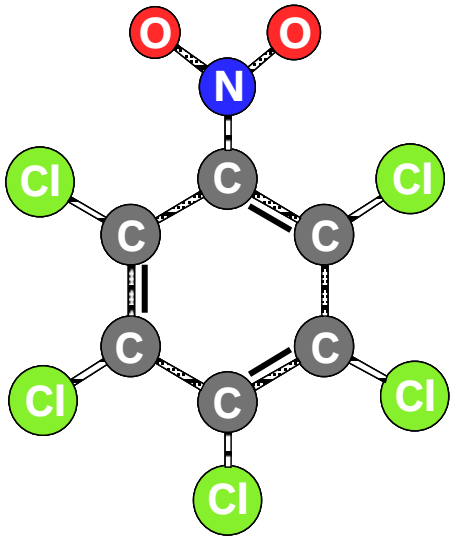


Figure 2.2. Structure of pentachloronitrobenzene (PCNB).

PCNB is included in the U.S. EPA's Toxicity Class III chemicals list and is among the thirty chemicals included in the U.S. EPA's list of "Waste Minimization Priority Chemicals", which includes chemicals found in hazardous waste and are documented contaminants of air, land, water, plants and animals (U.S. EPA, 2003a). The U.S. EPA has classified PCNB as a possible cancer causing substance (Howard, 1991). PCNB is regulated under the Toxic Substances Control Act of 1976 (TSCA), the Federal Insecticide, Fungicide, and Rodenticide Act of 1972 (FIFRA), the Resource Conservation and Recovery Act of 1976 (RCRA), and the Comprehensive Environmental Response, Compensation, and Liability Act of 1980 (CERCLA)(ATSDR, 2001).

The acute toxicity of PCNB is low according to assays conducted with mice (Searle, 1996; Courtney *et al.*, 1976; Marrs and Ballantyne, 2004), but PCNB may be hazardous to humans (Phillips, 2001; Tamura *et al.*, 1995). The estimated human lethal dose is 500-5000 mg/kg (Susarla *et al.*, 1996). According to the American Conference of Governmental Industrial Hygienists, chloronitrobenzenes (CNBs) and chloroanilines (CAs) are among industrial chemicals for which methemoglobin formation is the principal cause of toxicity (U.S. National Library of Medicine, 2002). Therefore, long-term exposure to PCNB may cause methemoglobinemia (Murthy and Kaufman, 1978). PCNB is either not mutagenic or weakly mutagenic based on the limited test information (EXTOXNET, 1993). PCNB has the ability to bind estrogen receptors and therefore it is a potential endocrine-disrupting compound (Zou *et al*. 2002) and is classified as an allergic contact dermatitis causing pesticide (Kamrin, 1997). Although skin irritation after a 48-hour contact was not observed in tests conducted on humans, a sensitivity reaction

has been reported in 20% of the subjects after a second skin exposure which indicates that allergic reactions to repeated exposure may occur (EXTOXNET, 1993).

The effects of long-term exposure of PCNB on humans are not very well known, but animal studies on exposure to PCNB via diet resulted in liver damage (FAO, 1970). According to an assay conducted on workers who had been exposed during the production of PCNB for 2.5 years (one person), 7 years (one person), 8.5 years (one person), or 11 years (10 persons) by monitoring twice-yearly their height, weight, vision, blood pressure, X-ray, hematological parameters (erythrocyte count, hemoglobin, hematocrit, total leukocyte count, hearing, blood chemistry, urinalysis, electrocardiography) no compound-related effects were observed (Clegg and Moretto, 1995). Another group of nine individuals, with exposure for three months (one person), one year (one person), two years (one person), three years (one person), four years (one person), seven years (one person), or 10 years (three persons), who were engaged in the manufacture of PCNB dust formulations were subjected to similar examinations and showed no compound-related abnormalities (Clegg and Moretto, 1995).

PCNB was found to be highly toxic to fish, with LC_{50} values of 0.55 mg/L in rainbow trout and 0.1 mg/L in bluegill sunfish (U.S. National Library of Medicine, 1995). Residues of PCNB were detected in aquatic plants and invertebrates. PCNB residues were also detected in bottom-feeding fish at concentrations as high as 16 µg/kg (Sutton *et al.,* 2004). De Wolf (1992) reported bioconcentration factor (BCF) values ranging from 363 to 1030 for guppies exposed to PCNB. Korte *et al*. (1978) reported PCNB BCF values of 14,000 and 20,000 in algae, and Wang *et al*. (1996) reported a PCNB BCF value of 4508 in *Chlorella fusca.*

2.3. Transformation Processes of HOCs

2.3.1. Abiotic Transformation

Abiotic reductive transformation of HOCs has been investigated by several researchers. van Eekert *et al.* (1998) demonstrated that heat-killed cells of methanogens and anaerobic mixed cultures catalyzed the dechlorination of carbon tetrachloride (CT) predominantly by forming similar products as the living cells. Egli *et al.* (1990) observed tetrachloromethane degradation in the cell-free extracts of *Acetobacterium woodii* similarly to that in whole cell but at a lower rate. They also reported that when *Methanobacterium thermoautotrophicum* or *Acetobacterium woodii* cultures were autoclaved, dechlorination was retained.

Abiotic dehalogenation can be performed by using electron transfer mediators/shuttles to catalyze such reductive transformation under anaerobic conditions. A number of studies reported the reductive dechlorination of a variety of chlorinated organic compounds, such as CT, chlorinated ethenes and ethenes, chlorobenzenes (CBs), and polychlorinated biphenyls (PCBs) utilizing porphyrin derivatives (i.e., vitamin B_{12}, coenzyme F_{430}, hematin, protoporphyrin, uroporphyrin, coproporphyrin, and hematoporphyrin). A variety of metals such as Co, Fe, Ni, Mg, and Mo are associated with reactive metalloporphyrins (Dror and Schlautman, 2003).

Vitamin B_{12}, a cobalt containing complex (cobalamin), is a common cofactor. The heat stable, cobalt-containing cofactor, vitamin B_{12}, was shown to directly catalyze the dechlorination of CT when supplied with an appropriate reducing agent such as Ti(III)-citrate, hydrogen sulfide, dithiothreitol or cysteine (Gantzer and Wackett, 1991; Krone *et al.,* 1991; Chiu and Reinhard, 1996; Assaf-Anid and Lin, 2002). Gantzer and Wackett

(1991) demonstrated that vitamin B_{12} has the capacity to mediate the eight-electron reduction of tetrachloroethene to ethene. They also reported that vitamin B_{12} catalyzed the reductive dechlorination of pentachlorobenzene (PeCB) to 1,2,4,5-tetrachlorobenzene (TeCB) (80%) and 1,2,3,5-TeCB (20%) and dechlorinated a small fraction (12%) of pentachlorophenol (PeCP) to a mixture of 2,3,4,6-tetrachlorophenol (TeCP) (50%) and 2,3,5,6-TeCP (50%). Holliger *et al.* (1993) observed 1,2-dichloroethane dechlorination in media which contained vitamin B_{12} (0.05 mg/L) without *Acetobacterium* sp. cells. However, dechlorination rates were almost 25-fold lower than in the presence of growing cells. Guerrero-Barajas and Field (2005) demonstrated over a 70-fold acceleration of CT conversion in heat killed sludge by the addition of cobalamins.

The nickel containing coenzyme F_{430} of methanogens was shown to act as a catalyst in the reductive dechlorination of CT to chloroform (CF), dichloromethane (DCM), chloromethane (CM), and methane (Gantzer and Wackett, 1991; Krone *et al.,* 1989). It was shown that oxidation of cobalamin and cofactor F_{430} have no impact on the dechlorination capacity once they are rereduced (Holliger *et al.*, 1992; Stroymeyer *et al.*, 1992; Koons *et al.*, 2001).

Recently, extracellular zinc-containing porphorinogen-type molecules found in *Methanosarcina thermophila* were shown to catalyze the reductive dechlorination of CT, CF, tetrachloroethene, and trichloroethene (Koons *et al.*, 2001; Baeseman and Novak, 2001). These cell exudates were effective at a temperature range from 35 to 65°C and the degradation rate increased with an increase of temperature from 35 to 65°C (Koons *et al*, 2001). Baeseman and Novak (2001) investigated the efficiency of cell exudates of

Methanosarcina thermophila at a temperature range form 4 to 23°C and they observed complete degradation of CT without production of any intermediate compounds.

Amonette *et al.* (2000) reported CT dechlorination to CF under anoxic conditions by Fe^{2+} that was sorbed to the surface of goethite (α-FeOOH). However, they did not observe any reaction in the presence of only Fe^{2+} without goethite. Iron-mediated, abiotic dechlorination was demonstrated for compounds such as CT and CF by several researchers (Matheson and Tratnyek, 1994; Kriegman-King and Reinhard, 1992; 1994). Klausen *et al.* (1995) reported the following trend in catalytic activity: magnetite >hematite ~ lepidocrocite > ferrihydrite for the reduction of monochlorinated nitrobenzene by Fe^{2+}. The reductive dechlorination of CT by the reducing ions $Fe^{2+}$and S^{2-} in homogeneous systems resulted in the formation of variable amounts of the mono- and di- chlorinated products, CF, and dichloromethane (Assaf-Anid and Lin, 2002). Assaf-Anid and Lin (2002) also observed dechlorination products in heterogeneous systems where freshly precipitated ferrous sulfide (FeS) (75 - 200 mM) acted as the bulk reductant. The addition of vitamin B_{12} (0 - 4 mM) resulted in an improvement of the dechlorination reaction (Assaf-Anid and Lin, 2002).

The nitro group is a strong, electron- withdrawing substituent with a strong deactivating effect on the benzene ring, whereas the chlorine groups are electron-withdrawing substituents which are relatively weak deactivating substituents of the benzene ring (Kemp and Vellaccio, 1980). Reduction of the nitro group and formation of the electron-donating amino group will lead to electronic and steric effects, which in turn are expected to affect the reductive dechlorination of CAs. It has been shown that substitution of an electron-withdrawing nitro group by an electron-donating amino group

strongly decreases the formation of electron donor-acceptor complexes by nitroaromatic compounds (Haderlein *et al.,* 2000). The strong electron-donating property of the amino group is expected to affect the sequential dechlorination of chlorinated anilines compared to that of chlorobenzenes.

Abiotic reduction of nitro groups by reducing agents (i.e., sulfide, cysteine) was previously investigated by Gorontzy *et al.* (1993) and they reported the abiotic reduction of the nitro to amino group of *o*-nitrophenol and 2,4-dinitrophenol in a phosphate buffer amended with 1.5 mM sulfide. Xin-hua *et al.* (2005) reported the reduction of *o*-chloronitrobenzene to *o*-chloroaniline followed by dechlorination to aniline by palladium-catalyzed Fe^0 particles. Elsner *et al.* (2004) observed that the surface-area-normalized rate constants for the abiotic reduction of 4-chloronitrobenzene (4-Cl-NB) by Fe^{2+} adsorbed onto various iron minerals increased in the following order: siderite < iron oxides < iron sulfides.

Klupinski *et al.* (2004) reported that the abiotic reduction of PCNB to PCA with Fe^{2+} was a surface-mediated reaction and the presence of goethite (α-FeOOH) and/or the formation of iron oxide nanoparticles significantly enhanced this reduction. In the case where mineral phases were not added, the PCNB to PCA transformation was very slow compared to the case where minerals were present and the transformation followed zero-order kinetics. For example, for the reaction condition with 0.85 mM Fe^{2+} in a pH 7 buffer, the zero-order PCNB transformation rate at an initial PCNB concentration of 1.225 μM was 1.15×10^{-3} μM/min ($t_{1/2}$ = 533 min) (i.e., over a 250-fold lower compared with the case where goethite was present).

2.3.2. Fungal Biotransformation

Use of fungi for soil bioremediation has become an active research area in recent years. It has been previously reported that fungi have the ability to degrade a diverse group of halogenated organic compounds that persist in the environment. Under carbon and/or nitrogen limiting conditions enzymes and factors secreted by fungi were reported to degrade lignin as well as xenobiotic compounds to carbon dioxide (Stahl and Aust, 1995).

Most of the early studies on the biodegradation of PCNB focused on using fungi and were conducted under aerobic/anoxic conditions. Steiman *et al.* (1992) examined the production of extracellular phenoloxidases and found that the most active groups were Mucedinaceae and Zygomycetes. However, no correlation was found between the production of extracellular phenoloxidases and the biodegradative capability of individual strains. Similarly, production of extracellular phenoloxidases by a number of fungal strains was not correlated with their capacity to degrade PCNB (Seiglemurandi *et al.*, 1992; Guiraud *et al.*, 1999). D'Annibale *et al.* (2005) worked on the potential of fungal bioaugmentation by filamentous fungi (white-rot fungi, in particular) of an aged contaminated soil taken from the ACNA site (Cengio, SV, Italy), a former industrial area highly contaminated by large-scale production of organic chemicals (i.e., PCA, 2,4-DCA, 2,6-DCA). They reported complete removal of dichlorinated anilines by white-rot fungi leading to the release of chloride ions but did not observe any PCA degradation. It is noteworthy that both lignin peroxidase and laccase were shown to be able to catalyze the oxidative dechlorination of both chlorophenols and chloroanilines (Dec and Bollag, 1995; Hammel and Tardone, 1988).

Biodegradation and biotransformation of chlorinated anilines which are the main residues of reductive biotransformation of PCNB by white rot fungi were previously reported (Arjmand and Sandermann, 1985; Chang and Bumpus, 1993; Morgan *et al.*, 1991). Demir *et al.,* (1998) reported the biological breakdown of both PeCP and PCNB using a white rot fungi (*Phanerochaete chrysosporium*) which was incubated in a culture medium of pH 5 and at 35°C along with basic nutrients and trace metals. The best PCNB degradation by the fungal species *Sporothrix cyanescens* was observed under carbon and nitrogen limitation (Lièvremont *et al.*, 1996a). Removal of more than 50% of 100 mg/L PCNB by three fungal species was attributed to biosorption and subsequent biodegradation (Lièvremont *et al.*, 1996b). During the biotransformation of PCNB by four soil micromycetes, six metabolites were identified according to three metabolic pathways (Mora Torres *et al.*, 1996): PCNB $\rightarrow$ PCA ($C_6Cl_5NH_2$) $\rightarrow$ TeCA ($C_6Cl_4HNH_2$); PCNB $\rightarrow$ pentachlorothiophenol (C_6Cl_5SH) $\rightarrow$ pentachlorothioanisole ($C_6Cl_5SCH_3$); and PCNB $\rightarrow$ PeCP (C_6Cl_5OH) $\rightarrow$ pentachloroanisole ($C_6Cl_5OCH_3$).

2.3.3. Reductive Biotransformation

Although, both abiotic and biotic transformation of pesticides are important factors in their overall environmental fate, by far the major route of pesticide detoxification is attributed to enzymatic transformation (van Eerd *et al.*, 2003). Microorganisms are the main mediators for the cycling and degradation of polyhalogenated organic compounds in the environment. It is crucial to understand the microbial processes for the biotransformation and degradation of these compounds. Polyhalogenated organic compounds tend to be resistant to biodegradation in aerobic

environments. Therefore, as the degree of halogenation increases, reduction of these compounds is more likely to occur than oxidation (Bossert *et al.*, 2003) (Figure 2.3). The physical size and shape of the halogen substituent may also affect reactivity of the compound because of steric constraints and may also affect uptake into cells and enzymatic attack during biodegradation (Häggblom and Bossert, 2003). Polyhalogenated compounds can be used as electron acceptors in thermodynamically favorable reactions (Dolfing and Beurskens, 1995; Fantroussi *et al.*, 1998; Fetzner and Lingens, 1994; Fetzner, 1998; Middeldorp *et al.*, 1999; Häggblom *et al.*, 2000). Under anaerobic conditions, the reductive transformation of nitroaromatic compounds proceeds with the reduction of one or more aryl nitro groups ($-NO_2$) and the formation of aryl amines. Nitroso (-NO) and hydroxylamino (-NHOH) intermediates, as well as condensation

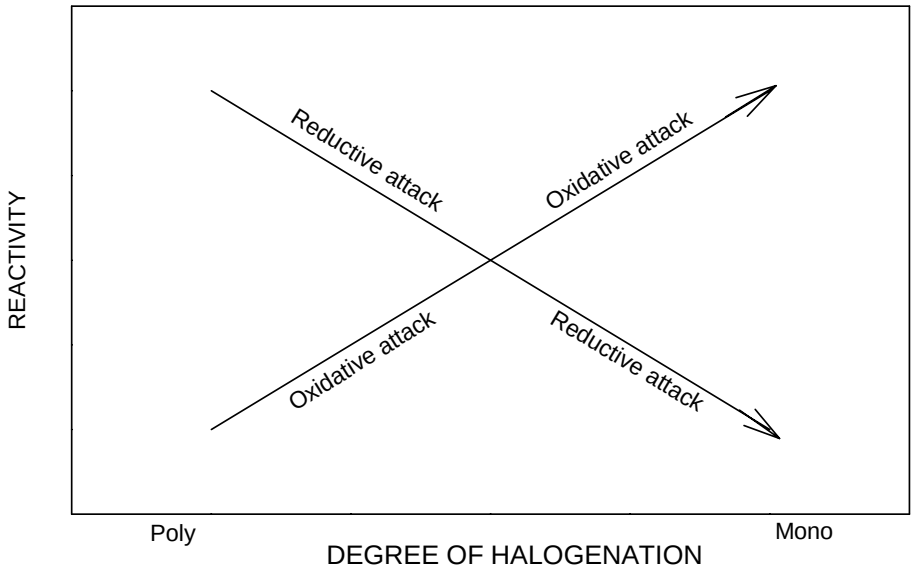


Figure 2.3. Oxidative versus reductive dehalogenation as a function of degree of halogenation (adapted from Bossert *et al.*, 2003).

products such as azoxy dimers (-N=NO-) have also been identified. In addition to the reduction of the nitro groups and formation of amino-derivatives, other reactions are possible, some of which result in the formation of polar compounds and oxidation products (Vorbeck *et al.*, 1994; 1998; Hughes *et al.*, 1998; Bhadra *et al.*, 1999; Larson *et al.*, 1999; Lenke *et al.*, 2000).

PCNB degradation was observed in a large number of soil samples screened for microorganisms capable of degrading PCNB, irrespective of previous application of PCNB to the field. An isolate with a relatively fast PCNB degradation rate was identified as *Pseudomonas aeruginosa*. This isolate degraded PNCB best under anoxic conditions and the principal metabolic product of PCNB was PCA, thus demonstrating that in anoxic soil environments the main degradation pathway of PCNB is its reduction to PCA (Tamura *et al.*, 1995). In theory, PCA may sequentially dechlorinate down to aniline. Figure 2.4 shows the theoretical reductive dechlorination products of PCA.

Co-metabolic mineralization of monochloronitrobenzenes was achieved by a coculture of *Pseudomonas putida* and a *Rhodococcus* sp. The former species converted the chloronitrobenzenes to chloro-hydroxyacetanilides by partial reduction and subsequent acetylation, and the later species mineralized the chloro-hydroxyacetanilides to CO_2, NH_4^+ and Cl^- (Park *et al.*, 1999). Sequential dehalogenation of CAs in microcosms developed with aquifer material was achieved under methanogenic, but not sulfidogenic conditions. Dechlorination at the *para* and *ortho* position of 2,3,4,5-TeCA resulted in the formation of 2,3,5-TrCA and eventually 3,5-DCA, whereas 3,4-DCA was transformed to 3-CA (Kuhn and Suflita, 1989).

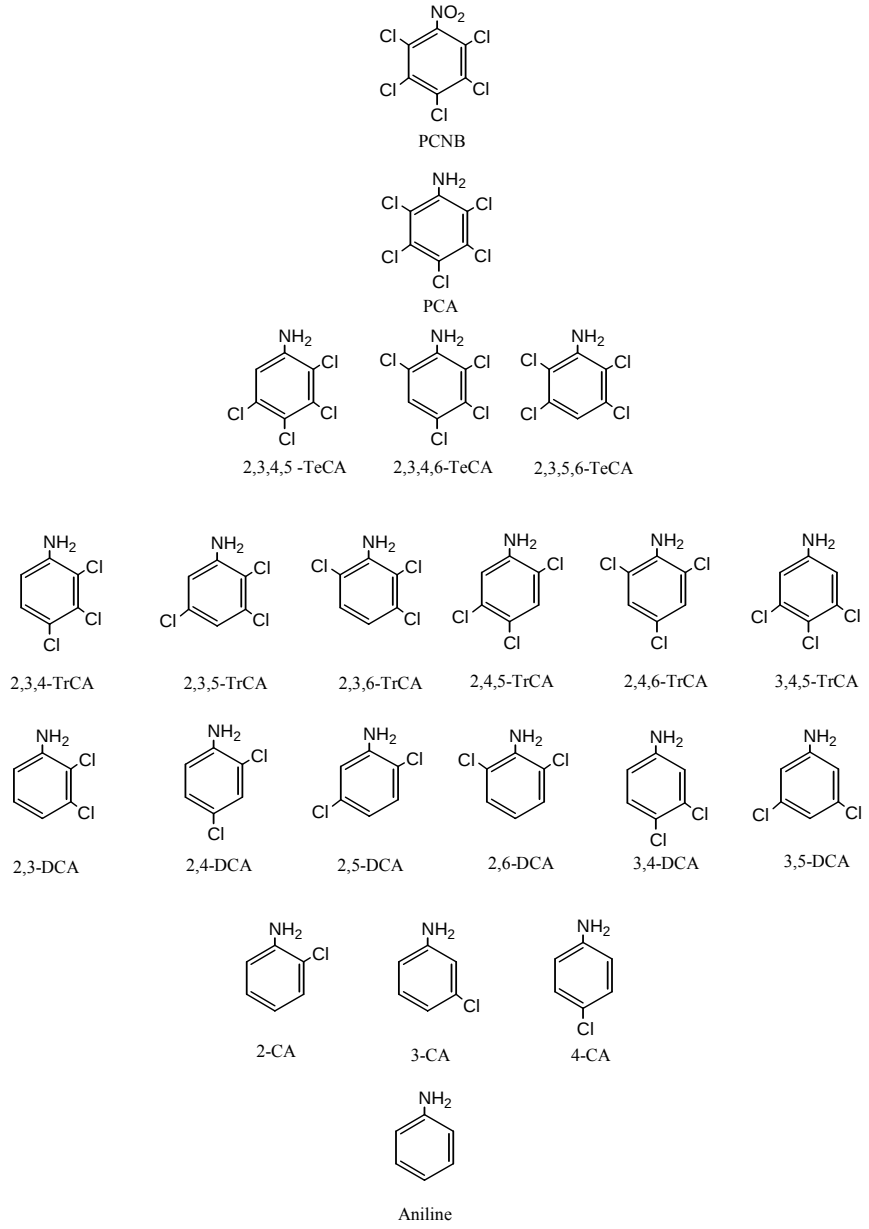


Figure 2.4. Possible compounds formed by the reductive transformation of PCNB to PCA and its sequential reductive dechlorination.

Table 2.1 shows the physicochemical properties of PCNB, PCA, representative CAs, and aniline, which are possible products resulting from the reductive biotransformation of PCNB. As expected, water solubility and vapor pressure increase, whereas the octanol-water partition coefficient decreases by several orders of magnitude with decreasing degree of chlorination. The combined effect of the chemicals' physicochemical properties is reflected on the media distribution estimated by the PBT Profiler which helps to identify the persistence, bioaccumulation, and toxicity potential of chemicals using a series of computer estimation methods based on chemical structure and physical/chemical properties (Table 2.2) (U.S. EPA, 2003b). The PBT Profiler uses the Level III multimedia fugacity model (Mackay *et al.*, 1992) to determine the fraction of the target compound in each medium (i.e., air, water, soil, and sediment). The PBT Profiler data demonstrate that as dechlorination of CAs proceeds, the chemical distribution shifts from the soil to the water media. Half-lives, bioconcentration factors and fish chronic toxicity values estimated using the PBT Profiler are also shown in Table 2.2. The persistent, bioaccumulative and toxic nature of the target compounds decrease with decreasing degree of chlorination. Table 2.3 shows previously reported toxicity values of chlorinated anilines. Argese *et al.* (2001) reported that chloroaniline congeners show a general trend of decreasing toxicity with decreasing degree of chlorination, although this trend is less marked for highly chlorinated compounds. Interestingly, Argese *et al.* (2001) and Ribo and Kaiser (1984) observed a decrease in the EC_{50} value of PCA when reduced to tetrachloroanilines. Tetrachloroanilines have only a slightly increased toxic effect compared to trichloroanilines. The EC_{50} values for trichloroanilines were about one order of magnitude lower than those of dichloroanilines and were rather

Table 2.1. Physicochemical properties of PCNB, representative chloroanilines, and aniline[a]

Properties	PCNB	PCA	2,3,5,6 TeCA	2,4,6 TrCA	3,5 DCA	4-CA	Aniline
Molecular weight	295.34	265.35	230.91	196.46	162.02	127.57	93.13
Melting Point (ºC)	144	235	107	78.5	52	72.5	-6
Boiling Point (ºC)	328			262	261	232	184.1
Specific gravity	1.718	1.756			1.58	1.17	1.0217
Water solubility (mg/L at 25ºC)	0.44	0.0298	9.49	40	784	3900	36000
Octanol-water part. Coeff. (Log P)	4.64	4.82	4.10	3.52	2.90	1.83	0.90
Vapor pressure (mm Hg at 25ºC)	5.00E-5[b]	3.50E-6	5.57E-7	2.18E-3[c]	2.12E-2	2.70E-2	4.90E-1
Henry's Law const. (atm m^3/mole at 25ºC)	4.42E-5	4.25E-7	5.74E-7	1.34E-6	1.58E-6	1.16E-6	2.02E-6
pKa	NA	-2.10[c]	-1.41[c]	-0.03	2.51	3.98	4.60

[a] Data from Syracuse Research Corporation (2003), unless otherwise indicated
[b] at 20ºC
[c] Reuther *et al.*, 1998

Table 2.2. Persistence, bioaccumulation and toxicity of the target compounds[a]

Compound	Media Distribution (%)				Half-Life (days)				BCF[b]	Fish ChV[c] (mg/L)
	Water	Soil	Sediment	Air	Water	Soil	Sediment	Air		
PCNB	4	86	9	1	**180**	**360**	**1600**	**2200**	750	**0.065**
PCA	4	82	14	0	**180**	**360**	**1600**	**12**	*1000*	**0.017**
2,3,5,6-TeCA	6	91	3	0	**180**	**360**	**1600**	**3.5**	290	**0.037**
2,4,6-TrCA	13	85	1	1	*60*	*120*	**540**	**15**	100	**0.079**
3,5-DA	20	80	0	0	38	*75*	**340**	0.3	34	*0.16*
4-CA	34	65	0	1	38	*75*	**340**	0.38	5.1	*0.33*
Aniline	45	55	0	0	15	30	*140*	0.14	3.2	*0.6*

Exceedence of EPA criteria
(Half-Life ≥ 2 months, persistent; BCF ≥ 1000, considered bioaccumulative; Fish ChV 0.1-10 mg/L, moderate concern)

Over exceedence of EPA criteria
(Half life ≥ 6 months, very persistent; BCF ≥ 1000, considered very bioaccumulative; Fish ChV ≤ 0.1 mg/L, high concern)

[a] Estimated values using the PBT Profiler
[b] BCF, bioconcentration factor
[c] Chronic toxicity value

Table 2.3. Reported toxicity values of chlorinated anilines (adapted from Argese *et al.*, 2001)[a]

Compounds	SMP log ($1/EC_{50}$) [b]	Microtox 30-min log ($1/EC_{50}$)[c]	*Poecilia reticulata* 14-d log ($1/EC_{50}$)[d]	*Tetrahymena pyriformis* 48-h log ($1/IGC_{50}$)[e]	Male Fischer 344 Rats 48-h BUN (%) (0.8 mmol CA/kg)[f]
2-CA	3.07	3.91	4.31	2.91	--
3-CA	3.13	3.96	3.98	3.09	--
4-CA	3.15	4.40	3.69	4.36	--
2,3-DCA	4.15	4.77	--	4.02	24
2,4-DCA	4.00	4.54	4.41	3.56	34
2,5-DCA	4.04	4.63	4.99	3.58	38
2,6-DCA	4.13	4.97	--	3.33	19
3,4-DCA	4.76	5.40	4.41	4.14	24
3,5-DCA	--	--	--	--	66
2,3,4-TrCA	5.23	4.92	5.15	4.35	--
2,3,5-TrCA	--	--	--	--	--
2,3,6-TrCA	--	--	--	--	--
2,4,5-TrCA	5.13	5.12	5.00	4.30	--
2,4,6-TrCA	4.64	4.63	--	4.01	--
3,4,5-TrCA	5.10	4.77	--	4.51	--
2,3,4,5-TeCA	5.28	5.37	5.81	4.96	--
2,3,4,6-TeCA	--	--	--	--	--
2,3,5,6-TeCA	5.24	5.16	--	4.76	--
PCA	5.20	4.35	--	--	--

[a] EC_{50}, LC_{50} and IGC_{50} values are in mol/L SMP-Submitochondrial particles; EC_{50}-50% effective concentration; IGC_{50}-50% inhibitory growth concentration; LC_{50}-50% lethal concentration; BUN-Blood Urea Nitrogen, for acute nephrotoxicity

[b] Argese *et al.*, 2001

[c] Ribo and Kaiser, 1984

[d] Hermens *et al.*, 1984

[e] Arnold *et al.*, 1990; Cajina-Quezada and Schultz, 1990

[f] Lo *et al.*, 1990

similar for all the isomers, with the exception of 2,4,6-trichloroaniline, which has a significantly lower toxicity. There is approximately one order of magnitude increase of EC_{50} values from mono- to di-chloroanilines and no significant difference in EC_{50} values among the monochloroanilines isomers, with only the 2-chloroaniline exhibiting slightly lower toxic effects. Within the group of dichlorinated anilines analyzed, 3,4-dichloroaniline was the most toxic compound, with an EC_{50} value substantially lower than those of the other isomers. Lo *et al.* (1990) studied the neuphrotoxic effect of the six dichlorinated aniline (DCA) isomers on male Fischer 344 Rats in vivo and in vitro. Based on the overall effect on renal function and morphology they reported an increase in the toxicity of DCA as follows: 2,3-DCA<2,6-DCA, 3,4-DCA, 2,4-DCA<2,5-DCA<3,5-DCA (Table 2.3). Although reductive biotransformation of PCNB results in a significant decrease in the toxicity of the parent compound, the products of reductive biotransformation (i.e., DCAs and CAs) are also relatively toxic (Monteiro *et al.,* 2006; Guilhermino *et al.,* 1994; Chhabra *et al.,* 1990). However, although di- and mono-chloroanilines are persistent under anoxic conditions (Kuhn and Suflita, 1989; Susarla et al, 1996), they are degradable under aerobic conditions (Reber *et al.,* 1979; Surovtseva *et al.,* 1996).

2.3.4. Biotransformation under Different Electron Accepting Conditions

Nitrate, sulfate, and iron are alternative electron acceptors commonly involved in anoxic/anaerobic subsurface processes (i.e., nitrate, sulfate, and iron reduction) and bioremediation applications.

Nitrate is a common electron acceptor in the environment, resulting from various industrial processes, such as fertilizer production and application, explosives manufacturing, coking, refining, food processing and the production of a wide range of organic compounds (Chaudhry and Beg, 1997). Agricultural activities, primarily row crop and livestock production is a major source of nitrate pollution. Due to its ubiquitous nature and high mobility in soil and sediment systems, nitrate is a highly available electron acceptor. Therefore, nitrate reduction is a widely occurring microbial process in both engineered and natural systems. In addition, nitrate reduction is a process which does not produce any secondary pollutant such as sulfide as in the case of sulfate reduction, and the growth rate of denitrifying bacteria is relatively fast. Dissimilatory nitrate reduction, a form of anaerobic respiration, is the reduction of NO_3^- to nitrogen oxides (i.e., NO_2^-, NO, N_2O) and N_2 catalyzed by four enzymes: nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase. A wide variety of facultative prokaryotes perform dissimilatory nitrate reduction, most of them belonging to the *Proteobacteria* (Madigan and Martinko, 2006). A number of obligate anaerobic, facultative anaerobic, and microaerophilic bacteria perform dissimilatory nitrate reduction to NH_4^+, mainly in carbon-rich, electron acceptor-poor environments (Tiedje, 1988). Brunet and GarciaGil (1996) reported that at extremely low concentrations of free S^{2-} (metal sulfides) nitrate was reduced via denitrification whereas at higher S^{2-} concentrations, dissimilatory nitrate reduction to ammonia (DNRA) and incomplete denitrification to gaseous nitrogen oxides took place. Denitrifiers can use a wide range of natural organic substrates as carbon and electron sources, and were also found to degrade several anthropogenic compounds (e.g., phenols and benzoates)(Yu and Smith, 2000).

Because of the increasing problem of nitrate pollution worldwide (Prasad, 1999), the occurrence of nitrate- and PCNB-contaminated sites is expected, especially in agricultural fields. In an overall methanogenic system, nitrate reduction can have a significant impact on both the carbon and electron flow, as well as microbial competition and inhibition, which in turn may have an adverse impact on contaminant reductive biotransformation, such as dechlorination. Several researchers previously reported biotransformation of halogenated organics by various denitrifying microorganisms (Bae *et al.*, 2002; 2004; Gerritse *et al.*, 1999; Häggblom and Young, 1999; Coshigano *et al*, 1994; Petersen *et al.*, 1994; Sherwood *et al.*, 1996). The degradation of 3,4-dichloroaniline by an anaerobic enrichment culture and a *Rhodococcus* sp. strain derived from this culture under nitrate reducing conditions was reported to proceed by reductive deamination and formation of 1,2-dihalobenzene (Travkin *et al.*, 2002). Travkin *et al.* (2002) isolated *Pseudomonas fluorescens* strain 26-K from coking industry wastewater which utilizes 3,4-DCA as the sole source of carbon, nitrogen, and energy. However, many studies have shown that nitrate inhibits the degradation of halogenated organic compounds by anaerobic microbial communities, specifically by inhibiting reductive dehalogenation (Milligan and Häggblom 1999; Genthner *et al.* 1989; Häggblom *et al.* 1993a,b; Picardal *et al.*, 1995). Nelson *et al.* (2002) reported inhibition of PCE dechlorination in a mixed H_2-fed culture which was amended with 640 μM NO_3^- . They also tested the inhibitory effect of denitrification intermediate products on dechlorination. Amendment of the mixed, PCE dechlorinating culture with 100 μM aqueous N_2O resulted in complete inhibition of PCE dechlorination.

Sulfate reducing bacteria are widespread in aquatic and terrestrial environments (Madigan and Martinko, 2006). Many researchers have reported either a decrease in the dechlorination rates or complete inhibition of dechlorination in the presence of sulfate (Boopathy and Peters, 2001; Harkness *et al.,* 1999). The reductive dechlorination and mineralization of chlorophenols under methanogenic conditions was completely inhibited when alternative electron acceptors such as sulfite and thiosulfate were added, whereas sulfate addition led to a decrease of the dechlorination rate (Häggblom *et al.*, 1993a). Nelson *et al.* (2002) reported complete inhibition of perchloroethene (PCE) dechlorination as well as methanogenesis in a mixed H_2-fed culture which was amended with 850 μM SO_4^{2-}, although the H_2 concentration in the culture was kept above the H_2 threshold for halorespirers (2 nM). When sulfate was completely reduced, significant CH_4 and TCE production was observed. Similarly, Shen and Sewell (2005) reported complete inhibition of PCE dechlorination to ethene in an enrichment culture in the presence of 1.8 mM sulfate leading to the conclusion that dechlorinating microorganisms compete for the same electron donor (H_2) with sulfate reducers. In contrast, Hoelen and Reinhard (2004) reported the reductive dehalogenation of PCE to ethene in the presence of sulfate (> 100 mg/L). Pavlostathis and Zhuang (1991) reported trichloroethene (TCE) reductive dechlorination to cis-1,2-dichloroethene (cDCE) by sulfate-reducing cultures enriched from a contaminated subsurface soil. However, the predominant biotransformation product was cDCE and they did not observe further dechlorination of cDCE. Chloronitrobenzenes (3-CNB, 3,4-DCNB, 2,3,4-TrCNB, and PCNB) were transformed in anaerobic, sulfidogenic estuarine sediments with pseudo-first-order rate constants ranging from 0.216 to 0.866 day^{-1}. Their transformation proceeded with

reduction of the nitro group resulting in the formation of the corresponding chloroanilines (CAs), which were further transformed via *ortho* and *para* dechlorination pathways (Susarla *et al.*, 1996). In contrast, CAs were sequentially dechlorinated in the same sulfidogenic estuarine sediments with pseudo-first-order rate constants ranging from 0.005 to 0.012 day^{-1} (Susarla *et al.*, 1997b).

Although ferric iron is by mass the most abundant alternative electron acceptor in anaerobic soil and sediment environments (Cornell and Schwertmann, 1996), it occurs mostly in the form of poorly bioavailable, insoluble oxides at neutral pH. It has been shown that when iron is bioavailable, iron reducing microorganisms outcompete sulfate reducers and methanogens (Lovley and Phillips, 1987). The metabolic diversity and ecological significance of dissimilatory iron reducers has increased the interest for these microorganisms for bioremediation applications (Lovley, 1993). The significant role of iron reducing bacteria in the cycling of organic matter and the biotransformation of organic contaminants has been shown (Lovley, 1991). The role of dissimilatory iron-reducing bacteria in the reduction of nitrobenzenes was elucidated (Heijman *et al*., 1995; Klausen *et al*., 1995). This transformation involves two coupled reactions, one biotic mediated by the iron-reducing bacteria, the other abiotic, where reduced iron [Fe^{2+}] serves as the intermediate electron donor and nitrobenzenes serve as the electron acceptor. Based on published reduction potentials of key redox couples of Fe^{3+}/Fe^{2+} and organic compounds (Haderlein and Schwarzenbach, 1995), polychlorinated organic compounds can be reduced by a number of Fe(II)-bearing compounds. Microbial dechlorination under iron-reducing conditions has been reported for a number of halogenated compounds, including CT (Picardal *et al.*, 1993), monochlorinated isomers

of phenol, and benzoate (Kazumi *et al*., 1995a; Kazumi *et al*., 1995b). The rate of vinyl chloride mineralization under iron-reducing conditions was four-times higher than that under methanogenic conditions (Bradley and Chapelle, 1996). An exhaustive literature review did not reveal any information on the definitive reductive dechlorination of polychloronitrobenzenes under iron-reducing conditions. The role of humic substances has long been recognized in electron-transfer reactions in general and more specifically in the biotransformation of recalcitrant compounds (Bradley *et al.*, 1998; Lovley *et al.*, 1998; Scott *et al.*, 1999). Humics could serve as carbon and energy sources in mixed, dissimilatory iron-reducing cultures, as electron-transfer mediators between Fe^{2+} and halogenated organic compounds (Haderlein and Schwarzenbach, 1995), as well as electron shuttles in the microbial reduction of insoluble Fe^{3+} compounds (He and Sanford, 2003; Komlos and Jaffé, 2004; Lovley *et al.*, 1996). Another way to enhance the bioavailability of insoluble Fe^{3+} is to increase its solubility with chelators, such as ethylene diamine tetraacetic acid (EDTA) (Lovley and Woodward, 1996). Reductive dehalogenation of polychlorinated organic compounds under iron-reducing conditions may take place by either one or both of the following mechanisms (Doikos, 1998): a) Co-metabolic biotransformation: both Fe^{3+} and the chlorinated organic compounds merely serve as electron acceptors (primary and secondary electron acceptors, respectively)(Mechanism I, Figure 2.5); b) Abiotic transformation: the reductive dechlorination is mediated abiotically, using Fe^{2+} as the terminal electron donor (i.e., as the reductant) which is biotically produced from the reduction of Fe^{3+} (Mechanism II, Figure 2.5). The difference between these two mechanisms is that in the first one, Fe^{3+} is reduced to Fe^{2+} without regeneration of Fe^{3+}, whereas, according to the second

mechanism, the reduction of the chlorinated organic contaminant(s) regenerates and continuously provides microbially available Fe^{3+}. In case of humic substances involvement in the reductive biotransformation process, these substances may serve as an electron shuttle between Fe^{2+} and the target chloroorganic compound, especially if the Fe^{2+} is surface-bound.

Figure 2.6 shows that the free energy released during the transformation of PCNB to PCA, as well as the sequential dechlorination of chlorinated anilines, is higher than that associated with metabolic processes such as sulfate reduction or methanogenesis. Despite the fact that dechlorination reactions are often more thermodynamically favorable than anaerobic metabolic processes such as sulfate reduction or methanogenesis, dechlorination in many cases takes place at the same time with other metabolic processes (Maitheepala and Doong, 2004; Vanderloop *et al*., 1999).

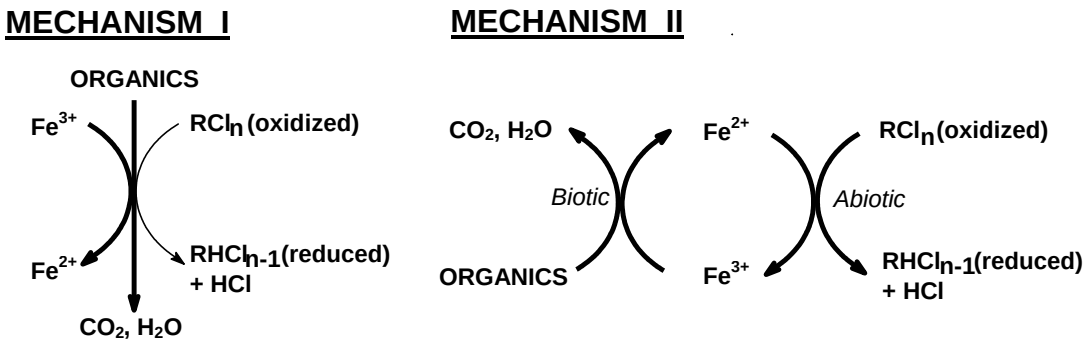


Figure 2.5. Mechanisms potentially involved in the reductive dechlorination of polychlorinated organic compounds under iron-reducing conditions.

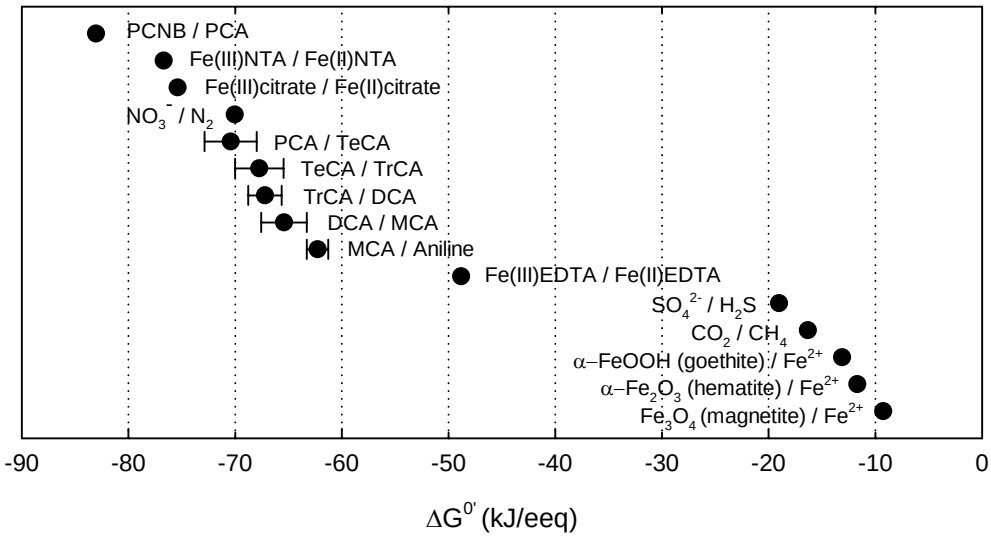


Figure 2.6. Free energy release during the biotransformation of PCNB and the sequential dechlorination of chlorinated anilines serving as electron acceptors as compared to the use of alternative electron acceptors such as nitrate, sulfate, iron and carbon dioxide (H_2 was used as the electron donor for all reactions; in the case of TeCA, TrCA, DCA and CA, mean free energy values of the isomers were used; redox potential data taken from references Madigan and Martinko, 2006; Thamdrup, 2000; Thomas *et al*., 1998; Wilson, 1978).

2.4. Prediction of Dechlorination Pathway of HOCs

Recent research interests have focused on the ability to predict the reductive dechlorination pathway of halogenated organic compounds. It is known that incomplete dechlorination may lead to the accumulation of intermediate products that may be more toxic than the parent compounds. In addition, the position of the chlorine atom for different isomers of a compound is a factor that determines its toxicity. Therefore, it is important to be able to predict the dehalogenation pathway. Pathways which result in products that are environmentally friendly, non toxic and easily degradable are shaught out for bioremediation applications. It should be emphasized that dechlorination may be specific to the conditions of the system such as electron donor, electron acceptor type and concentration, nutrient availability, pH, and temperature.

Dechlorination at different positions of the same congener yields different free energy. The existence of such differences suggests that certain dechlorination reactions will have preference over others. Such considerations make it possible to predict preferential dechlorination pathways for chlorinated organic compounds in anaerobic environments. Dolfing and Harrison (1993) put forward a hypothesis that microbially catalyzed dehalogenation reactions and pathways follow the redox potential of the couple that gives rise to the highest energy yield. This hypothesis was tested and validated for chlorinated benzenes (CBs) under anaerobic conditions (Beurskens *et al.*, 1994; 1995; Dolfing and Harrison, 1993). However, this hypothesis failed to predict the observed pathway for the dechlorination of chlorophenols (Masunaga *et al.*, 1996). Huang *et al.,* (1996) calculated Gibbs free energies of formation for 75 polychlorinated dibenzo-p-dioxins (PCDDs) using several semiempirical methods including the Modified Neglect

of Diatomic Differential Overlap (MNDO), Austin Model 1 (AM1), and MNDO–Parametric Method 3 (PM3), and Benson's group additivity method. Then, they used these results to predict reductive dechlorination pathways for PCDDs. The redox potentials that they calculated indicated that reductive dechlorination of chlorodibenzodioxins is an exergonic reaction. Because dechlorination at different positions of the same congener resulted different amounts of free energy, they concluded that energy differences between isomers can be used to predict the dechlorination pathway of PCDDs. Susarla *et al.* (1997a) estimated the Gibbs free energy of formation for chlorinated anilines with the Benson's method, and they determined the redox potentials using H_2 as the electron donor. The previously observed pathways for the dechlorination of chloroanilines (Kuhn and Suflita, 1989; Kuhn *et al.*, 1990) were compared with the redox potential of these reactions, and the results showed that based on redox potential values the pathway that yields the highest energy for a dechlorination reaction was experimentally the preferred one.

Studies on molecular orbitals involving the dechlorination process can provide some fundamental information that may help to understand the preferred dehalogenation reactions. Some useful generalizations concerning reductive dehalogenation of halogenated compounds can be made based on these theoretical studies.
Bylaska *et al.* (2005) used *ab initio* electronic structure methods to investigate the thermochemical properties (in the gas phase and in aqueous solution) for the reaction pathways of polychloroethenes under reducing conditions and estimated the energetics of several one-electron reductive pathways from computed thermochemical data. Similarly, Zhang *et al.* (2005) investigated the effect of the reaction energy and aqueous solvation

on the reaction pathway for C-Cl bond cleavage in CT using Hartree-Fock density functional theory, modified complete basis set *ab initio* methods, and a continuum conductor-like screening model. They observed that solvation significantly lowered the energies of the reaction products and reductive cleavage changed from an inner-sphere to an outer-sphere mechanism as the overall energy change for the reaction was increased. Heimstad *et al.* (2001) calculated theoretical molecular descriptors such as heat of formation with both molecular mechanics (MM3) and the semi empirical quantum mechanics model AM1 within MOPAC, and steric energy, for 36 polychlorinated bornanes, which are the majority compound class of the insecticide toxaphene. They also calculated ground state global negative energy [highest occupied orbital (HOMO) and lowest unoccupied orbital (LUMO) energies] and local (atomic charge and LUMO location) molecular reactivity descriptors of electron transfer reactions to understand differences in reactivity of certain chlorobornanes observed during reductive degradation. They observed that the electronaffinity (i.e., the negative energy of LUMO) of the compounds increased with increasing degree of chlorination. These investigators concluded that reactivity descriptors such as electronaffinity, hardness, LUMO location and atomic charges may guide reactions like dechlorination of polychlorinated bornanes in reductive environments. Lynam *et al.* (1998) reported that the HOMO–LUMO gap is an indicator of stability of a molecule for polychlorinated dioxins. They observed that the larger the gap the greater the stability of the molecule toward further reaction and the gap decreased with increasing extent of chlorination. They examined reductive dechlorination pathways from two different experimental studies using partial charges and estimated Gibbs free energy of dechlorination. They also found that for a given chlorinated dioxin,

when more than one dechlorination pathway was possible, dechlorination according to the most thermodynamically favored pathway occurred at the most positively charged carbon atom in the ring, which was usually a lateral carbon atom.

2.5. Problem Identification

Pesticides are a major source of environmental pollution due to their persistent properties. PCNB and its predominant (bio)transformation product PCA are persistent anthropogenic contaminants in the environment. PCNB is a suspected carcinogen, developmental, endocrine, gastrointestinal, liver, reproductive, skin, and sense organ toxicant. In addition, PCNB may also cause methemoglobinemia (Murthy and Kaufman, 1978). PCNB and PCA are very stable compounds which are not easily degraded under aerobic conditions but reductive biotransformation is a major mechanism for these compounds. Although considerable research has been conducted on the reductive biotransformation of chlorinated organic and nitroaromatic compounds, detailed information is lacking with regard to the biotransformation of chlorinated nitroaromatic compounds. Therefore, there is a need to investigate the biotransformation potential and kinetics of PCNB. In view of the fact that several electron acceptors are always present in the environment, the biotransformation potential of PCNB under different electron accepting conditions should also be taken into account. The results of the present study will have significant environmental implications in terms of the fate and biotransformation of PCNB, PCA, and less chlorinated anilines in subsurface systems such as soil and sediments. Such understanding will help with efforts related to the bioremediation of PCNB- and/or PCA-contaminated soil and sediment environments.

2.6. Research Objectives

On the basis of the literature information discussed above, research related to the fate and biotransformation of PCNB in anoxic/anaerobic microbial systems is very limited. Therefore, a research plan was developed to assess the potential for microbial reductive transformation of PCNB, and to determine the effect of environmental and physicochemical factors on the biotransformation process.

The specific objectives of this research were:

1) Assessment of the biotransformation potential of PCNB under fermentative/methanogenic, dissimilatory iron-reducing, as well as nitrate-reducing conditions with enrichment cultures derived from a contaminated sediment.
2) Physiological characterization of an enrichment PCNB-biotransforming fermentative/methanogenic culture, as well as assessment of possible inhibitory effects of PCNB on the mixed fermentative/methanogenic culture.
3) Systematic investigation of the pathway and kinetics of the sequential reductive transformation of PCNB, as well as the effect of environmental factors such as pH and temperature on the reductive biotransformation rate and extent of PCNB in the mixed, fermentative/methanogenic culture.
4) Thermodynamic analysis and molecular modeling of the sequential microbial dechlorination of PCA.

CHAPTER 3

ANALYTICAL METHODS AND GENERAL PROCEDURES

3.1. Analytical Methods

3.1.1. pH

Measurement of pH was performed using the potentiometric method with a digital pH/milivolt meter (Orion Digital pH/milivolt Meter, Model 611) and a gel-filled combination pH electrode (Fisher Scientific, Pittsburgh, PA). The meter was calibrated prior to each use using standard buffer solutions of pH 4, 7, and 10 (Fisher Scientific). Before and between sample readings, the electrode was rinsed with deionized water and gently dried with Kimpex (Fisher Scientific). The electrode was stored in a pH electrode storage solution (Fisher Scientific) of pH 4 when not in use. Although the sensitivity of the meter display was 0.01 units, measurements were recorded to only 0.1 pH units.

3.1.2. Oxidation-Reduction Potential (ORP)

Oxidation-reduction potential measurements were performed using an Orion Digital pH/milivolt Meter, Model 611 and a Sensorex combination ORP electrode (platinum electrode with an Ag/AgCl reference electrode in a 3.5 M KCl gel). The meter and electrode output were periodically checked using an ORP reference solution [0.10 M ferrous ammonia sulfate, 0.10 M ferric ammonium sulfate, and 1.0 M sulfuric acid (Light solution; APHA, 1998)]. The difference between the meter ORP reading and the theoretical value of the reference solution (455 mV at 25°C) was taken into account in all

sample ORP measurements. To obtain ORP values with reference to the standard hydrogen electrode (SHE), a correction factor of +220 mV was added to the instrument values. ORP measurements were conducted by taken a sample by syringe and transferring it into a 5-ml serum bottle in which the electrode fitted tightly. Then, the electrode was quickly inserted into the serum bottle. During the ORP measurement attention was paid not to include any air bubbles in the serum bottle and thus to minimize atmospheric oxidation. The ORP measurement was recorded when the instrument reading was not changing more than 1 mV/min.

3.1.3. Ammonia

Ammonia was measured with a gas-sensing, ammonia combination electrode (Denver Instruments, Denver, CO) connected to an Orion Digital pH/milivolt Meter, Model 611 according to the procedure described in Standard Methods (APHA, 1998). Aliquots of 10 ml of samples were placed in a glass 25 ml centrifuge tube and while mixing, the probe was immersed into the sample and then 10 μL of 10 N NaOH was added. The measurement was recorded when the instrument reading was not changing more than 1 mV/min. Aliquots of 10 ml of DI water were also used as blank samples. Ammonia calibrations were performed with a standard NH_4Cl solution (1 g/L).

3.1.4. Particulate Organic Carbon (POC)

Particulate organic carbon analysis was performed in order to quantify biomass levels in the enrichment cultures. POC analysis was accomplished using a Shimadzu TOC-5050A Total Organic Carbon (TOC) Analyzer equipped with a Shimadzu SSM-

5000A Solids Sample Module (SSM) and non-dispersive infrared (NDIR) detector. Culture samples of known volume (typically 2-5 mL) were vacuum filtered through 25 mm diameter Whatman GF/C glass fiber filters (1.2 µm nominal pore size). The filters were then rinsed twice with an equal amount of deionized water to remove any dissolved organic carbon or residual inorganic carbon, and then dried for 15 minutes in an oven set at 95ºC to remove moisture. The dried filters were placed in ceramic crucibles and combusted at 900ºC inside the SSM unit using ultra high purity oxygen. The evolved CO_2 was quantified using the response of the NDIR detector. Calibration for POC measurement was performed using certified ACS grade anhydrous dextrose powder (oven-dried and cooled in a desiccator) (Fisher Scientific). Calibration curves were prepared for range (x1) -low concentrations and range (x5) -high concentrations. If required, POC measurements performed for biomass estimation were converted to organic weight of biomass by assuming a biomass molecular formula of $C_5H_7O_2N$ (Rittmann and McCarty, 2001). Based on this formula, 60 mg POC represent 113 mg biomass organic matter (i.e., volatile suspended solids). The biomass concentration was converted to volatile suspended solids (VSS) units based on a linear correlation developed with the enrichment culture by measuring POC.

3.1.5. Gas Chromatography

3.1.5.1. Electron Capture Detection

Gas chromatography with electron capture detection (GC/ECD) was used for the quantification of PCNB and its transformation products. The concentration of chlorinated compounds in a solvent phase resulting from the liquid/liquid extraction of culture

samples (see Section 3.2.1, below) were measured using an HP 6890 Series (Hewlett Packard Co., Palo Alto, CA) gas chromatograph (GC) equipped with a micro electron capture detector (μ-ECD) and a 75-m 0.53-mm I.D. DB-624 fused silica column (0.3 µm film thickness) (J&W Scientific, Folsom, CA). Solvent samples were transferred to 2 ml amber glass vials and capped with Teflon-lined septa and aluminum crimps. The injection volume was 1 µl. The following oven temperature program was used: an initial setting of 100ºC was held for 4 minutes, and then increased at 2ºC/min to 210ºC and held for 61 minutes (total run 120 minutes). Helium was used as the carrier gas at a flow rate of 10 ml/min.

To maintain quality control of the data obtained with ECD analyses, 1,3,5-tribromobenzene (TrBB) was used as an internal standard in both calibration standards and sample extracts. The retention time of PCNB and each of the chlorinated anilines, and TrBB was determined by injecting a methanol standard containing one compound at a time. Once the elution sequence was determined, the ECD response was calibrated using serial dilutions of a composite standard of the analytes and the internal standard. (the calibration curves used in this study are shown in Appendix A, Figures A.1 through A5). A sample chromatogram which shows the elution order and retention time of each compound is presented in Figure 3.1. Only two of the target compounds, 2,5- and 2,4-dichloroaniline, could not be separated during the GC analysis due to their structural similarity.

To maintain a consistent baseline of the μ-ECD signal output, the column was routinely conditioned by varying the oven program final temperature hold time. The required run time to quantify all 16 chloroanilines, PCNB, and the TrBB internal standard

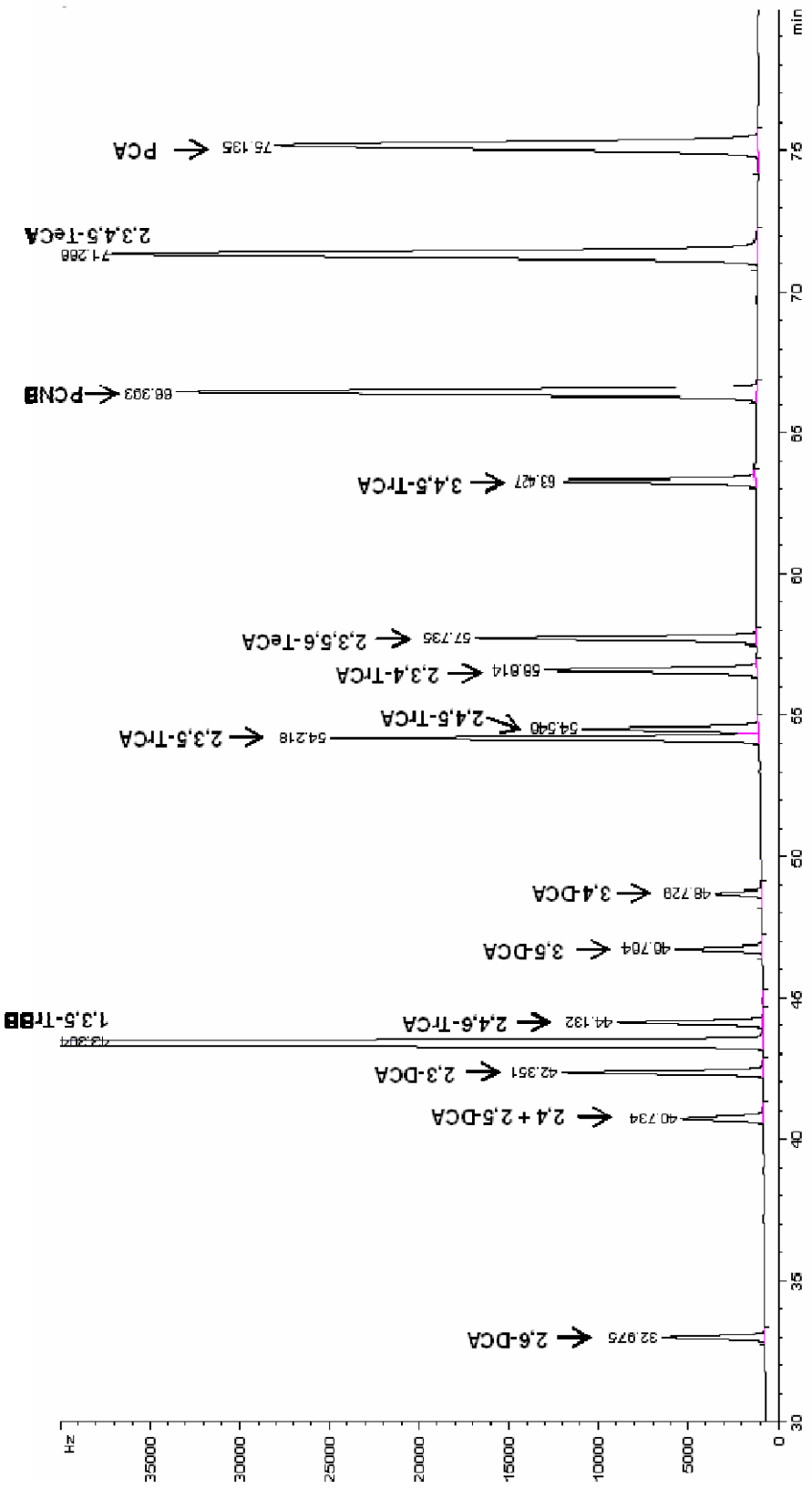


Figure 3.1. Sample μ-ECD output chromatogram showing each analyte elution order and its retention time.

was 80 minutes. Pure isooctane solutions were also run using a 120-minute program before and after sample injection. Liquid/liquid extracts (see Section 3.2.1, below) were run at 120 minutes to clean the column after each sample and return ECD response to its normal baseline value for the next injection.

The overall consistency of the μ-ECD output was monitored over time using a constant concentration of the TrBB internal standard for each analysis. Small variations in TrBB concentration observed from sample to sample (due to slight variations in the 1-µL volumes delivered by the autosampler) were used to normalize the response for all analytes in each sample using the following relationship.

$$Analyte\ Concentration = Analyte\ Conc_{sample} x (\frac{TrBB\ Conc_{average}}{TrBB\ Conc_{sample}})$$

The minimum detection limits for chlorinated compounds were as follows: PCNB, 7 nM; PCA, 4 nM; tetrachloroanilines, 9 nM; trichloroanilines, 25 nM; dichloroanilines, 0.15 µM; monochloroanilines 16 mM. 2,5-DCA and 2,4-DCA coeluted and could not be separated by GC/ECD analysis.

3.1.5.2. Thermal Conductivity Detection

The gas composition was determined by a GC unit (Agilent Technologies, Model 6890N; Agilent Technologies, Inc., Palo Alto, CA) equipped with two columns and two thermal conductivity detectors. Methane (CH_4), nitrogen (N_2) and nitric oxide (NO) were separated with a 15 m HP-Molesieve fused silica, 0.53 mm i.d. column (Agilent Technologies, Inc.); carbon dioxide (CO_2) and nitrous oxide (N_2O) were separated with a

25 m CP-PoraPLOT Q fused silica, 0.53 mm i.d. column (Varian, Inc., Palo Alto, CA). CH_4 and NO have the same retention time during the GC analysis. Therefore, in samples where NO measurements were desired, attention was paid to not have any CH_4 present. Both columns were operated with helium as the carrier gas at a constant flow rate of 6 mL/min. The 10:1 split injector was maintained at 150ºC, and the detector temperature was set at 150ºC. All gas analyses were performed by injecting a 100 µL gas sample. The minimum detection limits for NO and N_2O were 0.5 mL/L and 0.007 mL/L, respectively.

Hydrogen (H_2) was measured with a GC unit (Hewlett Packard, HP 5980 Series) equipped with a thermal conductivity detector and a 10-m Chrompack Molsieve 5A fused silica, 0.53-mm I.D. column. The carrier gas was N_2 at a constant pressure of 70 psi. The output signal of the TCD (on low sensitivity) was monitored using a Hewlett Packard HP 3396 Series II integrator. H_2 calibrations were performed with low and high sensitivity options that can be adjusted on the instrument. The GC minimum detection limit for H_2 was 500 ppmv.

3.1.5.3. Flame Ionization Detection

Dissolved methanol and VFAs (acetic, propionic, iso-butyric, n-butyric, iso-valeric, and n-valeric acids) concentrations were measured using a Hewlett Packard 5890 Series II gas chromatography unit equipped with a flame ionization (FID) detector and a 30-m Restek 11025 Stabilawax-DA, 0.53-mm I.D. column (Restek Corp., Bellefonte, PA). The inlet and detector temperatures were both at 300°C. Nitrogen was used as the carrier gas at 4.5 mL/min. The H_2 and air flow rates were 40 mL/min, and 450 mL/min, respectively. The following oven temperature program was used: 90°C for 0.5 min,

2°C/min to 100°C, 6°C/min to 120°C, 10°C/min to 230°C and held for 30 min. The total run time was 30 min.

Aqueous samples were first centrifuged at 12000 rpm for 5 minutes then acidified with a 1:2 ratio (acid:sample) with 2.5% v/v phosphoric acid (H_3PO_4) and filtered through 0.2 µm Whatman syringe filters (Fisher Scientific) into 1.5-mL glass autosampler vials. Then the vials were capped with Teflon-lined septa and aluminum crimp caps for automatic injection of 1-µL volumes of each sample into the GC. Calibration curves were prepared by using pure standards dissolved in deionized water containing 0.83% phosphoric acid.

3.1.6. High Performance Liquid Chromatography (HPLC)

Aniline, monochlorinated and dichlorinated anilines were also measured with a high performance liquid chromatography (HPLC) unit (Hewlett Packard Co., HP Model 8453), which was equipped with a Zorbax SB-C18 column (3 x 250 mm, 5 µm) (Agilent Technologies, New Castle, DE). The column compartment temperature was set at 40ºC and the injection volume was 10 µL. The eluent mixture was an aqueous solution of 25 mM phosphate buffer (pH = 2.5) (52%) and methanol (48%) with a flow rate of 0.35 mL/min. The 25 mM phosphate buffer was prepared from a stock solution of 1 M phosphate buffer (mixture of 250 ml 1M H_3PO_4 and 460 ml 1 M KH_2PO_4) prepared with nanopure DI water. The eluent was filtered throughout a 0.20 µm filter with a Teflon filtration unit. Spectrophotometric detection was set at 203 nm. Samples used in HPLC analyses were filtered through 0.20 µm PTFE syringe filters (Cole-Parmer, Vernon Hills, IL) using glass syringes. The HPLC minimum detection limit for dichlorinated and

monochlorinated anilines was 4 µM, whereas that for aniline was 6.4 µM. 2,5-DCA and 2,4-DCA were separated during HPLC analysis. Sample calibration curves are shown in Appendix A, Figure A.6 and A.7. A sample chromatogram which shows the elution order and retention time of each compound is presented in Figure 3.2.

Glucose was measured with an HP Series 1050 HPLC (Hewlett Packard) unit equipped with an Aminex HPX-87H-resin packed column (Bio-Rad, Richmond, CA). The column was maintained at 65ºC in an external column heater (Eppendorf CH-430 heater with an Eppendorf TC-50 temperature controller). The output was processed using a Hewlett Packard HP3396 Series II integrator (Hewlett Packard, Palo Alto, CA). The eluent was 0.01 N H_2SO_4 at a flow rate of 0.6 mL/min. Culture samples were centrifuged and then acidified with 0.2 N H_2SO_4 in a 1:1 ratio, and filtered through 0.2 µm membrane filters. Calibration curves were prepared using acidified standards prepared with a pure glucose solution. The minimum detection limit was 10 mg glucose/L.

3.1.7. Total Gas Production

Gas production was measured by periodically connecting the culture headspace via a needle to an acid-brine (10% NaCl w/v, 2% H_2SO_4 v/v) solution filled graduated burette and recording the volume of displaced solution, after adjusting to atmospheric pressure. Depending on the culture size, different volume burette manometers were used (25, 250, or 2000 mL). The smaller burette was used for serum bottle and serum tube experiments, while the larger manometers were used for 2 to 9-L culture bottles.

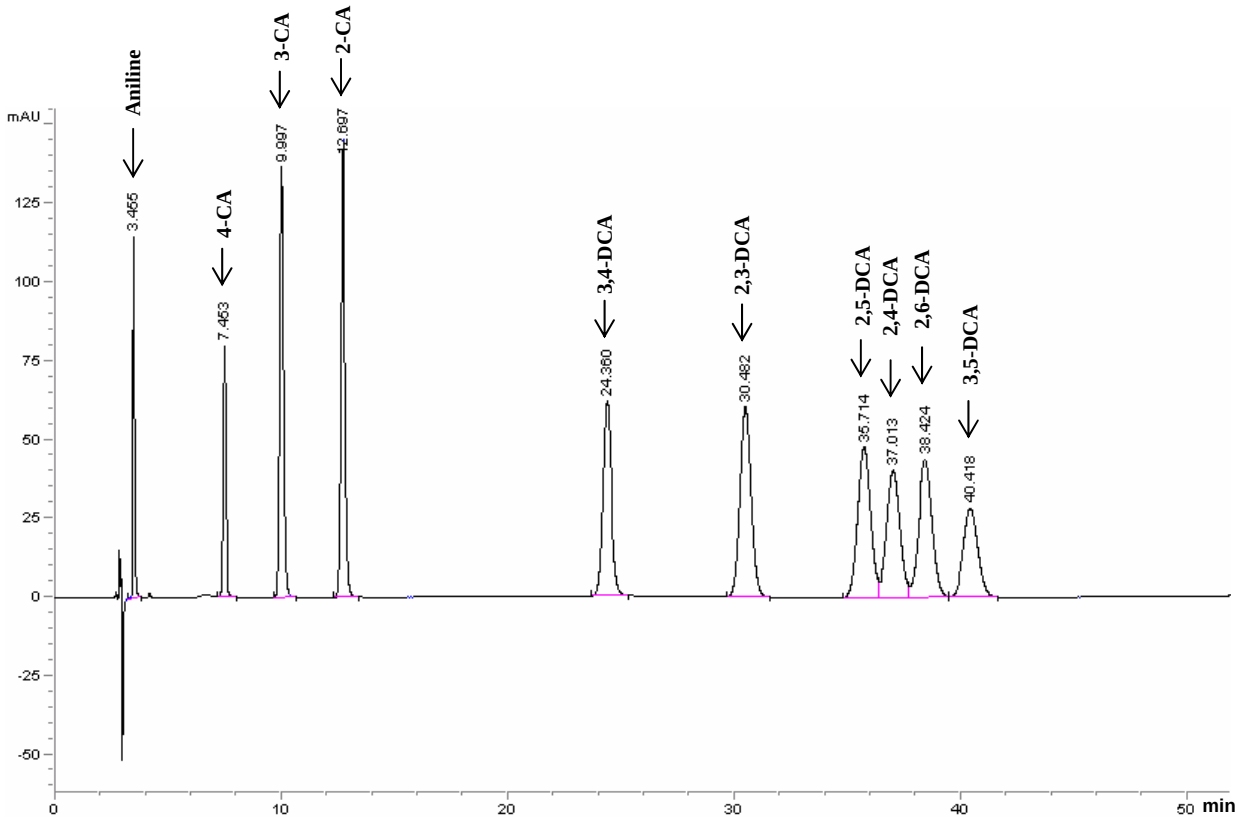


Figure 3.2. Sample HPLC output chromatogram showing each analyte elution order and its retention time.

3.1.8. Ion Chromatography

Nitrate (NO_3^-) and nitrite (NO_2^-) concentrations were determined using a Dionex DX-100 ion chromatography unit (Dionex Coorporation, Sunnyvale, CA, USA) equipped with a conductivity detector, a Dionex IonPac AG14A (4x50 mm) precolumn, and a Dionex IonPac AS14A (4x250 mm) analytical column. The unit was operated in autosuppression mode with 1 mM $NaHCO_3$/8 mM Na_2CO_3 eluent with a flow rate of 1 mL/min. Calibration curves were prepared using standards prepared by dissolving reagent grade sodium salts of each analyte in deionized (DI) water. All standards and samples were filtered through 0.2 µm membrane filters prior to injection. The injection volume was 0.2 mL. The minimum detection limits for NO_3^- and NO_2^- were 0.05 and 0.1 mg N/L, respectively.

3.1.9. Iron

Fe^{2+} was quantified with the method described by Kazumi *et al.* (1995b). Aliquots of 1 mL well-mixed culture were added to 20 mL 0.5 N HCl in 28 mL serum tubes capped with Teflon-lined septa and aluminum crimps. During the sampling care was taken not to expose the samples to oxygen and cause oxidation of Fe^{2+} to Fe^{3+}. After 10 min of acidification, an aliquot of 100 µL was filtered and added to a serum tube containing 5 mL of ferrozine solution [1 g/L ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine) in 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonicacid) buffer; pH=7]. The resulting solution was vortexed for 10 s to develop the characteristic magenta color of the Fe^{2+}-ferrozine complex and its absorbance was measured at 562 nM using an HP 8453 UV-Visible spectrophotometer (Hewlett-

Packard Co.). Calibration curves were prepared using a ferrous solution by dissolving reagent-grade ferrous ammonium sulfate sodium salt (green powder) – $(NH_4)_2Fe(SO_4)_2.6H_2O$ (Sigma Aldrich, St. Louis, MO) in deionized water and in autoclaved culture media (see Appendix A, Figure A.8). There was no difference in terms of calibration curves between standards prepared in DI water and autoclaved culture media. A 0.5 N HCl solution was used as the blank for these measurements.

Total iron was quantified with the same method of Fe^{2+} measurement. However, in this method, instead of a ferrozine solution, an hydroxylamine ferrozine buffer [10 g hydroxylamine (Sigma Aldrich) in 1 L ferrozine solution] was used which reduces ferric iron (Fe^{3+}) to Fe^{2+}. Fe^{3+} was determined by difference measurements after reduction of iron(III) to Fe^{2+} with hydroxylamine.

3.1.10. Centrifugation

A Beckman Avanti JE centrifuge was used to separate solvent phase from liquid phase. Glass centrifuge tubes (25 mL capacity, Corning) were used with a Beckman JA-25.50 rotor along with special holders designed for this purpose. Depending on the sample, the time and speed of the centrifugation varied.

3.2. General Procedures

3.2.1. Liquid/Liquid Extraction

The quantification of dissolved chlorinated organic compounds in aqueous samples was performed using a liquid/liquid extraction technique based on U.S. EPA methodology (U.S. EPA, 1986). The extractions were carried out in 25-mL glass

PYREX® round bottom nongraduated centrifuge tubes (Corning, NY). Analytes were extracted using isooctane containing 0.5 mg/L of 1,3,5-tribromobenzene (1,3,5-TrBB) internal standard. To perform the extraction, 2 mL of the solvent were transferred into the serum tube, followed by 5 to 20 mL (depending on the concentration of the analytes) of aqueous sample. The tube was capped with a Teflon-lined rubber stopper and sealed with an aluminum crimp. The samples were then vigorously shaken by hand for 2 minutes, and then mixed on a vortex mixer for another minute. The extraction tubes were centrifuged at 3500 rpm for 20 to 30 minutes (depending on how fast the solvent emulsion was separated), to obtain a clear solvent layer. Finally, 1 to 1.2-mL of the solvent extract was transferred to a 1.5-mL glass amber autosampler vial using a glass Pasteur pipette, the vial was capped with a Teflon-lined septum and an aluminum crimp, and analyzed by GC-ECD as described in Section 3.1.5.1, above.

The extraction efficiency of the liquid/liquid extraction method was assessed using aqueous standards run through the same extraction procedure as the experimental samples. One concentration of standard was prepared in deionized water and another in autoclaved culture media to test the effect of media on the extraction efficiency. The rest of the standards were prepared in deionized water because no difference was observed between the standards prepared in deionized water and autoclaved culture media. To prepare these standards, known concentrations of all chloroanilines and PCNB were dissolved in methanol. Three independent stock standards were prepared, and several dilutions of each stock solution were performed. The same amount of methanol was associated with each sample. The efficiency of the solvent extraction procedure was assessed at a concentration range of 0.03-40 µM PCNB and chloroanilines for an

extraction period of 2 min, 1 h, 1 d and 3 d. Aqueous standard samples were prepared as triplicates at each concentration level used in the calibration.

The samples were transferred to glass centrifuge tubes. The tubes were then thoroughly mixed and allowed to equilibrate for one hour. After equilibration, 2 mL of isooctane (with 0.5 mg/L 1,3,5-TrBB internal standard) was injected into each tube, and the extraction procedure performed as described above for four different extraction periods. Each of the samples was analyzed by GC-μ-ECD and calibration curves were generated using the calculated aqueous concentration and the μ-ECD area response for each analyte in the sample extract. Using the mass calibration of the μ-ECD (described above), liquid/liquid extraction efficiencies for each analyte were also calculated. Figure 3.3 shows the average recoveries observed for PCNB, PCA and less chlorinated anilines by liquid/liquid extraction. The highest recoveries were observed for the lowest aqueous concentrations and tended to drop as the mass contaminant in the tubes increased. ECD area responses for the aqueous standards and the concentrations of contaminants in experimental samples were corrected for autosampler injection volume variations using the area response of TrBB for each sample as described above.

3.2.2. Culture Media

Anaerobic cultures were developed and sustained in growth media which supplied all necessary nutrients, trace metals, and vitamins. The composition of culture media is shown in Tables 3.1 and 3.2. Resazurin was added as a redox indicator, which is colorless when reduced and bright pink when oxidized (at neutral pH; at low pH values it is blue

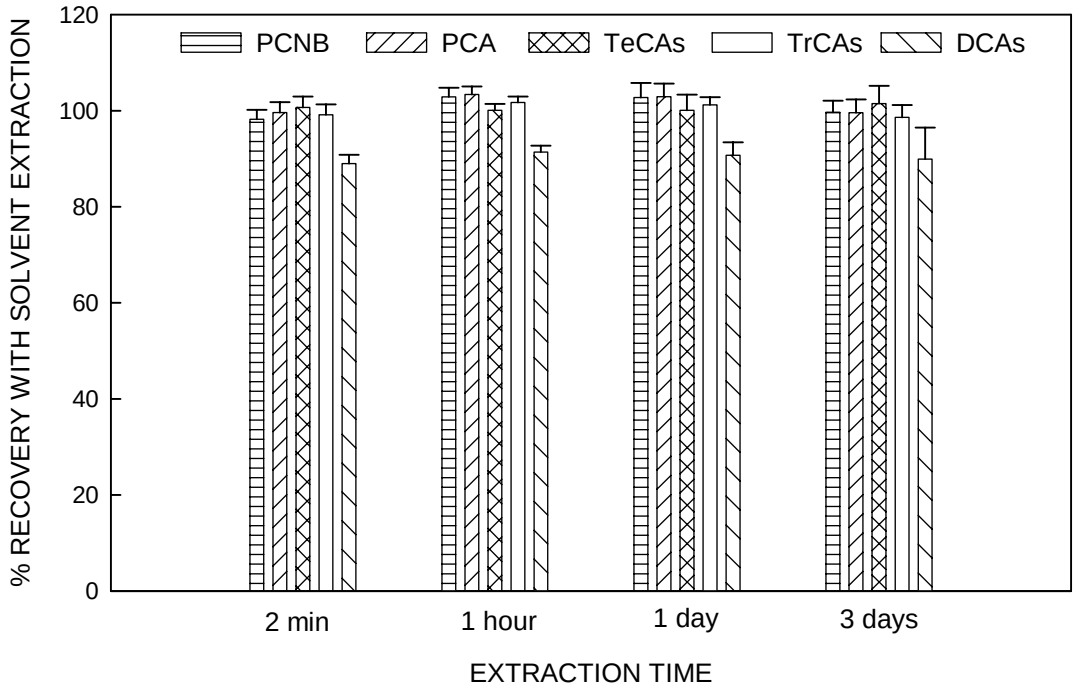


Figure 3.3. Liquid/liquid extraction efficiency of PCNB, PCA, and CAs as a function of extraction period (mean recoverage at the range of initial compound concentrations from 0.03 to 40 μM).

when oxidized). Resazurin was used in the media as a visual aid so that it could be quickly confirmed when the media were anaerobic.

Culture media were prepared by adding the first 8 ingredients in Table 3.1 to 8-L DI water in 9-L Pyrex serum bottles. The media bottles were covered with aluminum foil and steam autoclaved (121°C; 45 min). Stoppers fitted with Nalgene rigid tubing for headspace gas exchange and dispensing the media were also autoclaved wrapped in aluminum foil alongside the media bottles.

After autoclaving, the stoppers were clammed in place on top of the media bottles which were then flushed with either nitrogen or helium (through the Nalgene tube reaching the bottom of the bottle – later to be used for dispensing media) for at least 1 hour while being mixed using magnetic stir plates. This step was performed to strip most of the dissolved oxygen from the media as it cooled. After flushing, and while the media were still warm, sodium sulfide was added as a reducing agent. Finally, when the media were fully cooled, vitamins and sodium bicarbonate were added and the media were thoroughly mixed. The pH was checked to confirm that it was in the range of 7.3 to 7.5. The media bottles were covered with black bags in order to avoid light which may have a detrimental effect on the vitamins.

Denitrifying culture media was also prepared following the same procedures. Table 3.2 shows the media composition for the denitrifying cultures.

3.2.3. Amorphous Ferric Oxyhydroxide (FeOOH) Preparation

A stock solution of the less bioavailable amorphous ferric oxyhydroxide (FeOOH) was prepared by dissolving a known amount of $FeCl_3$ (200 mM) in deionized water. The

Table 3.1. Mixed fermentative/methanogenic culture media composition

Compound	**Concentration**
K_2HPO_4	0.9 g/L
KH_2PO_4	0.5 g/L
NH_4Cl	0.5 g/L
$CaCl_2 \cdot 2H_2O$	0.1 g/L
$MgCl_2 \cdot 6H_2O$	0.2 g/L
$FeCl_2 \cdot 4H_2O$	0.1 g/L
Trace metal stock solution	1 mL/L
Resazurin Stock	2 mL/L
Vitamin stock solution	0.2 mL/L
$NaHCO_3$	1.2 g/L
$Na_2S \cdot 9H_2O$	0.5 g/L

Trace metal stock solution	**Concentration, g/L**
$ZnCl_2$	0.5
$MnCl_2 \cdot 4H_2O$	0.3
H_3BO_3	3
$CoCl_2 \cdot 6H_2O$	2
$CuCl_2 \cdot 2H_2O$	0.1
$NiSO_4 \cdot 6H_2O$	0.2
$Na_2MoO_4 \cdot 2H_2O$	0.3

Vitamin stock solution	**Concentration, g/L**
Biotin	0.2
Folic Acid	0.2
Pyridoxine Hydrochloride	1
Riboflavin	0.5
Thiamine	0.5
Nicotinic Acid	0.5
Pantothenic Acid	0.5
Vitamin B_{12}	0.01
p-Aminobenzoic Acid	0.5
Thioctic Acid	0.5
Resazurin stock solution	1 g/L

Table 3.2. Denitrifying culture media composition

Compound	Concentration
K_2HPO_4	0.9 g/L
KH_2PO_4	0.5 g/L
NH_4Cl	0.5 g/L
$CaCl_2 \cdot 2H_2O$	0.1 g/L
$MgCl_2 \cdot 6H_2O$	0.2 g/L
$FeCl_2 \cdot 4H_2O$	0.1 g/L
Trace metal stock solution	1 mL/L[a]
Vitamin stock solution	0.2 mL/L[a]

[a] For the composition of the trace metal and vitamin stock solutions see Table 3.1

pH of the solution was then carefully adjusted to 7 by slowly adding 1 N NaOH. The resulting solution was kept with mixing while its pH was adjusted. Sufficient attention was paid to not go over a pH value of 7. The resulting suspension was centrifuged and the supernatant was carefully decanted. The DI washing step was repeated until the chloride concentration in the supernatant decreased below 1 mM. In order to eliminate oxygen interference, the solution was transferred to a serum bottle which was flushed with He for 30 minutes and then the suspension was stored under refrigeration.

3.2.4. Ferric EDTA Preparation

A stock solution of completely bioavailable iron in the form of ferric EDTA was prepared by dissolving 500 mM of Fe^{3+}-EDTA ($C_{10}H_{12}N_2NaFeO_8$) (Sigma Aldrich, St. Louis, MO) in deionized water. The pH of the solution was carefully adjusted to 7 by slowly adding 1N NaOH. In order to eliminate oxygen interference, the solution was transferred to a serum bottle which was flushed with He for 30 minutes, and then the suspension was stored under refrigeration.

3.2.5. Glassware Washing

Glassware used for liquid/liquid extraction, or in assays with cultures amended with chlorinated compounds was cleaned very carefully to prevent carryover of chlorinated organic residues from one assay to another. First, the glassware was rinsed with isooctane, then with methanol, and then soaked in a detergent (Sparkleen 1 for manual washing; Fisher Scientific) bath overnight. Each glass piece was then scrubbed with a nylon brush, rinsed well with tap water and then deionized water. After washing, all glass pieces were baked at 300ºC for at least 1 hour, and after cooling, they were kept in covered containers to eliminate any contamination

CHAPTER 4

PHASES OF STUDY

The goal of this research was to investigate the potential of the microbial transformation of PCNB and the environmental and physicochemical factors that control this biotransformation process. The experimental plan was divided into five phases. In Phase 1, an enrichment culture that transforms PCNB under fermentative/methanogenic conditions was developed. The PCNB-biotransforming culture was physiologically characterized based on the use of inhibitors for specific microbial groups and molecular characterization of the culture was performed (Phase 2). Phase 3 focused on the pathway analysis and kinetics of the PCNB reductive transformation process as well as the effect of various environmental factors (i.e., pH and temperature) on the biotransformation of PCNB. The effect of alternative electron acceptors (i.e., iron and nitrate) was assessed (Phase 4). Finally, experimental results related to observed dechlorination reactions were linked to molecular modeling (Phase 5). For each phase, a brief description follows.

4.1. Phase 1: Development of a PCNB-Biotransforming, Enrichment Culture

A mixed, fermentative/methanogenic, PCNB-biotransforming culture was developed in this phase of the research. The enrichment culture was derived from a contaminated sediment from Bayou d'Inde, Louisiana and culture development was performed at 22ºC to better simulate natural conditions. A sediment-free culture was obtained after multiple culture transfers. The microbial activity of the culture was

monitored by measuring pH, glucose, total gas, H_2, methane, and carbon dioxide production (Chapter 5)

4.2. Phase 2: Reductive Biotransformation of PCNB and Physiological Characterization of the PCNB-Biotransforming, Enrichment Culture

The major metabolic processes in the enriched culture fed with glucose and PCNB were fermentation, methanogenesis and reductive dechlorination. In order to determine the role and/or effect of these metabolic processes on the reductive biotransformation of PCNB, these processes were inhibited with selective metabolic inhibitors. Different electron donors were also used to assess the contribution of specific microbial groups. For example, in order to eliminate fermentation processes, acetate and H_2 were used as electron donors. 16S rRNA gene amplicons obtained with universal bacterial primers were used as templates for PCR with primer pairs that specifically targeted the 16S rRNA genes of known reductively dechlorinating organisms. The concentration effects of PCNB and biomass on the rate of PCNB biotransformation was investigated. The inhibitory effect of PCNB and its biotransformation products were assessed by the direct comparison between contaminant amended and unamended cultures (Chapter 6).

4.3. Phase 3: Pathway Analysis and Kinetics of the Sequential Dechlorination of PCA

The microbial reductive dechlorination kinetics of PCA, and all CAs were investigated with the fermentative/methanogenic enrichmet culture (Chapter 7). The

dechlorination of each parent compound and the production and disappearance of chlorinated aniline intermediates were simulated using a branched-chain Michaelis-Menten kinetic model. In addition to the kinetics, the effect of physicochemical factors such as temperature and pH on the biotransformation of PCNB was investigated. The effect of temperature on the PCA dechlorination rate was modeled using an Arrhenius relationship which accounts for both enzyme activation and deactivation (Chapter 7).

4.4. Phase 4: Effect of Alternative Electron Acceptors on the Biotransformation of PCNB

In order to assess the effect of alternative electron acceptors on the reductive biotransformation of PCNB, the enrichment fermentative/methanogenic culture was amended with different electron acceptors such as nitrate and iron. The influence of iron reduction on the reductive biotransformation of PCNB in a mixed, methanogenic enriched culture, and the effect of humics on iron reduction and the sequential reductive dechlorination of PCA were investigated (Chapter 8). The effect of nitrate at different initial concentrations on the biotransformation of PCNB was assessed with the fermentative/methanogenic PCNB biotransforming culture. The Effect of PCNB on nitrate reduction was also investigated in two cultures developed from the estuarine sediment as PCNB-acclimated and PCNB-free, under denitrifying conditions (Chapter 9).

4.5. Phase 5: Thermodynamic Analysis and Molecular Modeling of the Sequential Dechlorination of PCA

A semi-empirical molecular model (MOPAC) was used to optimize molecular geometries and estimate values of heat of formation and entropy for PCNB, PCA, and

CAs as well as their electronic properties. Correlations between the observed dechlorination pathway based on free energy considerations of compounds formed and electronic properties of C-Cl bond sites of parent compounds were assessed (Chapter 10).

CHAPTER 5

DEVELOPMENT OF A PENTACHLORONITROBENZENE-TRANSFORMING ENRICHMENT CULTURE

5.1. Introduction

Although there are a few reports in the literature on the microbial reductive transformation of PCNB (Tamura *et al.*, 1995; Susarla *et al.*, 1996; Murthy and Kaufman, 1978; Torres *et al.*, 1996; Murphy *et al.*, 1982), there is a lack of information on the biodegradation potential and environmental consequences of PCNB and its metabolites under anoxic/anaerobic conditions typically encountered in water-saturated soils and sediments. In order to systematically investigate the biotransformation potential of PCNB, a sediment-free enrichment culture was developed. The biotransformation potential of PCNB as well as various conditions that may affect such biotransformation were investigated with the sediment-free culture. The specific objectives of the research reported in this chapter were to: a) develop a PCNB-transforming mixed culture; b) assess the reductive biotransformation of PCNB in the mixed, fermentative/methanogenic culture; and c) assess the rate and extent of the abiotic transformation of PCNB under various conditions.

5.2. Materials and Methods

5.2.1. Culture Development

The enrichment culture was developed from a contaminated sediment obtained from Bayou d'Inde, a tributary of Calcasieu River near Lake Charles, LA. The location and details on the sediment sampling and analysis have been reported elsewhere (Prytula and Pavlostathis, 1996; Gess and Pavlostathis, 1997). Industrial wastes, including petroleum hydrocarbons, polycyclic aromatic hydrocarbons, and polychlorinated aromatic and aliphatic organic compounds have been discharged into this canal. However, PCNB and PCA were not detected in the sediment sample used as inoculum in this study. The culture was initiated by diluting 80 g of sediment in 1.8 L of mineral culture media (Chapter 3; Table 3.1) in a N_2-flushed, 2-L glass flask reactor, capped with a Teflon-lined stopper. The culture was fed weekly with glucose, yeast extract and a PCNB/methanol solution. Glucose, yeast extract and methanol served as the carbon source and electron donor. At the beginning of each 7-day feeding cycle, glucose, yeast extract, and PCNB in methanol were added resulting in initial concentrations of 333 mg/L, 17 mg/L, 0.09 μM and 53 mg/L, respectively. The culture was kept in the dark in a 22ºC constant temperature room, was stirred once a day using a Teflon-coated stirring bar over a magnetic stir plate, and its pH was kept at ca. 7 with $NaHCO_3$ addition. The reason for the mixing of the culture only once a day for a few minutes was due to promote larger floc formation that may help to develop efficient biomass for the reductive biotransformation of PCNB.

The microbial activity of the culture was monitored by measuring gas production and composition, pH and depletion of glucose and methanol as well as chlorinated

compounds. After several feeding cycles, a culture transfer took place by diluting 100 mL of the first generation culture in 1.8 L mineral media in a N_2-flushed, 2 L glass flask reactor, capped with a Teflon-lined stopper (second generation culture). In order to prevent sediment transfer, the culture was transferred to the new reactor after 30 s settling time. Finally, after several weekly feeding cycles, a similar culture transfer took place in 2 L of mineral media in a N_2-flushed, 9 L glass reactor, capped with a Teflon-lined stopper and its contents were gradually increased up to 6 L (third generation culture). The culture was maintained with weekly electron donor and PCNB addition. Every two weeks, 1 L of the reactor content was withdrawn while the culture was completely mixed and an equal volume of fresh anaerobic autoclaved culture media was added. Every two weeks when the culture was fed with only electron donor and PCNB, additional $NaHCO_3$ (0.5 g/L) was added as a powder to keep the pH around 7. The retention time for the culture was 84 days. Batch assays conducted under fermentative/methanogenic conditions were performed with the sediment-free, third generation mixed culture.

5.2.2. Biotransformation of PCNB

The biotransformation of PCNB by the enrichment culture was investigated in two separate assays using 160 mL serum bottles. Culture series were prepared with an initial PCNB concentration of 3 µM as triplicates. All serum bottles had the same amount of carbon sources. The initial biomass concentration in the serum bottles was 330 ± 25 mg/L (mean ± stand. dev.; n = 3) expressed as particulate organic carbon (POC). The serum bottles were sealed with Teflon-lined septa and flushed with nitrogen gas. For each culture series, one serum bottle was used for gas sampling and the remaining two serum

bottles were used for liquid sampling. The chemical oxygen demand (COD) of the glucose and methanol added to each serum bottle was 43 and 272 mg, respectively. All serum bottles were incubated in a 22ºC constant temperature room and were manually shaken once a day.

5.2.3. Abiotic PCNB Transformation Assay

Because of the potential of several culture media components (i.e., sulfide, Fe^{2+}, vitamin B_{12}), as well as culture products to abiotically mediate the reductive transformation of PCNB, an abiotic assay was conducted using 160 mL serum bottles. Three types of abiotic controls were used: autoclaved culture media, deionized (DI) water, and autoclaved culture. Two autoclaved culture media controls were set up with an initial PCNB concentration of 0.09 µM at two sulfide concentrations (3 and 67 mg/L total S), resulting at a pH of 7.82 and 7.85, respectively. The autoclaved culture media were transferred anaerobically with a syringe to previously N_2-flushed serum bottles, capped with a Teflon-lined stoppers. The culture media (see Chapter 3; Table 3.1) contained 28.1 mg/L (503 µM) Fe^{2+} and 2 µg/L (1.5 nM) vitamin B_{12}. The higher sulfide concentration corresponds to the $Na_2S{\cdot}9H_2O$ concentration in the culture media used in this study.

In order to assess the effect of sulfide alone on the transformation of PCNB to PCA, two controls were set up with an initial PCNB concentration of 0.09 µM at two sulfide concentrations (3 and 67 mg/L total S) in DI water. Before adding the sulfide solution and PCNB in to the DI water, the serum bottles containing the DI water were capped with a Teflon-lined stopper and were flushed for 20 minutes with N_2 under 15 psi. The resulting pH of 3 and 67 mg/L total S was 9.96 and 11.30, respectively.

In order to be able to compare the data obtained with the active culture and to take into account potentially important reductants that may be formed by the culture, another set of abiotic controls was setup with autoclaved culture. Aliquots of the active culture were transferred to 160 mL serum bottles and amended with glucose and PCNB dissolved in methanol. After 12 d of incubation, the serum bottles were autoclaved twice in two consecutive days at 121°C for 30 min. Then, two sets of triplicate autoclaved bottles were amended with PCNB resulting in an initial PCNB concentration of 0.12 and 3 µM, respectively.

All abiotic controls were incubated in a 22ºC constant temperature room and were manually shaken once a day. The concentration of chlorinated compounds was monitored over time by periodically withdrawing samples for liquid/liquid extraction.

5.3. Results and Discussion

5.3.1. Enrichment Culture

Transformation of PCNB to PCA occurred immediately in the sediment microcosm (first generation culture), but dechlorination of PCA to less chlorinated anilines (2,3,5,6-TeCA; 2,3,5-TrCA) was not observed until 35 d of incubation (Figure 5.1A). As discussed in Chapter 2, PCA has the same acute toxicity as PCNB, but is more hydrophobic than PCNB. Thus, this potentially toxic metabolite may accumulate in anoxic systems that do not support reductive dechlorination. Faster biotransformation of PCNB to PCA and also to less chlorinated anilines was observed in the second generation culture (Figure 5.1B). Because of the relatively high GC/ECD minimum detection limit of 2,4-DCA and 2,5-DCA (0.15 µM), accumulation of these compounds in the culture

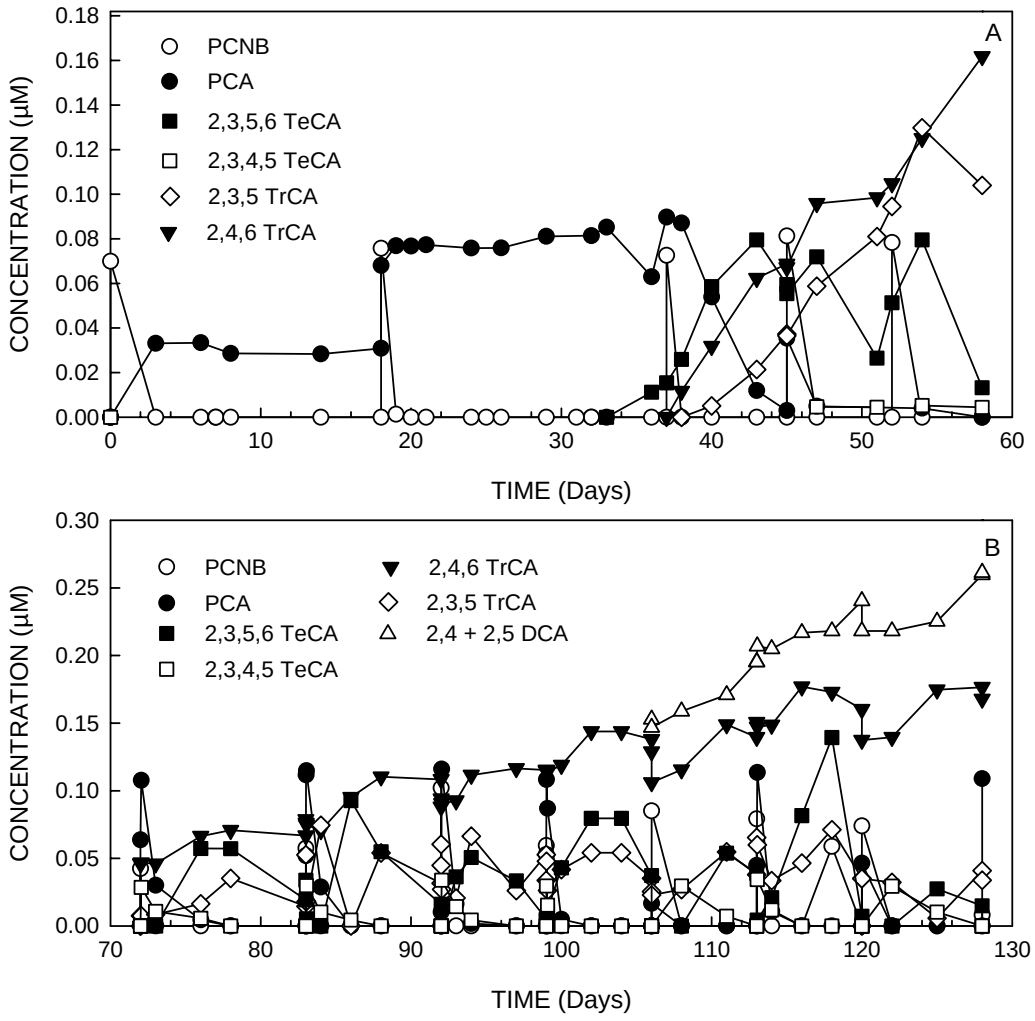


Figure 5.1. PCNB and transformation products in the first generation (A) and second generation culture (B)(multiple feeding cycles).

was observed only after 105 d of incubation following several feeding cycles. The PCNB transformation pathway in the third generation culture was very similar to that of the second generation culture. In every feeding cycle, glucose and methanol were consumed within 1 d. Propionate and acetate produced as a result of glucose fermentation were both consumed by the 7th day of the feeding cycle. The steady-state pH and biomass concentration of the third generation enrichment culture were 7.0 ± 0.3 and 315 ± 40 mg POC/L, respectively (mean ± stand. dev.; n = 3). The CH_4 and CO_2 partial pressure was 0.71 ± 0.01 and 0.29 ± 0.01, respectively, and H_2 was not detected at the end of each feeding cycle.

5.3.2 Biotransformation of PCNB

The concentration profiles of PCNB and its biotransformation products during a typical batch biotransformation assay conducted with the PCNB-enriched culture at an initial PCNB concentration of 3 μM is shown in Figure 5.2. The pseudo-first-order rate constant of the PCNB to PCA transformation was 14.1 ± 0.1 d^{-1}. Among the eighteen theoretically possible transformation products of PCNB to the level of monochloroanilines, ten products were observed during the biotransformation of PCNB by the enrichment culture (Figure 5.3). First, PCNB was transformed to PCA and then, 2,3,4,5-TeCA and 2,3,5,6-TeCA were two tetrachloroaniline isomers observed. 2,3,5-TrCA, and 2,4,5-TrCA were the major trichloroaniline isomers formed. 2,5-DCA and 3,5-DCA were the two dichloroaniline isomers observed in the enrichment culture. Because of the relatively high minimum detection limit of monochloroanilines by GC/ECD, during the PCNB biotransformation assay monochloroanilines could not be

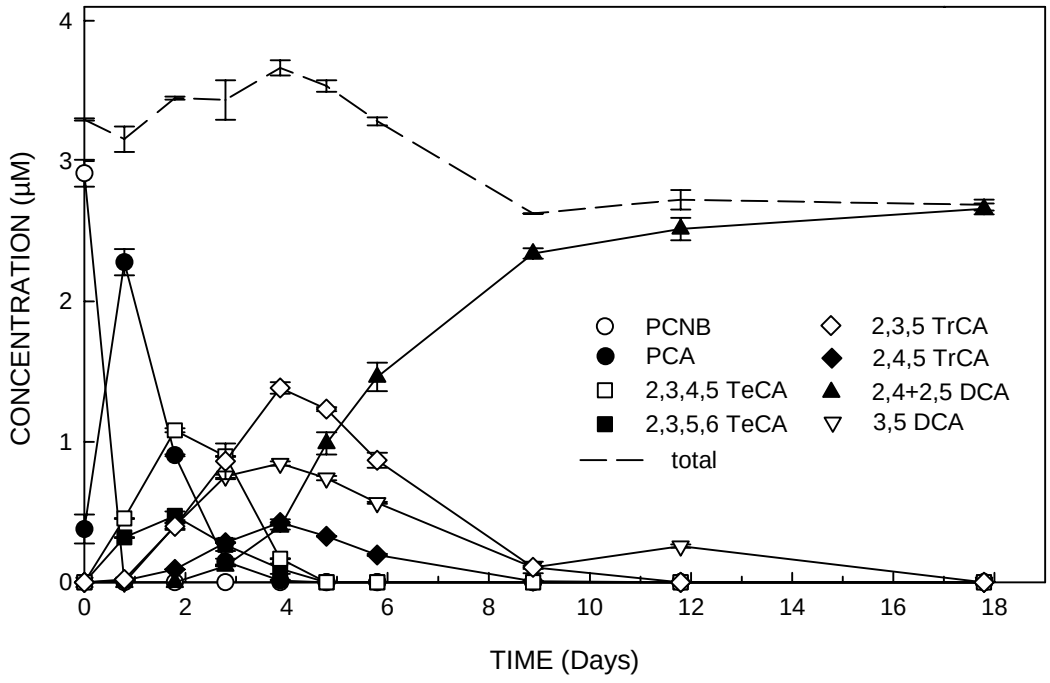


Figure 5.2. Time course of PCNB and its biotransformation products during a typical batch biotransformation assay conducted with the PCNB-enriched, mixed culture (Error bars represent mean values ± one standard deviation).

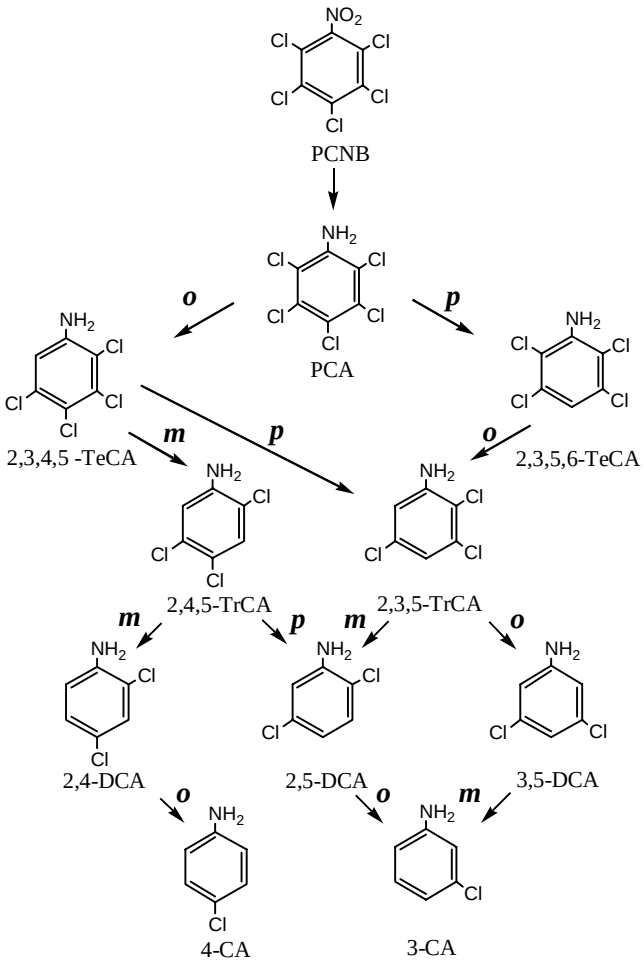


Figure 5.3. PCNB biotransformation products observed in the enrichment culture (***o***, ***m***, ***p***: *ortho-*, *meta-*, *para*-dechlorination).

quantified. However, HPLC analysis performed after several feeding cycles of the enrichment culture showed that the monochloroanilines 3-CA and 4-CA had accumulated in the culture.

Based on our results, dechlorination of PCA proceeded mainly by *para* and *ortho* dechlorination, although *meta* dechlorination was also observed leading to the formation of some di- and mono-chloroanilines. Susarla *et al.,* (1996) observed the following PCNB biotransformation pathway in assays conducted with a contaminated sediment under sulfidogenic conditions: PCNB $\rightarrow$ PCA $\rightarrow$ 2,3,4,5-TeCA $\rightarrow$ 3,4,5-TrCA $\rightarrow$ 3,5-DCA $\rightarrow$ 3-CA with traces of 2-CA and 4-CA. These reactions represent dechlorination at the *ortho, ortho, para,* and *meta* positions, respectively. In a subsequent study on the reductive dechlorination of chloroanilines by the same contaminated sediment under sulfidogenic conditions, Susarla *et al.,* (1997a) observed the reductive dechlorination of 2,3,4,5-TeCA at the *para* and *ortho* positions to form 2,3,5-TrCA and 3,5-DCA, and then 3-CA was formed by *meta* dechlorination. This pathway was the same as that starting with PCNB, except that the trichloroaniline isomer was different than that observed in their first study. Similar results were reported by Kuhn and Suflita (1989) on the sequential dechlorination of 2,3,4,5-TeCA at the *para* and *ortho* positions to form 2,3,5-TrCA and then 3,5-DCA, but further dechlorination of 3,5-DCA was not observed under methanogenic conditions in their study. Dechlorination of 3,4-DCA at the *para* position resulted in the formation of 3-CA and none of the three monochloroaniline isomers were dechlorinated in these assays under methanogenic conditions. Kuhn and Suflita (1989) concluded that reductive dechlorination of chloroanilines was only observed if these compounds had at least two chlorine substituents in adjacent positions. However, when

methanogenic aquifer slurries were amended repeatedly with 2,3,4,5-TeCA, in addition to the formation of 2,3,5-TrCA via *para* dechlorination, 2,4,5-TrCA was also produced via *meta* dechlorination (Kuhn and Suflita, 1989). Struijs and Rogers (1989) observed reductive dechlorination of a series of dichloroanilines to monochloroanilines in anoxic pond sediment slurries, but the resulting monochloroanilines were not transformed further under the conditions of their study. Similarly, monochloroanilines formed as a result of reductive dechlorination of 2,3,4-TrCA spiked in flooded soil suspensions and incubated under anaerobic conditions were not further dechlorinated (Pardue *et al.*, 1996). Our observations of only minor production of monochloroanilines and the absence of aniline during the reductive transformation of PCNB and the sequential dechlorination of the resulting PCA are consistent with previously conducted studies with respect to the relatively recalcitrant nature of monochloroanilines under methanogenic conditions.

Based on calculated standard free energy for the reductive dechlorination of chloroanilines using H_2 as the electron donor, Susarla *et al.*, (1997b) reported that under standard conditions, dechlorination of chloroanilines results in available free energy in the range of 123 to 146 kJ/mol of chlorine removed. Based on redox potentials using the H^+/H_2 couple as the electron donor, the same researchers noted that the sequential reductive dechlorination of 2,3,4,5-TeCA to 3-CA observed in their study, as well as that observed by Kuhn and Suflita (1989), followed the route by which reactions with a higher standard redox potential (E_o') are preferred over those reactions with lower redox potentials. However, it should be pointed out that, all dechlorination reactions are highly exergonic at standard conditions and therefore, the criterion that relatively higher energy release favors a particular dechlorination reaction may not be valid. Beyond the

thermodynamic potential, without any doubt, other factors (e.g., kinetic, steric, enzymatic specificity, etc.) play an important role in microbially-mediated dehalogenation reactions, as discussed below (Pavlostathis and Prytula, 2000).

Previously observed reductive dechlorination pathways for chlorophenols under sulfidogenic conditions primarily followed the route of *ortho*-dechlorination (Masunaga *et al.*, 1996). However, when the above discussed hypothesis relative to dechlorination pathways consistent with the highest redox potential was compared with observed, microbially-mediated dechlorination pathways of chlorophenols, just the opposite was found to be true, i.e., the least thermodynamically favorable pathways were observed (Masunaga *et al.*, 1996). In contrast to these results, the sequential reductive dechlorination of pentachlorophenol proceeded mainly via *para* and then *ortho* dechlorination (Magar *et al.*, 1999). As Kuhn and Suflita (1989) have pointed out, in addition to free energy considerations, two additional factors may govern aryl halide removal: a) increased positive polarization of the Cl-substituted carbons in two or more adjacent positions on the aromatic ring may result in destabilization of the halogen-carbon bonds, thus making them more labile; b) due to resonance effects of electron-donating groups containing an unshared pair of electrons (e.g., $-NH_2$, -OH), the *ortho* and *para* positions of the aromatic ring are more susceptible to electrophilic attack resulting in the preferential release of halides from these positions. The predominant pathway for the sequential reductive dechlorination of hexachlorobenzene (HCB) by a sediment-derived, HCB-enriched, mixed methanogenic culture (HCB $\rightarrow$ PCB $\rightarrow$ 1,2,3,5-TeCB $\rightarrow$ 1,3,5-TrCB $\rightarrow$ 1,3-DCB) followed the above-discussed preferential route of *para*, *ortho*, and *ortho* dechlorination (for the last three reactions), although low concentrations of

other congeners resulting from *meta* dechlorination were also observed (Pavlostathis and Prytula, 2000).

5.3.3. Abiotic Transformation of PCNB

The abiotic transformation of PCNB in the first abiotic set was monitored over a 2-month period. Complete transformation of 0.09 μM PCNB to PCA was observed in the autoclaved culture media controls within 5 days at a sulfide concentration of 67 mg/L (Figure 5.4A), whereas the same transformation required 35 days at a sulfide concentration of 3 mg/L (Figure 5.4B). The pseudo-first-order rate constants for the PCNB transformation at 3 and 67 mg/L sulfide in autoclaved culture media were 0.144 ± 0.006 and 0.851 ± 0.004 d^{-1} (mean ± stand. error), respectively.

Transformation of PCNB to PCA in the DI water/sulfide controls was observed at both the higher and lower sulfide concentration (0.006 ± 0.001 and 0.107 ± 0.008 d^{-1}, respectively; mean ± stand. error)(Figure 5.4C and 5.4D). However, the PCNB transformation rates were much lower than the ones observed in the autoclaved culture media abiotic controls (0.144 ± 0.006 and 0.851 ± 0.004 d^{-1}, respectively).

Complete transformation of PCNB to PCA in the autoclaved culture controls occurred in 6 h and 1 day for an initial PCNB concentration of 0.12 and 3 μM, respectively (Figure 5.5A and 5.5B) with the corresponding pseudo-first-order rate constants of 40.8 ± 3.7 and 1.7 ± 0.17 d^{-1} (mean ± stand. error), respectively. Therefore, biotically generated reductants by the enrichment culture before autoclaving enhanced the rate of PCNB to PCA transformation observed in the autoclaved culture media controls by about 48-fold. It is noteworthy that the pseudo-first-order rate constants for the

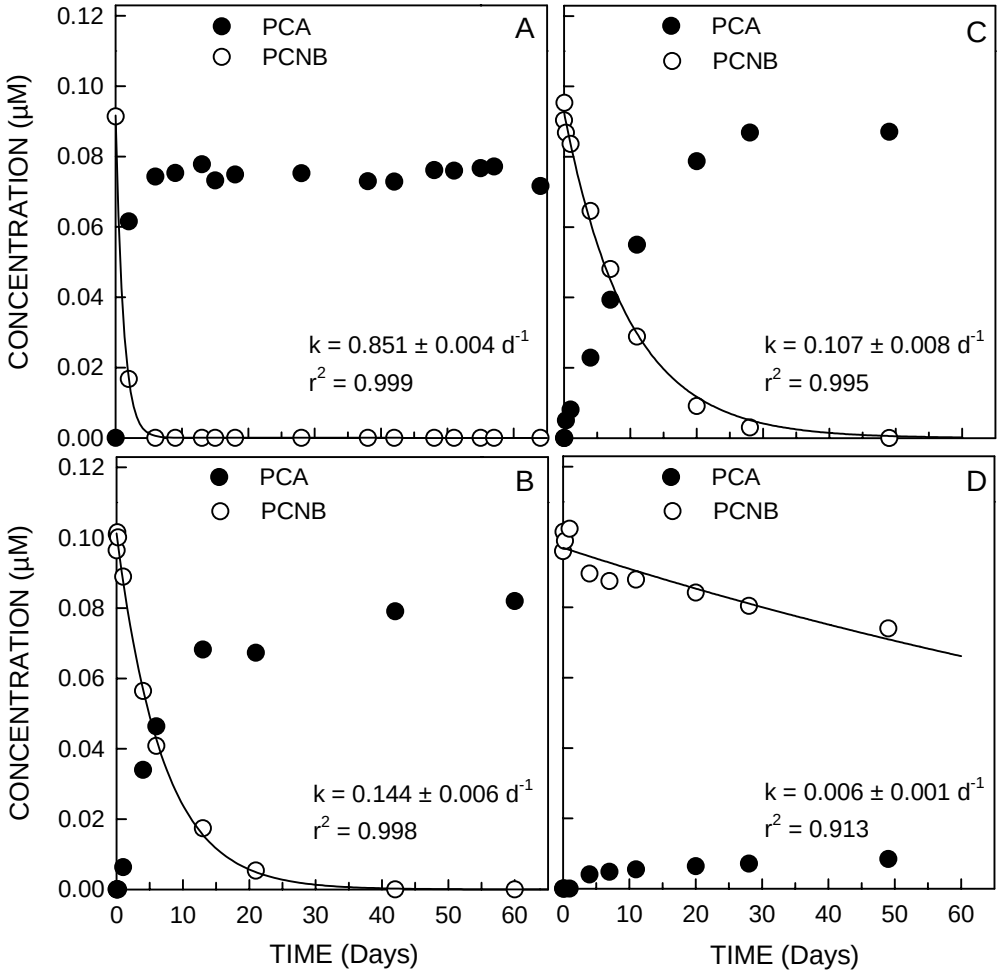


Figure 5.4. Abiotic transformation of PCNB in autoclaved culture media with 67 mg/L sulfide-S (= 0.5 g/L $Na_2S{\cdot}6H_2O$), pH = 7.85 (A) and 3 mg/L sulfide-S (= 0.027 g/L $Na_2S{\cdot}6H_2O$), pH = 7.82 (B), as well as in DI water with 67 mg/L sulfide-S, pH = 11.30 (C) and 3 mg/L sulfide-S, pH = 9.96 (D). The culture media contained 67 mg/L sulfide-S (Lines are first-order fits to the PCNB data).

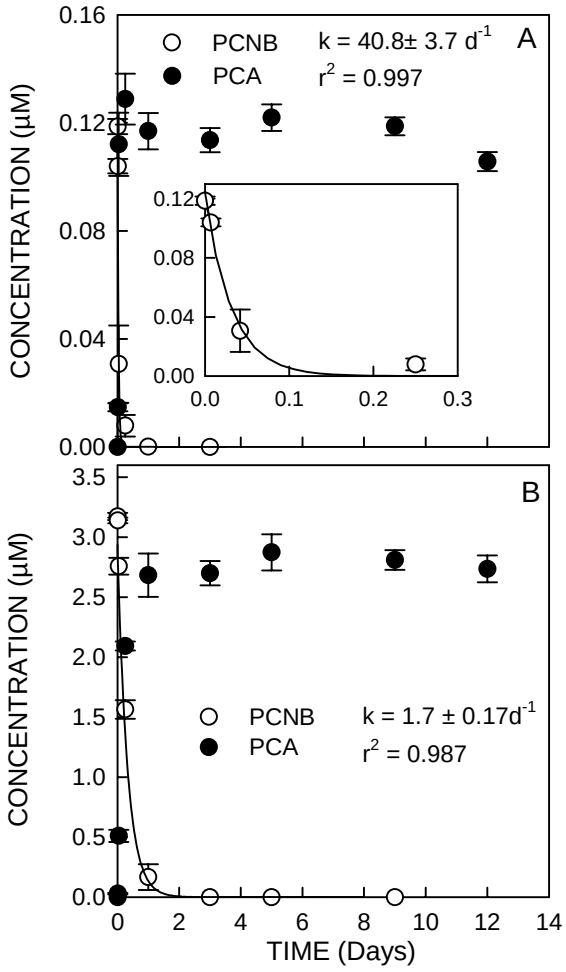


Figure 5.5. Abiotic transformation of PCNB in autoclaved culture controls at an initial PCNB concentration of 0.12 µM (A) and 3 µM (B)(Lines are first-order fits to the PCNB data)(Error bars represent mean values ± one standard deviation; *n* = 3).

transformation of PCNB to PCA by the active culture at an initial PCNB concentration of 0.12 and 3 μM were 40 ± 0.5 and 14.1 ± 0.1 d^{-1} (mean ± stand. error), respectively, which are very similar to the rates obtained with the autoclaved culture controls. Thus, the rate of abiotic transformation of PCNB to PCA depends on the reducing agent (sulfide) concentration and the presence of certain culture media components (e.g., Fe^{2+}, vitamin B_{12}). More importantly, biotically generated reductants and/or other factors have the highest effect on the PCNB to PCA transformation rate. Dechlorination of PCA was not observed in any of the abiotic controls over a 25-d incubation period.

Similar to our results, Cheng *et al.* (1996) reported the reduction of 2,4-dinitrotoluene in the presence of sulfide minerals. Fe^{2+} associated with iron-containing minerals is known to catalyze abiotic nitroaryl reduction. Klupinski *et al.* (2004) reported a pseudo-first-order rate constant of 0.326 min^{-1} ($t_{1/2}$ = 2.1 min) for the abiotic reduction of PCNB (0.849 μM) under the following reaction conditions: Fe^{2+}, 527 μM; goethite, 100 ± 11 mg/L; NaCl, 200 mM; and pH, 7.18. The same researchers reported that in the case where mineral phases were not added, the PCNB to PCA transformation was very slow compared to the case where minerals were present and the transformation followed zero-order kinetics. For example, for the reaction condition with 0.85 mM Fe^{2+} in a pH 7 buffer, the zero-order PCNB transformation rate at an initial PCNB concentration of 1.225 μM was 1.15 x 10^{-3} μM/min ($t_{1/2}$ = 533 min), i.e., over a 250-fold lower compared with the case where goethite was present. Elsner *et al.* (2004) observed that the surface-area-normalized rate constants for the abiotic reduction of 4-chloronitrobenzene (4-Cl-NB) by Fe^{2+} adsorbed onto various iron minerals increased in the following order: siderite < iron oxides < iron sulfides. For the case of goethite, they reported a surface-

normalized pseudo-first-order rate constant of 1.9 L/h · m^2 (= 0.79 min^{-1}; $t_{1/2}$ = 0.9 min) under the following conditions: Fe^{2+}, 1 mM; goethite, 1.54 g/L (25 m^2/L surface area); and pH 7.2; initial 4-Cl-NB concentrations 0.5-3 μM. The abiotic PCNB reduction rates observed in our study in the absence of iron solid surfaces (homogeneous system) were two to three orders of magnitude lower than those reported by Klupinski *et al.* (2004) for PCNB reduction in the presence of goethite and for the reduction of 4-Cl-NB in the presence of goethite or mackinawite (FeS) reported by Elsner *et al.* (2004). These results indicate the catalytic role of iron solid surfaces in the abiotic reduction of nitroaromatic compounds in heterogeneous anoxic systems.

Vitamins, such as B_{12}, which was present in the culture media used in this study, also have the potential to reduce nitroaromatic and chlorinated compounds (Brearley *et al.*, 1971; Assaf-Anid *et al.*, 1992; Ahuja *et al.*, 2004). However, abiotic dechlorination of PCA did not occur in the presence of only autoclaved culture media and sulfide in our study. Similarly, dechlorination of PCA resulting from the abiotic reduction of PCNB in the presence of Fe^{2+}/goethite was not observed by Klupinski *et al.*, (2004). It should be noted that the vitamin B_{12} concentration in our culture media (1.5 nM) was more than six orders of magnitude lower than in previous studies reporting the catalytic effect of vitamin B_{12} in the reduction of chloro- and nitro-organic compounds. Thus, vitamin B_{12} may not have contributed significantly to the reduction of PCNB observed in this study.

5.4. Summary

The microbial reductive transformation of PCNB was investigated with a mixed, methanogenic culture developed from a contaminated estuarine sediment. PCNB was transformed to PCA under both biotic and abiotic reductive conditions. However, the

sequential reductive dechlorination of PCA was only achieved in the presence of anaerobic biological activity, but required culture enrichment over a relatively long acclimation period (about 35 d) under methanogenic conditions (i.e., strict anaerobic, low redox potential conditions). Given the fact that PCA is also toxic, its formation and persistence in anoxic natural environments, where dechlorination of PCA does not take place may pose a potential environmental risk. The predominant PCA dechlorination products under methanogenic conditions were di- and mono-chloroanilines, which accumulated. Thus, such products appear to be relatively recalcitrant under methanogenic conditions. Due to the fact that there is about a 1800- to 9800-fold increase in aqueous solubility and 55- to 340-fold decrease in the octanol-water partition coefficient between PCA and di- or mono-chloroanilines, respectively, microbial reductive dechlorination leads to an increased mobility of the PCA biotransformation products. PCNB was also abiotically transformed to PCA in autoclaved culture media, which contained relatively low concentrations of sulfide, Fe^{2+} and vitamin B_{12}, but at much lower rates as compared to the biotic assays. In contrast, the rate of PCNB to PCA transformation in autoclaved culture controls was similar to the rates observed in the active enrichment culture, indicating that biotically-derived reductants and/or other factors facilitated the observed transformation of PCNB to PCA. Dechlorination of PCA was not observed in any of the abiotic controls.

CHAPTER 6

BIOTRANSFORMATION OF PENTACHLORONITROBENZENE UNDER FERMENTATIVE/METHANOGENIC CONDITIONS

6.1. Introduction

The third generation sediment-free, enrichment culture described in Chapter 5 was used to investigate the reductive biotransformation of PCNB under fermentative/methanogenic conditions, as well as to physiologically characterize it. The mixed culture mediates three main processes: fermentation, methanogenesis and dechlorination. As described in Chapter 5, microbial transformation of PCNB was achieved under fermentative/methanogenic conditions. It has been reported that PCNB transformation takes place under both methanogenic and sulfidogenic conditions (Susarla *et al*., 1996; 1997b; Kuhn and Suflita, 1989). However, to our knowledge, physiological characterization of a PCNB-biotransforming mixed culture has not previously been performed.

The specific objectives of the research reported in this chapter were to assess: a) the effect of initial biomass concentration on PCNB biotransformation; b) the effect of various electron donors on PCNB biotransformation; c) possible inhibitory effects of PCNB on the mixed, methanogenic culture; d) the effect of 2-bromoethanesulfonate (BES), an inhibitor of methanogenesis, as well as sodium azide, a general bacteriostatic agent, on the reductive biotransformation of PCNB in the mixed,

fermentative/methanogenic culture. Finally, a 16S rRNA gene-based community analysis of the mixed, enrichment culture was undertaken.

6.2. Materials and Methods

6.2.1. Effect of Initial Biomass Concentration on PCA Dechlorination

The effect of the initial biomass concentration on the PCA dechlorination kinetics was investigated in a batch assay. Four culture series were prepared with initial biomass concentration of 257 ± 16, 195 ± 7, 122 ± 1, and 61 ± 3 mg POC/L (mean ± standard deviation; $n = 3$) by diluting the enrichment culture with anaerobic culture media. The assay was conducted in triplicate 160 mL serum bottles with an initial PCA concentration of 3 µM. The chemical oxygen demand (COD) of the glucose and methanol added to each serum bottle was 43 and 272 mg, respectively. All serum bottles were incubated in a 22ºC constant temperature room and were manually shaken once a day. The cultures' activity was monitored by measuring gas production and composition, chlorinated compounds, VFAs, and pH.

6.2.2. Effect of Initial PCNB Concentration on its Biotransformation

The effect of the initial PCNB concentration on its biotransformation kinetics was investigated in a batch assay. Five culture series were prepared with initial PCNB concentrations of 0, 0.09, 3, 10 and 40 µM. The assay was conducted in triplicate 160 mL serum bottles that were sealed with Teflon-lined septa and flushed with nitrogen. The initial biomass concentration in these cultures was 330 ± 25 mg POC/L (mean ± standard deviation; $n = 3$). All serum bottles had the same amount of carbon sources. Four PCNB

stock solutions were prepared in methanol at different concentrations. Thus, the same amount of methanol (1535 mg/L) was added to each serum bottle. All serum bottles were incubated in a 22ºC constant temperature room and were manually shaken once a day. The cultures' activity was monitored by measuring gas production and composition, chlorinated compounds, VFAs, and pH.

6.2.3. Electron Donor Type and PCNB Biotransformation

Because of the very low solubility of PCNB (0.44 mg/L at 25°C) this compound was introduced to the cultures as dissolved in methanol during most of the batch assays and in the stock culture. In order to investigate the effect of methanol on PCA dechlorination as the only electron donor, two series of cultures were setup in 160-mL serum bottles. The total liquid volume in these cultures was 120 mL. One of the culture series was amended with 3 µM PCA dissolved in methanol (1535 mg/L) and the other culture series were amended with 3 µM PCA dissolved in methanol (1535 mg/L), glucose (333 mg/L), and yeast extract (17 mg/L). In each culture series, one of the serum bottles was used for liquid and the other serum bottle was used for gas sampling. All serum bottles were incubated in a 22ºC constant temperature room and were manually shaken once a day. Liquid and gas samples were periodically taken to monitor gas composition, VFAs, chlorinated compounds and pH.

In order to test the biotransformation potential of PCNB with H_2 and acetate as the only electron donors, approximately 4 µM of PCNB was introduced to the culture (120 mL). PCNB was transferred to each 160 mL serum bottle in crystalline form and the serum bottles were sealed with Teflon-lined septa and flushed with nitrogen gas. Culture

aliquots were then transferred to the serum bottles anaerobically and amended with acetate (10 mM) or H_2 (20 mL). H_2 was added to the 40 mL head space of the cultures by a syringe which was flushed with H_2. The initial H_2 concentration in the headspace was estimated to be between 300,000 and 350,000 ppmv, based on the known volume of H_2 added. The pH was kept around 7 with $NaHCO_3$ addition which also provided the carbon source for these cultures. Both culture series were incubated upside down to eliminate possible gas loses from the septa. In order to eliminate shortage of electron donor, the cultures were amended with acetate or H_2 three times during the one month incubation period.

6.2.4. Effect of Inhibitors on the PCNB Biotransformation

Two inhibitors were used in order to assess the relation between PCNB biotransformation and microbial activity in the mixed culture. BES, a structural analogue of coenzyme M (CoM), the last enzyme in the methanogenic pathway mediating the conversion of methyl to methane, and a potent inhibitor of methanogenesis, was added to culture samples at initial BES concentrations of 25, 50 and 75 mM. BES is a water-soluble compound and is effective at relatively low concentrations (<1 mM); however, it is often necessary to use much higher concentrations (>10 mM) to fully inhibit methanogenesis and to eliminate possible degradation, transport, and resistance problems (Oremland and Capone, 1988). Sodium azide, a general bacteriostatic agent, was added to another set of cultures at an initial concentration of 200 mg/L. Before the addition of electron donor and PCNB, the inhibitor-amended cultures were incubated for a day in

order to enhance the effectiveness of the two inhibitors. The initial biomass concentration in these cultures was 310 ± 20 mg POC/L.

The assay was conducted in triplicate 160 mL serum bottles which were sealed with Teflon-lined septa and flushed with nitrogen gas. For each culture series, one serum bottle was used for gas sampling and the remaining two serum bottles were used for liquid sampling. The chemical oxygen demand (COD) of the glucose and methanol added to each serum bottle was 43 and 272 mg, respectively. All serum bottles were incubated in a 22ºC constant temperature room and were manually shaken once a day.

6.2.5. 16S rRNA Gene-Based Community Analysis

Genomic DNA of the PCNB-biotransforming, fermentative/methanogenic, sediment-free culture was extracted using two extraction kits: QIAamp DNA Mini Kit (Qiagen, Valencia, CA) and Mo Bio UltraClean Soil DNA Kit (MO BIO Laboratories, Inc., West Carlsbad, CA). The DNA purification procedure was performed according to the manufacturers' recommendations. The quality of the extracted genomic DNA was verified on 1.5% agarose gels prepared in TAE buffer (Tris-base, 48.4 g; glacial acetic acid, 10.9 g; EDTA, 2.92 g; dissolved in 1L DI water). The DNA was quantified spectrophotometrically at 260 and 280 nm by diluting the samples at a ratio of 1:10 (sample:DI water). The purity of the DNA was verified by the ratio of 260 to 280 which should be approximately 1. To increase the detection sensitivity of dechlorinating bacterial populations, a nested PCR approach was used. An initial amplification of the communities' 16S rRNA genes was performed using universal bacterial primers 8F and 1541R and conditions as described by Löffler *et al*. (2000). Then, 1:100 and 1:1000

dilutions of the 16S rRNA gene amplicons were used as templates for primer pairs that specifically target the 16S rRNA genes of targeted dechlorinating bacterial groups as previously published by Löffler *et al*., (2000). Following amplification, the presence of known groups of dechlorinating bacteria was explored with 16S rRNA gene-targeted primers specific for members of the *Dehalococcoides*, *Dehalobacter*, *Desulfuromonas*, *Geobacter*, and *Anaeromyxobacter* groups (Lévesque *et al*., 1997; Löffler *et al*., 2000, 2005; Schlötelburg *et al*. 2002; He *et al*., 2003a, 2003b; Petrie *et al*., 2003).

6.3. Results and Discussion

6.3.1. Effect of Initial Biomass Concentration on PCA Dechlorination

The effect of the initial biomass concentration on the PCA dechlorination rate was investigated with the mixed enrichment culture at an initial PCA concentration of 3 µM. The initial and final pH values in this assay were 7.2 and 6.8, respectively. Figure 6.1 shows the cumulative methane production as well as PCA dechlorination profiles in these culture series. Methane production profiles showed that the rate of methanogenesis increased with increasing initial biomass concentration, but the extent of the produced cumulative methane was the same for all cultures (Figure 6.1A). PCA dechlorination took place in all culture series, but at different rates (Figure 6.1B). To compare initial PCA dechlorination rates at different initial biomass concentrations, linear regressions were performed and the initial volumetric dechlorination rates are summarized in Table 6.1. The PCA dechlorination rate values for the three diluted culture series relative to the undiluted culture were 0.8, 0.57, and 0.27, corresponding to the three initial culture levels of 75, 50, and 25%, respectively. Thus, the relative PCA transformation rates for all

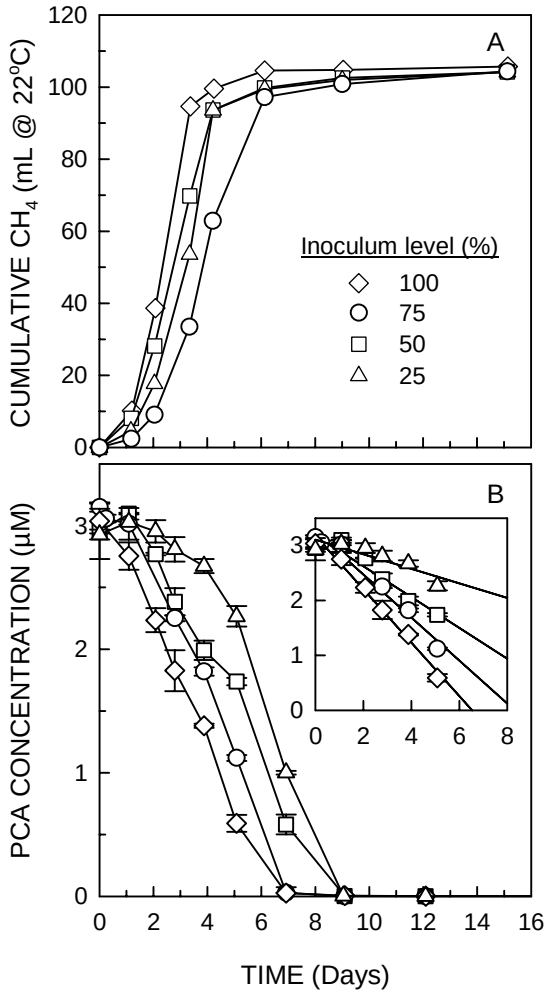


Figure 6.1. Effect of biomass concentration on cumulative methane production (A) and PCA dechlorination (B).

Table 6.1. Initial volumetric PCA transformation rates as a function of initial total biomass concentration[a]

Initial biomass (mg VSS/L)	Initial PCA transformation rate (μmol PCA/L·d)	r^2	Relative PCA transformation rate[c]	Biomass-normalized rate (μmol PCA/mg VSS·d)
514 ± 32	0.49 ± 0.04[b]	0.986	1	$1.01 \pm 0.08 \times 10^{-3}$
390 ± 14	0.39 ± 0.04	0.973	0.80	$1.04 \pm 0.05 \times 10^{-3}$
244 ± 2	0.28 ± 0.04	0.905	0.57	$1.16 \pm 0.01 \times 10^{-3}$
122 ± 6	0.13 ± 0.04	0.766	0.27	$1.14 \pm 0.08 \times 10^{-3}$

[a] Experimental conditions: initial PCA concentration 3 μM, incubation temperature 22°C, and pH range 6.8 to 7.2. [b] Mean ± standard deviation ($n \geq 4$) [c] Rates normalized to that of the undiluted (i.e., 100%) culture.

culture series were directly proportional to the initial biomass level (Table 6.1). The net increase in the total biomass concentration over the 15-d incubation period was less than 20% of the initially added biomass in each culture series. The PCA removal rates were progressively slower as the initial inoculum concentration decreased, but in all cases complete PCA removal was accomplished within 9 days of incubation. These results indicate that the maximum dechlorination rate at given experimental conditions was proportional to the biomass concentration (i.e., $k' = kX$; where k'= the maximum CA dechlorination rate for a constant biomass concentration; k= the maximum CA dechlorination rate per unit biomass; X= the biomass concentration). Similar to our results, Rhee *et al*. (2001) reported that the dechlorination rates of polychlorinated

biphenyls (PCBs) were closely related to the population size of dechlorinating microorganisms. At Aroclor concentrations below 0.14 μmol/g sediment, dechlorination as well as growth of dechlorinating species was not observed. Similarly, the dechlorination rate of hexachlorobenzene (HCB) was shown to be directly proportional to the biomass level of a HCB-dechlorinating enrichment culture (Pavlostathis and Prytula, 2000).

6.3.2. Effect of Initial PCNB Concentration on its Biotransformation

The effect of initial PCNB concentration on its biotransformation was investigated with the mixed enrichment culture. The potential inhibitory effect of PCNB on the PCNB-enriched and dechlorinating mixed culture was also investigated in the same batch assay. The highest PCNB concentration used in this study is approximately 27 times higher than its aqueous solubility. The initial and final pH values for this assay were 7.0 and 6.8, respectively. After 10 days of incubation, the cultures were fed again with only glucose and methanol as carbon source to eliminate any limitation of electron donor at the higher PCNB concentration cultures and to achieve complete biotransformation. PCNB was not added in this second feeding. The effect of the initial PCNB concentration on the total gas, methane, and carbon dioxide production is shown in Table 6.2. Methane, carbon dioxide, and biomass production were very similar in all cultures regardless of the initial PCNB concentration. H_2 was also monitored, but concentrations were very low and around the minimum detection limit (400 ppmv in the gas phase). Thus, PCNB up to 40 μM did not have any significant effect on the activity of the mixed culture. Data for the detailed comparison between the PCNB-free, control

Table 6.2. Gas production and biomass concentration in the enrichment culture amended with different initial PCNB concentrations[a]

Initial PCNB (μM)	Initial biomass concentration (mg POC/L)	Final biomass concentration (mg POC/L)	Total gas production (%)[c]	Methane production (%)[c]	Carbon dioxide production (%)[c]
0	342 ± 11[b]	572 ± 5	100	100	100
0.09	349 ± 10	565 ± 2	93.3	92.8	101
3	353 ± 11	566 ± 8	95.3	93.7	100
10	340 ± 7	560 ± 7	101	97.7	100
40	352 ± 5	572 ± 10	101	97.8	101

[a] After 25 d of incubation. [b] Mean ± standard deviation (n = 3). [c] Expressed as a fraction of the PCNB-free, control culture.

culture and the 40 μM PCNB-amended culture are shown in Figure 6.2. Glucose was depleted in all five cultures within 7 h. Methanol was consumed at a slower rate than glucose and was completely depleted within 5 days. The production and consumption of VFAs were not significantly affected by the PCNB addition at four initial concentrations. In all cultures, the propionate concentration was higher than that of acetate, but both VFAs were completely consumed in all cultures and no inhibitory effect was observed (Figure 6.2). However, after the second feeding with only carbon source, a higher concentration of acetate was observed in the culture series amended with 40 μM PCNB, which may be the result of low and transient inhibition of acetoclastic methanogens by

the accumulated di- and mono-chloroanilines (Figure 6.2E). Kim *et al*. (1996) reported that the three monochloroaniline isomers at concentrations of 400 mg/L and above had a significant impact on the fermentation of ethanol to acetate and H_2, inhibited acetoclastic methanogenesis, but hydrogenotrophic methanogenesis was affected to a lesser extent.

The effect of the initial PCNB concentration on the rate and extent of its biotransformation is shown in Figure 6.3. The pseudo-first-order rate constants for the PCNB to PCA transformation in the cultures with an initial PCNB concentration of 0.09, 3, 10, and 40 μM were (mean ± stand. error) 39.6 ± 0.1, 14.1 ± 0.1, 5.6 ± 0.4, and 0.8 ± 0.2 d^{-1}, respectively. Thus, increased initial PCNB concentrations resulted in decreased pseudo-first-order rates of PCNB transformation to PCA. PCA was depleted in 4 days in the culture with an initial PCNB concentration of 3 μM, whereas it took 6 and 15 days for the complete depletion of PCA at PCNB initial concentrations of 10 and 40 μM, respectively. At the highest initial PCNB concentration tested (40 μM), PCA dechlorination was very slow for about 10 days (Figure 6.3D), indicating that PCA dechlorination may have been inhibited by the relatively high initial PCNB and/or PCA concentrations. However, after the complete transformation of PCNB to PCA, the dechlorination pathway was similar in all culture series. At the end of the 21-day incubation period, the predominant products were dichloroanilines with traces of monochloroanilines. According to HPLC analysis conducted after a 90-day incubation period of the 40 μM PCNB-amended culture, the mole distribution of the accumulated compounds in this culture was as follows: 2,5-DCA, 65%; 2,4-DCA, 9%; 4-CA, 23%; and 3-CA, 3%. However, the sum of these products corresponded to 60% of the initially added PCNB. Because of the relatively high extraction efficiency (see Analytical

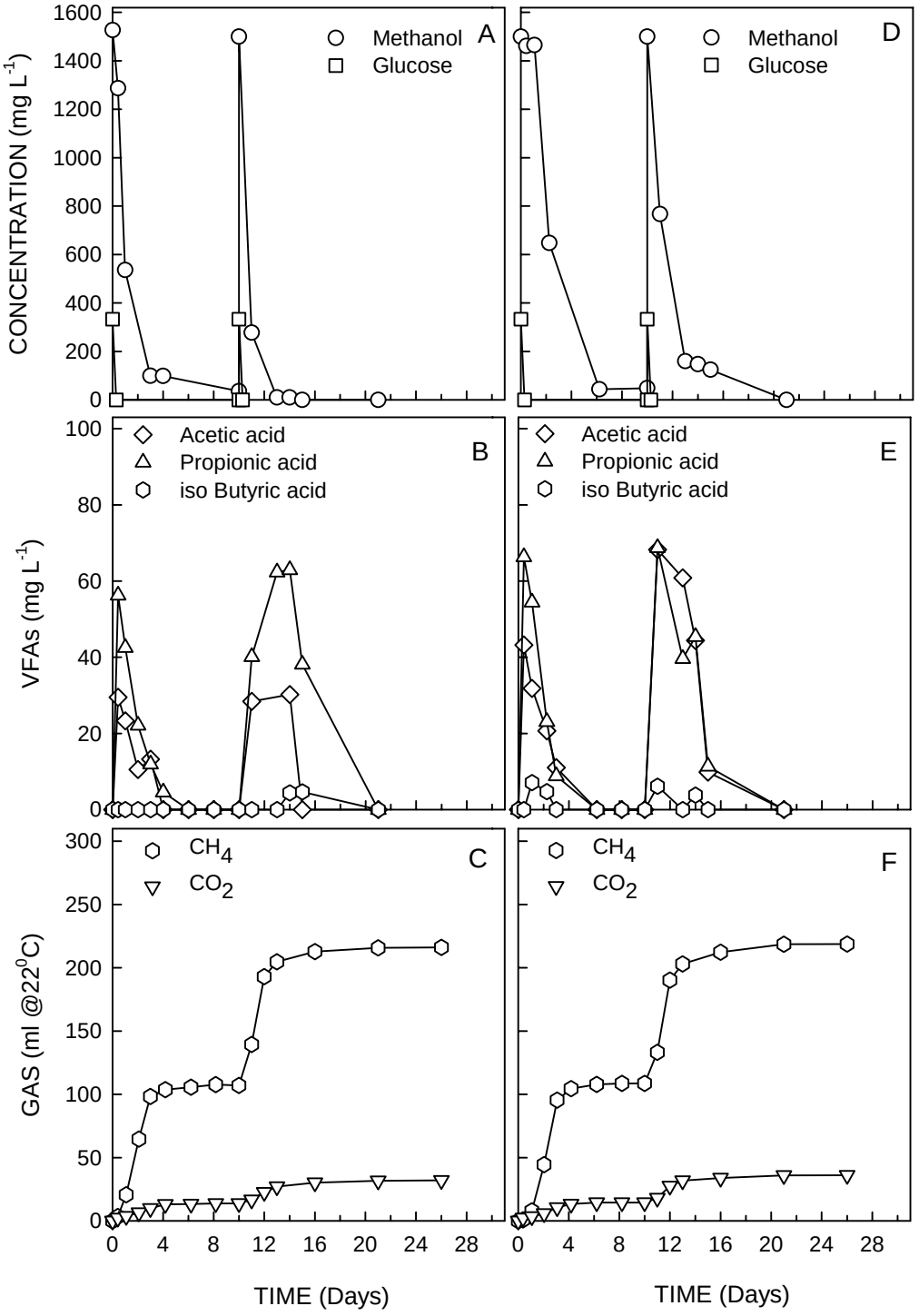


Figure 6.2. Comparison of methanol and glucose consumption, VFAs production and consumption, as well as cumulative methane and carbon dioxide production in the PCNB-free (control) culture (A, B, and C) and the PCNB-amended culture (40 µM PCNB; D, E, and F)(Both cultures were fed again with only glucose and methanol at 10 d of incubation).

Methods, Chapter 3), the low mass balance is attributed to possible losses and/or sorption of transformation products through and/or on the Teflon-lined septa, which were repeatedly perforated as a result of multiple samplings during the long incubation period.

According to electron equivalence calculations for the control and the four PCNB-amended cultures, the fraction of electron equivalents (eeq) of the added carbon source (glucose and methanol) accounted in the main products was as follows (mean ± stand. dev.; n = 5): biomass, 0.12 ± 0.003; methane, 0.88 ± 0.007. The fraction of the added electron donors (glucose and methanol) which was used for the observed reductive transformation of PCNB was only 1.9×10^{-6} to 8.6×10^{-4}. These results agree with previous observations where the fraction of electrons used by mixed cultures for the reductive dechlorination of haloorganic compounds was relatively very small as compared to that in halorespiring pure cultures (Prytula and Pavlostathis, 1996; Pavlostathis and Prytula, 2000; Löffler *et al*., 1999; Pavlostathis *et al*., 2003). It has been observed that as much as 70% of the total eeq was used by halorespiring pure cultures during the dechlorination of chlorinated solvents at much higher concentrations compared to the PCNB concentrations used in this study (Löffler *et al*., 1999).

6.3.3. Electron Donor Type and PCNB Biotransformation

Throughout the incubation period, the pH values in the cultures amended with methanol and methanol plus glucose were 7.1 ± 0.2 and 6.7 ± 0.1, respectively. Cumulative CH_4 and CO_2 production in these culture series is shown in Figure 6.4. Based on the theoretical COD to methane conversion (1 g COD converted to 350 mL CH_4 at

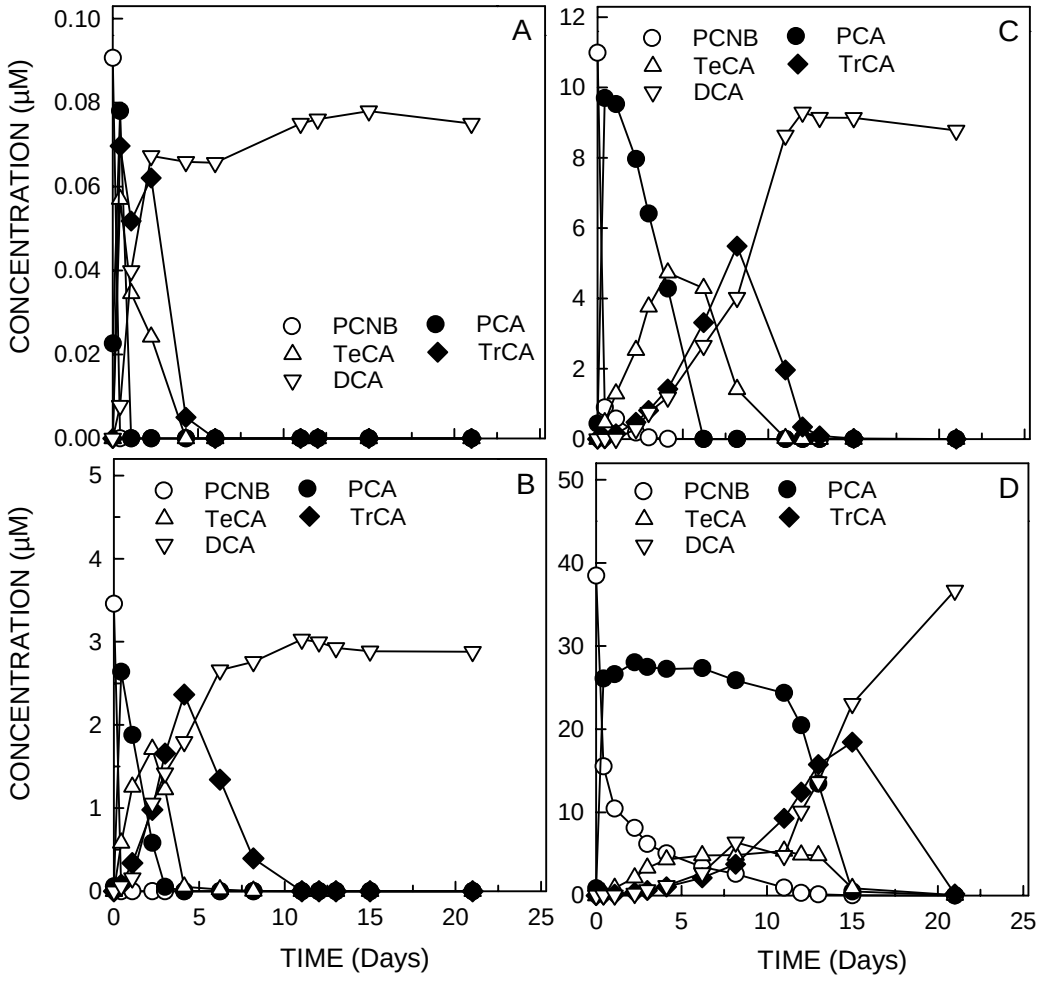


Figure 6.3. Effect of initial PCNB concentration on its biotransformation rate and product distribution (Initial PCNB concentration, µM: A, 0.09; B, 3; C, 10; and D, 40).

STP) for the cultures fed with only methanol (285 mg COD) and methanol plus glucose (328 mg COD), the expected cumulative CH_4 production in each culture was 108 mL and 124 mL, respectively. Thus, the cumulative methane production in both cultures matched to expected methane production Figure 6.4. Methanol was consumed within 4 days of incubation in the methanol-fed and methanol plus glucose-fed cultures. Acetate and propionate production was observed in the glucose-fed culture. Methanol and all the VFAs were consumed in 4 days. VFAs were not detected in the cultures amended with only methanol as the electron donor. Complete transformation of PCA to less chlorinated anilines took 7 days in the cultures amended with glucose plus methanol. However, PCA transformation in the methanol-amended cultures was much slower as compared with that in the glucose plus methanol-amended cultures (Figure 6.5). Although the dechlorination rates were slower in the cultures amended with only methanol, both cultures dechlorinated PCA down to dichlorinated anilines.

The biotransformation potential of PCNB with acetate and H_2 as the electron donor was investigated by adding PCNB in crystalline form. The results of this assay are shown in Figure 6.6. Because of the slow dissolution of PCNB, the transformation rates in the cultures fed with acetate and H_2 were significantly slower compared to the culture fed with glucose plus methanol, where PCNB was added dissolved in methanol.

In the cultures where PCNB was added in crystalline form, the measured concentration of PCA was very low, indicating that the sequential PCA dechlorination was faster than the PCNB dissolution rate. In order to have excess electron donor in these cultures, acetate and H_2 were added three times during the incubation period. Although the initial PCNB concentration was approximately the same in all culture series, higher

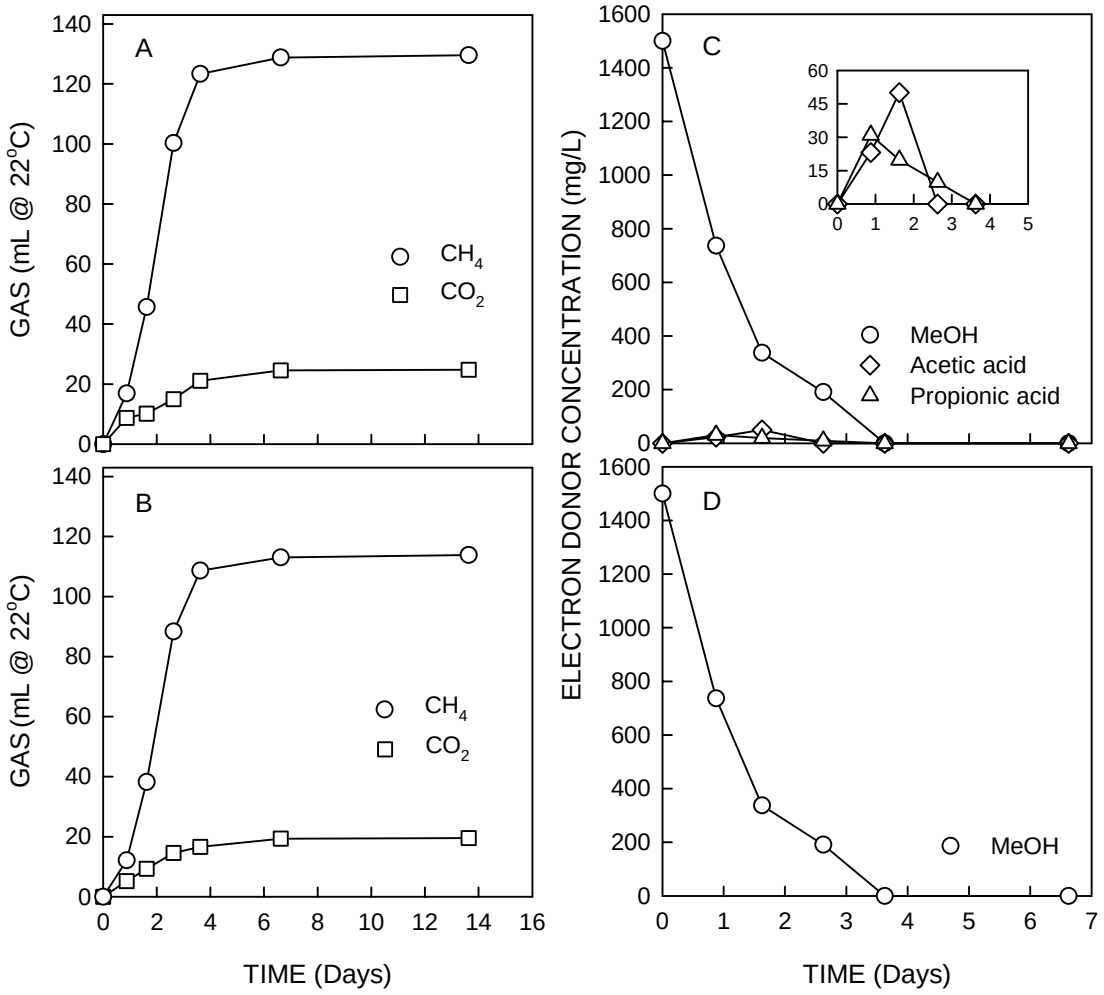


Figure 6.4. Comparison of methane and carbon dioxide production in the cultures fed with glucose plus methanol (A) and only methanol (B); as well as VFAs production and methanol consumption in the cultures fed with glucose plus methanol (C) and only methanol (D).

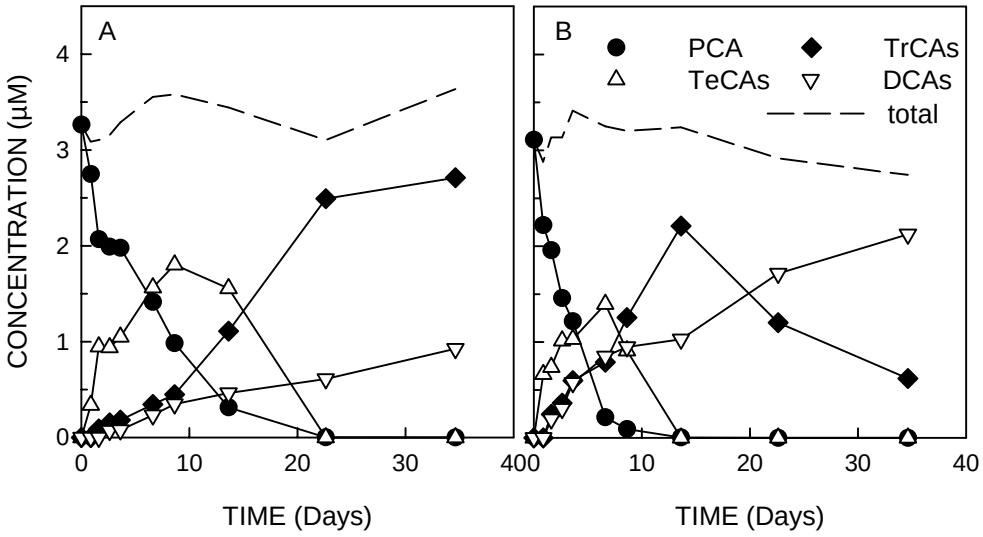


Figure 6.5. Effect of electron donor on the PCNB biotransformation rate and product distribution (A, methanol-fed; B, glucose plus methanol-fed).

dechlorination rates were observed in the culture amended with H_2 as compared to the acetate-amended culture. In the acetate-fed culture, complete dechlorination of TrCAs was observed in 25 days whereas, in the H_2-fed culture, complete dechlorination of TrCAs required only 15 days. In both cultures the predominant end products were DCAs. The dechlorination pattern in these cultures matched the dechlorination pattern of the control culture which was fed with glucose and PCNB dissolved in methanol (Figure 6.6).

It was concluded that there was not any significant difference in the PCNB biotransformation products among the cultures amended with methanol, methanol plus glucose, acetate, or H_2. However, a significant difference was observed in these culture

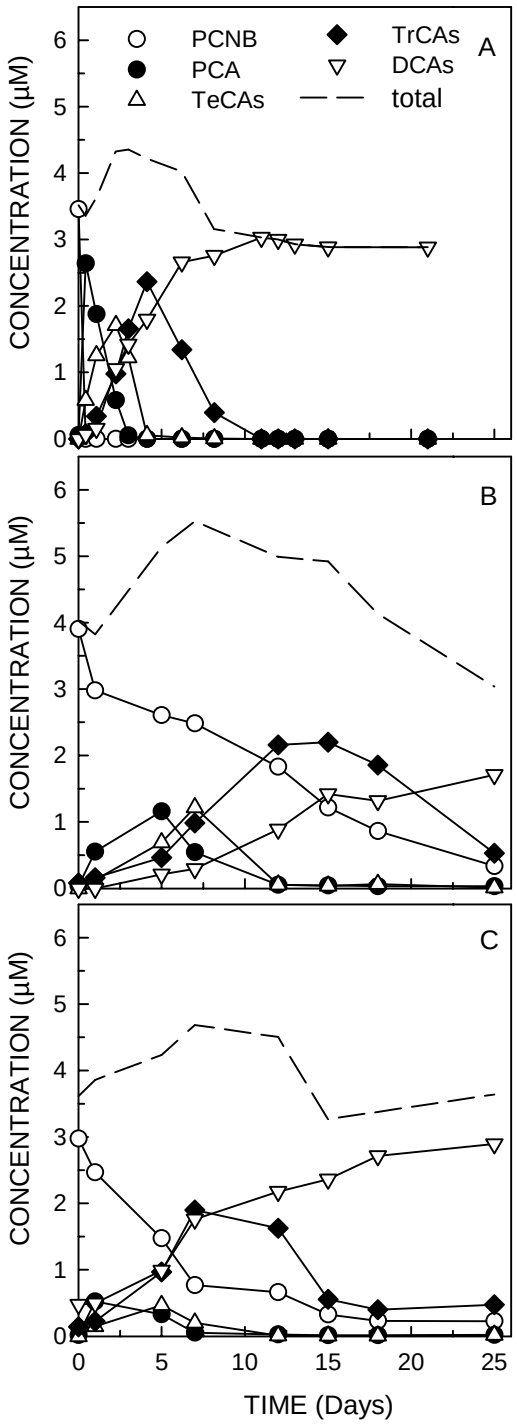


Figure 6.6. Effect of electron donor type on the PCNB biotransformation rate and product distribution (A, glucose plus methanol-fed; B, acetate-fed; C, H_2-fed).

series in terms of PCNB biotransformation as well as PCA dechlorination rates. Therefore, the availability of PCNB is an important parameter that affects its biotransformation rate and extent.

It is possible that an endogenous source of electrons, such as microbial death and decay, provided sufficient reducing equivalents to sustain the sequential dechlorination of PCA beyond the incubation time corresponding to the depletion of the exogenous electron donor (i.e., glucose, acetate, methanol, or H_2). Wrenn and Rittmann (1995) reported that reductive dehalogenation occurred in the absence of exogenous electron donors. Decaying biomass was found to be an excellent donor for dehalogenation because of the slow release of H_2 resulting from biomass decay (Yang and McCarty, 2000). Lee *et al*. (2004) pointed out the significant role of decay of a glucose-grown culture in the reductive dehalogenation of PCE. According to simulations that they performed they reported a 5 and 14% decrease in the rate of PCE and cDCE dechlorination to ethane when the biomass decay was not taken into account, respectively.

6.3.4. Effect of Inhibitors on the PCNB Biotransformation

Specific inhibitors are often used in dechlorination studies in order to identify and isolate the microorganisms responsible for the dechlorination reactions. In this assay the effect of BES, an inhibitor of methanogenesis, at three concentrations (25, 50 and 75 mM) and sodium azide (200 mg/L), a general bacteriostatic agent, on the mixed, dechlorinating culture was investigated. Cumulative methane production profiles in the

control, the azide-amended, and the BES-amended cultures are shown in Figure 6.7. Methane was not observed in any BES-amended culture as a result of the complete inhibition of methanogenesis through acetate and methanol in the presence of BES, even at the lower concentration of 25 mM BES. Similarly, methane production was not observed in the azide-amended culture.

A detailed comparison between the azide-amended and the 25 mM BES-amended cultures is presented in Figure 6.8. Glucose was consumed in all cultures within 7 h of incubation (Figure 6.8B and 6.8D). However, methanol consumption in the BES-amended cultures was much slower as compared to that in the BES-free, control culture. Methanol consumption was not observed in the sodium azide-amended culture (Figure 6.8B). In the three BES-amended cultures, acetate accumulation exceeded that of

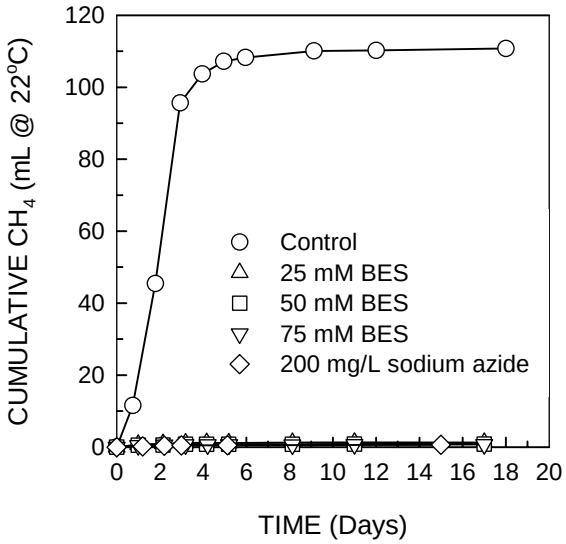


Figure 6.7. Cumulative methane production in the control culture (without any inhibitor amendment), in the cultures amended with 25, 50, and 75 mM BES or 200 mg/L sodium azide.

propionate and reached about 900 mg/L in the 25 mM BES-amended culture compared to 600 mg/L in the 75 mM BES-amended culture. It is possible that non-methanogenic bacteria, which either produce or consume acetate, were indirectly affected by BES. Similarly, in the azide-amended culture the amount of acetic and propionic acids produced matched approximately the amount of glucose consumed (in terms of eeq).

In terms of PCNB transformation (both rate and products), no significant difference was observed in the three BES-amended cultures as compared to the control culture. The sequential dechlorination profiles for the control culture, and the three BES-amended cultures were very similar to that shown in Figure 6.8C. These results indicate that BES did not affect the dechlorination process. Transformation of PCNB to PCA took place in the sodium azide-amended culture, but PCA dechlorination was not observed (Figure 6.8A). The PCNB to PCA transformation rate in the sodium azide-amended culture was much higher than that observed in the autoclaved culture media control, but similar to that observed with the autoclaved culture controls, indicating again that this transformation is biotically-mediated and enhanced by reductants and/or other factors produced by the enrichment culture before autoclaving or addition of azide.

Although BES is considered to be a specific inhibitor of methanogenesis (it is a structural analog of CoM), it has been reported that nonmethanogens, which reductively dechlorinate polychlorinated biphenyls (Ye *et al*., 1999) and chlorinated ethenes (Löffler *et al*., 1997) were also inhibited by BES. In contrast, Chiu and Lee (2001) reported that, although long-term exposure to BES altered the bacterial community structure of an anaerobic, trichloroethene (TCE)-dechlorinating culture, it did not inhibit the dechlorination of TCE or other chlorinated ethenes; however, BES exposure resulted in

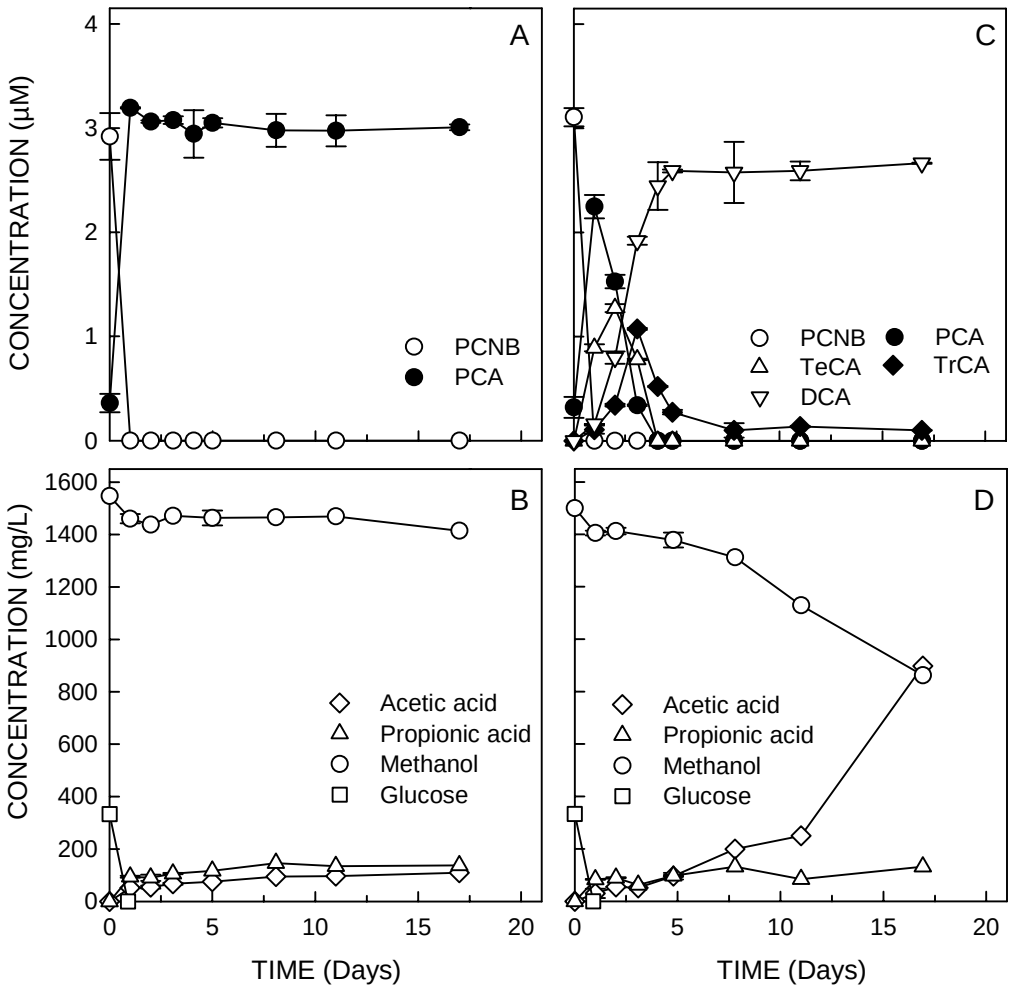


Figure 6.8. Effect of inhibitors on PCNB transformation, methanol and glucose consumption and VFAs production (A and B: 200 mg/L sodium azide-amended culture; C and D: 25 mM BES-amended culture)(Error bars represent mean values ± one standard deviation).

the formation of different intermediates and end products. Therefore, BES inhibition of both methanogenesis and dechlorination cannot be used as a proof of the methanogens' involvement in dechlorination reactions. In the present study, although methanogens were completely inhibited by BES, the rate of PCNB transformation to PCA and its dechlorination pathway were not affected. These results indicate that methanogens were not directly involved in the sequential dechlorination of PCA. There is a possibility, however, that halorespiring species may exist in the enrichment culture, which may be responsible for the observed reductive dechlorination reactions.

6.3.5. 16S rRNA Gene-Based Microbial Community Analysis

The DNA concentrations extracted by the Qiagen DNA kit from duplicate culture samples were 51.1 and 64.9 ng/mL with a 260/280 ratio of 1.51 and 1.48, respectively. The DNA concentrations extracted by the Mo Bio Ultraclean soil DNA from duplicate culture samples were 146 and 46.8 ng/mL with a 260/280 ratio of 1.35 and 3.85, espectively. In order to test the quality of the extracted DNA, the isolated DNA was used as template in PCR with 16S rRNA universal bacterial primers (Figure 6.9). Because of the high DNA recovery, DNA extracted with the Mo Bio Ultraclean soil DNA kit was used for characterization of the culture.

1:100 and 1:1000 dilutions of the 16S rRNA gene amplicons obtained with the universal primers was used as templates in a second round of PCR (i.e., nested PCR) for primer pairs that specifically select the 16S rRNA genes of targeted dechlorinating bacterial groups. The results for the presence of known groups of dechlorinating bacteria (*Dehalococcoides, Dehalobacter, Desulfuromonas, Geobacter*, and *Anaeromyxobacter*)

(Lévesque *et al*., 1997; Löffler *et al*., 2000, 2005; Schlötelburg *et al*. 2002; He *et al*., 2003a, 2003b; Petrie *et al*., 2003) are shown in Figure 6.10. Based on 16S rRNA gene-based analysis, among the dechlorinating bacterial groups tested (*Dehalococcoides, Dehalobacter, Desulfuromonas, Geobacter*, and *Anaeromyxobacter*) only *Dehalococcoides* was detected in the mixed fermentative/methanogenic culture. However, false negatives results cannot be excluded, and other dechlorinating bacteria may be present. In any case, *Dehalococcoides* could be responsible for the observed reductive dechlorination of PCA in the mixed enrichment culture. Although *Dehalococcoides* was detected in the mixed, enrichment culture, it is not known at present which dechlorination reactions in the observed sequential dechlorination of PCA are mediated by *Dehalococcoides*. The possibility of the existence of other dechlorinating organisms in the mixed culture should not be discounted.

6.4. Summary

Batch PCA dechlorination assays conducted with different initial biomass concentrations resulted in PCA dechlorination rates which were directly proportional to the initial biomass concentration. Glucose fermentation, methanogenesis and dechlorination were not inhibited at an initial PCNB concentration up to 40 μM, which is 27 times higher than its aqueous solubility. Batch PCNB biotransformation assays conducted with different electron donors showed that methanol, acetate as well as H_2 can be used as an electron donor by the enrichment culture and support PCA dechlorination. Addition of 25 mM 2-bromoethanesulfonate (BES) to the PCNB-amended culture

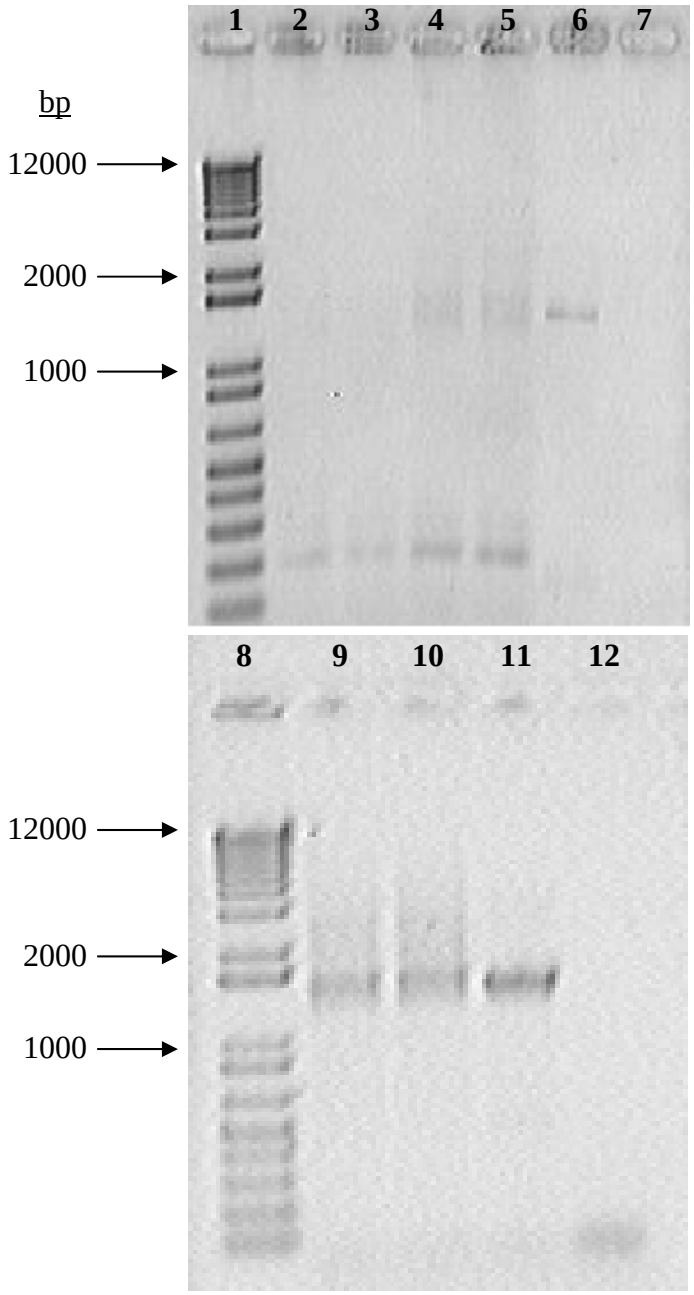


Figure 6.9. Universal bacterial 16S rRNA gene-targeted PCR primers. Lanes 1 and 8, DNA size marker 1Kb Plus (Invitrogen); lanes 2, 3 and 3,4, DNA extracted with Qiagen DNA kit, sample-A and sample-B, respectively; lanes 6 and 11 positive controls (BAV1); lanes 9 and 10, DNA extracted with the Mo Bio Ultraclean soil DNA kit, sample-A and sample-B, respectively; lanes 7 and 12, negative controls.

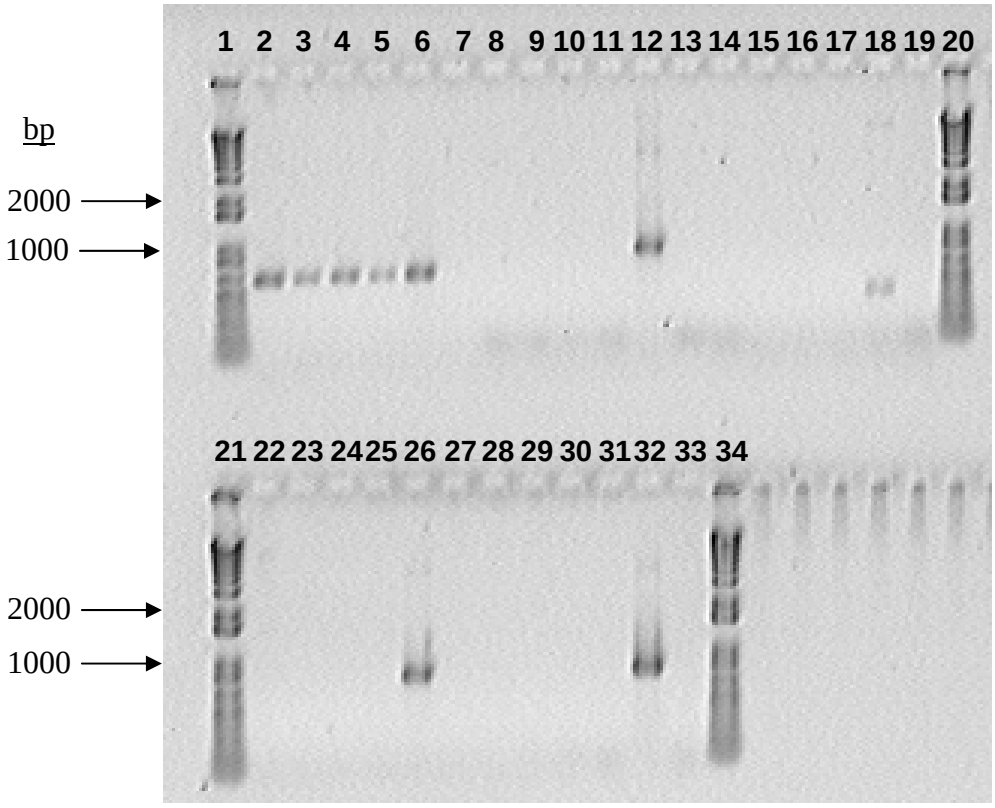


Figure 6.10. Detection of the presence of known groups of dechlorinating bacteria. Lanes 1, 20, 21, and 34, DNA size marker 1Kb Plus (Invitrogen); lanes 2,3 and 4,5, *Dehalococcoides* 16S rRNA gene-targeted PCR products of 1:100 and 1:1000 diluted sample-A and sample-B, respectively; lane 6 positive control (BAV1); lane 8,9 and 10,11, *Dehalobacter* 16S rRNA gene-targeted PCR products of 1:100 and 1:1000 diluted sample-A and sample-B, respectively; lane 12 positive control; lanes 14, 15 and 16, 17, *Anaeromyxobacter* 16S rRNA gene-targeted PCR products of 1:100 and 1:1000 diluted sample-A and sample-B, respectively; lane 18 positive control; lanes 22, 23 and 24, 25, *Geobacter* 16S rRNA gene-targeted PCR products of 1:100 and 1:1000 diluted sample-A and sample-B, respectively; lane 26 positive control; lanes 28, 29 and 30, 31, *Desulfuromonas* 16S rRNA gene-targeted PCR products of 1:100 and 1:1000 diluted sample-A and sample-B, respectively; lane 32 positive control; lanes 7, 19, 27, and 33 negative controls.

resulted in the complete inhibition of methanogenesis, but the biotransformation of PCNB to PCA and its sequential dechlorination pathway were not affected. Therefore, methanogens were not directly responsible for the observed PCA dechlorination in the enrichment culture. Addition of sodium azide (200 mg/L) to the PCNB-amended culture resulted in complete inhibition of methanogenesis; transformation of PCNB to PCA took place, but PCA dechlorination was not observed. *Dehalococcoides* could be responsible for the observed PCA dechlorination in the mixed enrichment culture.

CHAPTER 7

PATHWAY ANALYSIS AND KINETICS OF THE SEQUENTIAL DECHLORINATION OF PENTACHLOROANILINE

7.1. Introduction

In contrast to available information regarding the (bio)transformation kinetics of PCNB to PCA under anoxic conditions (Murthy and Kaufman, 1978; Klupinski *et al.*, 2004), little is known about the dechlorination kinetics of PCA and all CA congeners. The transformation of PCNB to PCA under abiotic conditions, as well as the sequential microbial reductive dechlorination pathway of PCA using the fermentative/methanogenic enrichment culture was reported in Chapters 5 and 6. Abiotic reduction of PCNB to PCA by Fe^{2+} associated with iron-containing minerals or sulfide-containing culture media was shown in Chapter 5, but dechlorination of PCA was not observed under these conditions (Chapter 5; Klupinski *et al.*, 2004). The focus of the work reported in this chapter was on the sequential reductive dechlorination kinetics of PCA and all available CAs under fermentative/methanogenic conditions.

Incubation temperature and pH are expected to have a significant effect on PCNB (bio)transformation. To our knowledge, this important area of research has not previously been explored. Nearly all laboratory studies of microbial PCNB transformation reported to date have been conducted at 22 to 30°C and at circumneutral pH (Tamura *et al.*,1995; Susarla *et al.*, 1996). However, depending on depth, location and climate, PCNB-

contaminated soil and sediment environments in a wide range of temperature (Wu *et al.*, 1996, 1997) and pH values (Boyd, 1995) are encountered.

The objective of the research reported here was to systematically assess the reductive dechlorination kinetics of PCA and all available CA congeners in the mixed, fermentative/methanogenic enrichment culture. Based on the independently estimated values of the kinetic parameters for individual CAs and the observed dechlorination pathway, an overall kinetic model was developed which successfully described the sequential reductive dechlorination of PCA. In addition, the effect of pH and temperature on the reductive biotransformation rate and extent of PCNB as well as the sequential dechlorination pattern of PCA in the mixed, fermentative/methanogenic culture was investigated. The kinetics of the sequential dechlorination of PCA under different incubation temperature and pH values were also simulated using a branched-chain Michaelis-Menten kinetic model.

7.2. Materials and Methods

7.2.1. Kinetic Assay

The sequential dechlorination kinetics of PCA and all available CAs by the enrichment culture were investigated. Each of the CAs -- from PCA to the three monochlorinated aniline isomers -- were tested individually as the primary compound in separate culture series at an initial concentration of about 3 μM. Because of detection limitations, monochlorinated anilines were added at an initial concentration of about 10 μM. All serum bottles had the same amount of carbon sources (glucose and methanol). The COD of the glucose and methanol added to each serum bottle was 86 and 272 mg,

respectively. The initial biomass concentration in the serum bottles was 268 ± 15 mg POC/L (mean ± standard deviation; $n = 3$). All assays were conducted in 160 mL serum bottles that were sealed with Teflon-lined septa and flushed with nitrogen gas. All serum bottles were incubated in a 22ºC constant temperature room and were manually shaken once a day. Liquid and gas samples were periodically taken to monitor chlorinated compounds, pH, volatile fatty acids (VFAs), and gas composition.

7.2.2. Kinetic Modeling

It is known that the rate of microbial dechlorination can be affected by different factors such as electron acceptor and donor concentration and their bioavailability, availability of nutrients, physicochemical factors (i.e., temperature, pH, salinity, pressure), the toxicity of chlorinated compounds and biomass concentration (Fennell and Gossett, 1998, 1999; Rhee *et al.*, 2001; Cupples *et al.*, 2004). Some factors may affect the rate of microbial uptake (e.g., biomass concentration), and other factors may alter the rate of contaminant transport to the microorganisms (e.g., bioavailability) (Bosma *et al.*, 1997). The PCA concentration tested in this study was not inhibitory to the mixed, enrichment culture (Chapter 6). In the case of sequential reductive dechlorination, the potential exists for competition among multiple chlorinated compounds as has been reported before (Cupples *et al.,* 2004; Yu *et al.,* 2005; Magar *et al.,* 1999). For the simulation of the sequential dechlorination of PCA in the present study, competitive effects among the CAs were ignored as justified in the Results and Discussion section. The concentration of PCA tested in this study was higher than its aqueous solubility. Therefore, in order to eliminate bioavailability limitations, PCA was introduced to the

culture dissolved in methanol. As shown in Chapter 6, methanol consumption was slow and lasted for at least 5 days of incubation. During this incubation time, the added PCA was completely transformed to less chlorinated anilines which are soluble at the tested concentration. All nutrients were added to the culture in excess to eliminate any limitations. Assays were conducted at a constant temperature (22°C), the culture pH was kept around 7 by adding sodium bicarbonate. Under these experimental conditions, the dechlorination rate of CAs can be described by a Michaelis-Menten equation:

$$-\frac{dC}{dt} = \frac{kXC}{K_C + C}\frac{D}{K_D + D} \tag{7-1}$$

where C is the concentration of CA (μM), k is the CA dechlorination rate per unit biomass (μmol/mg biomass·d), X is the biomass concentration (mg/L), K_C is the half-velocity coefficient for CA dechlorination (μM), D is the electron donor concentration (mM), and K_D is the half-velocity coefficient for electron donor utilization (mM). The concentration of the exogenous electron donor (i.e., glucose and methanol) was kept in excess to eliminate any limitation related to the availability of reducing equivalents during the batch dechlorination assays.

Based on recent findings that many microbial dechlorination reactions are through energy metabolism (i.e., halorespiration), a second equation which describes the net growth rate of the dechlorinating species using the CAs as electron acceptors (i.e., substrates) could be used in conjunction with equation 7-1 as previously described (Cupples *et al.*, 2004; Yu *et al.*, 2005):

$$\frac{dX}{dt} = Y\frac{dC}{dt} - k_d X \qquad (7\text{-}2)$$

where X is the biomass concentration (mg VSS/L), C is the concentration of CA (μM), Y is the biomass growth rate (= 0.008 mg VSS/μmol of Cl^- removed), k_d is the decay constant (= 0.024 d^{-1}). Y and k_d values were as those cited in Yu *et al.,* 2005 obtained from literature (Fennell and Gossett, 1998; Maymo-Gatell *et al.,* 1997). However, in the present study, an average and constant biomass concentration was assumed during the batch assays based on the following reasoning. During the batch assays, the total biomass increase was less than 20% by the end of the relatively short incubation period. In addition, a relatively high initial dechlorinating biomass concentration was expected because of the long-term culture enrichment with PCNB. Because the specific CA-dechlorinating biomass concentration could not be measured and modest growth of dechlorinators was expected during the relatively short incubation period, the following simplified equation was used to model the dechlorination rate of CAs:

$$-\frac{dC}{dt} = \frac{k'C}{K_C + C} \qquad (7\text{-}3)$$

where $k' = kX$ (μM/d) ≈ constant, is the CA dechlorination rate for a constant biomass concentration and other imposed experimental conditions. Additional justification for assuming an average and constant biomass concentration is presented in the Results and Discussion section.

Integration of equation 7-3 yields

$$C = C_0 - K_C \ln(C / C_0) - k't \tag{7-4}$$

A nonlinear regression procedure based on the Marquardt-Levenberg algorithm using the SigmaPlot Version 8.02 software (Richmond, CA) was used to fit equation 7-4 to each CA time course data and estimates of *k'* and K_C were obtained. A half-lifetime equation was derived by evaluating equation 7-4 for the condition of $C = 0.5\ C_0$ and was used to calculate half-lifetime of CAs:

$$t_h = \frac{0.5C_0 - K_C \ln\ (0.5)}{k'} \tag{7-5}$$

A sequential chloroaniline dechlorination model was developed based on equation 7-3 and by accounting for the degree of branching and product distribution in each dechlorination step. The resulting system of the simultaneous ordinary differential equations was solved numerically using MATLAB Version 7, ode23 suite which uses a Runge-Kutta solver (MATLAB, 2004).

7.2.3. Temperature Assay

The effect of temperature on PCNB (bio)transformation and PCA dechlorination was assessed using culture series amended with PCNB and incubated at 4, 15, 22, 35, and 45°C. Assays were conducted in 160-mL serum bottles which were sealed with Teflon-lined septa and flushed with nitrogen gas. Before the addition of electron donor and PCNB, all cultures were incubated at their respective temperature for one day. All

cultures were then amended with the same initial amount of glucose (330 mg/L), yeast extract (17 mg/L), and PCNB (3 μM) dissolved in methanol (234 μL resulting in 1535 mg/L). In order to avoid any electron donor limitation and to achieve complete biotransformation, the cultures were fed again with only glucose and methanol at the following incubation times: 6 days for the 22°C cultures; 16 days for the 15°C cultures; 6 and 16 days for the 35°C cultures. PCNB was not added during all subsequent electron donor additions. The initial pH of the cultures varied from 6.8 to 7 and the initial biomass concentration was 320 ± 10 mg POC/L (mean ± stand. dev.; $n = 3$). All cultures were incubated in the dark and manually shaken once a day. Liquid and gas samples were periodically taken to monitor chlorinated compounds, pH, volatile fatty acids (VFAs), and gas composition.

7.2.4. pH Assay

The effect of pH on PCNB (bio)transformation was assessed using a culture series with the following pH values (mean ± stand. dev.; $n = 3$): 2.7 ± 0.1, 4.1 ± 0.1, 6.2 ± 0.1, 6.6 ± 0.1, 7.2 ± 0.1, and 7.6 ± 0.1. The initial pH was adjusted using 1 N HCl or 1 N NaOH filter sterilized stock solutions. pH buffers were not used in these culture series as the goal of this assay was also to assess pH changes during incubation and their effect on PCNB biotransformation. All cultures were amended with the same initial amount of glucose (330 mg/L), yeast extract (17 mg/L), and PCNB (3 μM) dissolved in methanol (234 μL resulting in 1535 mg/L). The initial biomass concentration in these cultures was 285 ± 15 mg POC/L (mean ± stand. dev.; $n = 3$). All cultures were incubated in a 22°C constant temperature room in the dark and were manually shaken once a day. Liquid and

gas samples were periodically taken to monitor chlorinated compounds, pH, volatile fatty acids (VFAs), and gas composition.

7.3. Results and Discussion

7.3.1. Dechlorination of Individual Chloroanilines

The dechlorination pathways and kinetics of all available CAs from PCA to the three monochlorinated aniline isomers (seventeen compounds in total) were evaluated with the sediment-free enrichment culture. Two CAs, 2,3,4,6-TeCA, and 2,3,6-TrCA were not tested because they were not available. However, based on chromatographic evidence as well as mass balance calculations, these CAs were not observed during the sequential dechlorination of PCA. Dechlorination of PCA, two tetrachloroanilines, and five trichloroanilines was observed. However, none of the six dichloroanilines and three monochloranilines was dechlorinated by the enrichment culture during the relatively short batch assays. A very low level of 3,5-DCA dechlorination was observed resulting in the production of 3-CA production, both in the short-term batch assays and in the enrichment culture. For each CA tested, the dechlorination reactions mediated by the enrichment culture as well as the observed product distribution are summarized in Table 7.1. The dechlorination product distribution was calculated based on the mole fractions among all dechlorination products measured during the dechlorination of individual CAs.

The sequential dechlorination profiles of PCA and its dechlorination products (i.e., 2,3,4,5-TeCA, 2,3,5,6-TeCA, 2,3,5-TrCA, 2,4,5-TrCA, and 3,4,5-TrCA) obtained in separate enrichment culture series for each chlorinated aniline congener are shown in Figure 7.1. During the dechlorination of PCA, 2,3,5-TrCA was the predominant

Table 7.1. Dechlorination reactions carried out by the mixed, enrichment culture and product distribution

Parent compound	Possible products[a]	Position of Cl removed	Observed product distribution (%)[b]
PCA	2,3,4,5-TeCA	*ortho*	44
	2,3,5,6-TeCA	***para***	**56**
	2,3,4,6-TeCA	*meta*	0
2,3,4,5-TeCA	2,3,4-TrCA	*meta*	0
	2,3,5-TrCA	***para***	**50**
	2,4,5-TrCA	*meta*	34
	3,4,5-TrCA	*ortho*	16
2,3,5,6-TeCA	**2,3,5-TrCA**	***ortho***	**100**
	2,3,6-TrCA	*meta*	0
2,3,4-TrCA	2,3-DCA	*para*	5
	2,4-DCA	***meta***	**95**
	3,4-DCA	*ortho*	0
2,3,5-TrCA	2,3-DCA	*meta*	0
	2,5-DCA	***meta***	**100**
	3,5-DCA	*ortho*	0
2,4,5-TrCA	2,4-DCA	*meta*	0
	2,5-DCA	***para***	**100**
	3,4-DCA	*ortho*	0
2,4,6-TrCA	**2,4-DCA**	***ortho***	**89**
	2,6-DCA	*para*	11
3,4,5-TrCA	3,4-DCA	*meta*	0[c]
	3,5-DCA	***para***	**100**

[a] After one dechlorination step (predominant reaction and product shown in boldface type). [b] Relative fractions among observed dechlorination products (molar basis). [c] Traces of 3,4-DCA were detected.

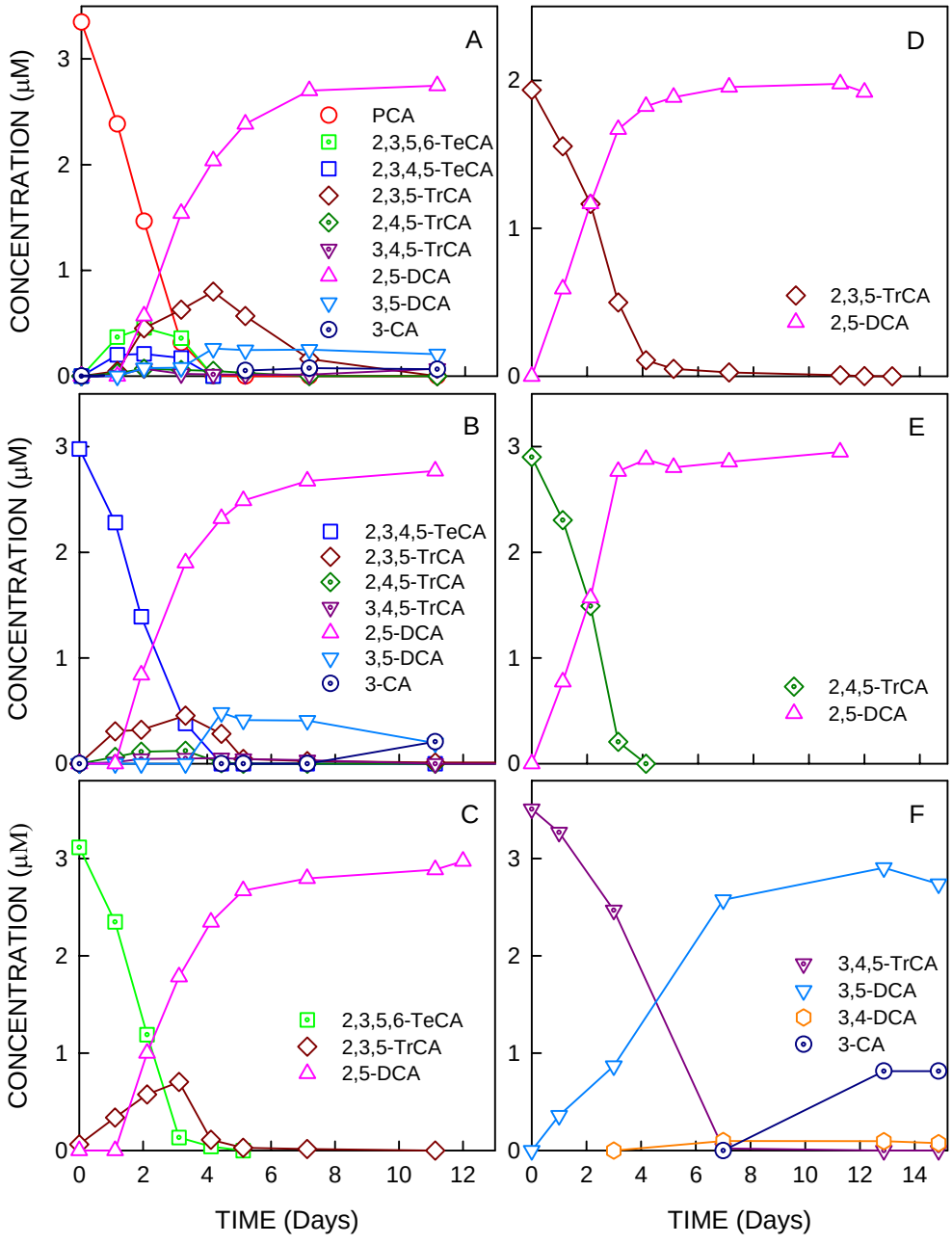


Figure 7.1. Time course of PCA (A), 2,3,4,5-TeCA (B), 2,3,5,6-TeCA (C), 2,3,5-TrCA (D), 2,4,5-TrCA (E), and 3,4,5-TrCA (F) and their dechlorination products during the batch dechlorination assays conducted with each chlorinated aniline tested independently using the PCNB-enriched culture.

intermediate while 2,5-DCA was the predominant end product. Two trichloroanilines not observed as intermediates during the biotransformation of PCA in batch assays (i.e., 2,3,4-TrCA and 2,4,6-TrCA) were transformed (Figure 7.2). For all di- and mono-chlorinated aniline isomers tested, dechlorination of the parent compound was not observed during the 30 days incubation period (Figure 7.3 and 7.4) with the exception of 3,5-DCA, where very low degree of dechlorination resulted in the formation of 3-CA. On the basis of these results it was again confirmed that the predominant PCA dechlorination pathway at 22^{o}C was as follows: PCA → 2,3,4,5- and 2,3,5,6-TeCA → 2,4,5- and 2,3,5-TrCA→ 2,5-DCA → 3-CA (low levels).

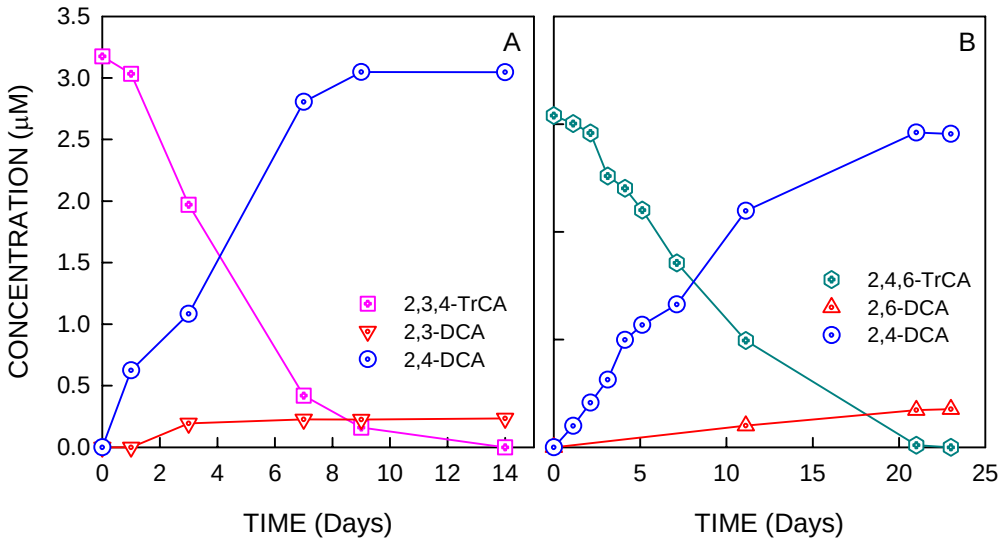


Figure 7.2. Time course of 2,3,4-TrCA (A), 2,4,6-TrCA (B) and their dechlorination products during the batch dechlorination assays conducted with each chlorinated aniline tested independently using the PCNB-enriched culture.

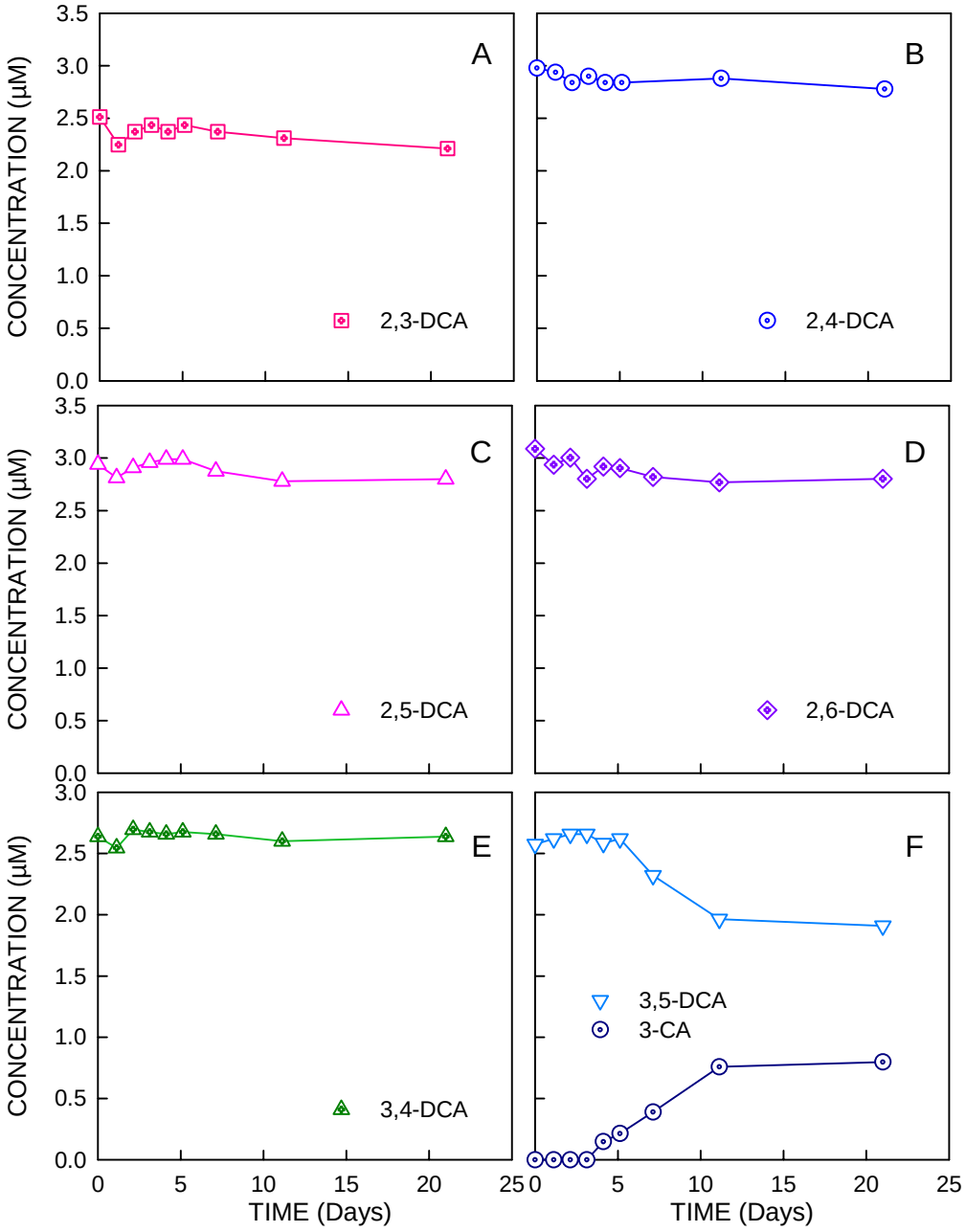


Figure 7.3. Time course of 2,3-DCA (A), 2,4-DCA (B), 2,5-DCA (C), 2,6-DCA (D), 3,4-DCA (E), and 3,5-DCA (F) and their dechlorination products during the batch dechlorination assays conducted with each chlorinated aniline tested independently using the mixed, fermentative/methanogenic enrichment culture.

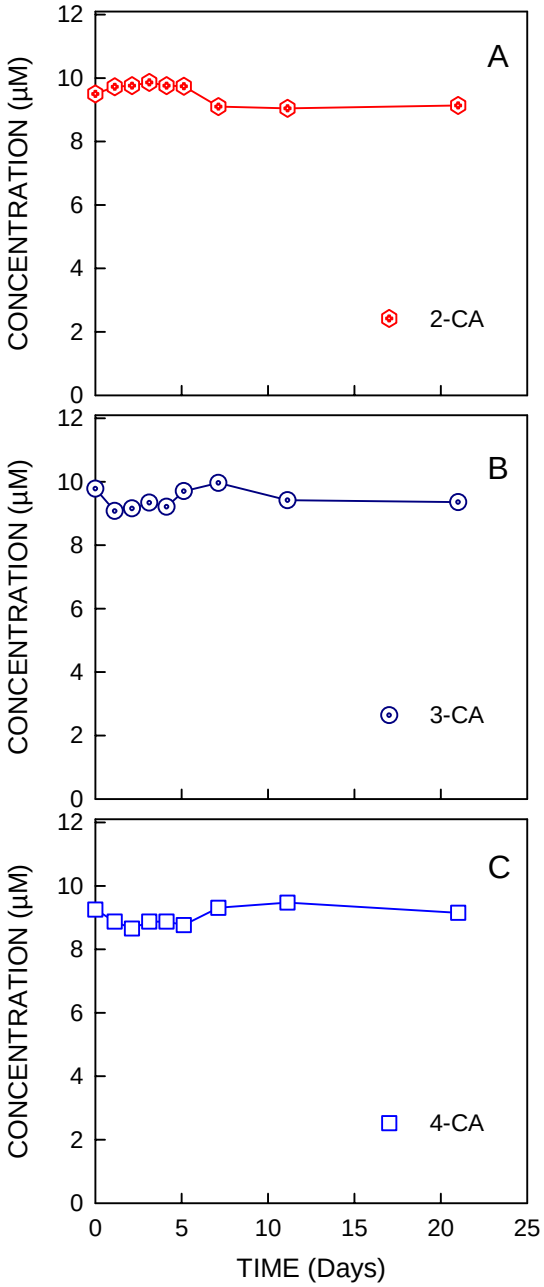


Figure 7.4. Time course of 2-CA (A), 3-CA (B), and 4-CA (C) during the batch dechlorination assays conducted with each chlorinated aniline tested independently using the mixed, fermentative/methanogenic enrichment culture.

Susarla *et al.* (1996) observed the following PCA dechlorination pathway in assays conducted with a contaminated sediment under sulfidogenic conditions: PCA → 2,3,4,5-TeCA → 3,4,5-TrCA → 3,5-DCA → 3-CA with traces of 2-CA and 4-CA. These reactions represent dechlorination at the *ortho*, *ortho*, *para*, and *meta* positions, respectively. In a subsequent study, they observed the reductive dechlorination of 2,3,4,5-TeCA at the *para* and *ortho* positions to form 2,3,5-TrCA and 3,5-DCA, and then 3-CA was formed by *meta* dechlorination (Susarla *et al.,* 1997). Susarla et al. (1996) observed the following 2,3,4-TrCA biotransformation pathway in assays conducted with a contaminated sediment under sulfidogenic conditions: 2,3,4-TrCA → 3,4-DCA → 3-CA. These reactions represent dechlorination at the *ortho* and *para* positions, respectively. In the current study, under fermentative/methanogenic conditions, the following dechlorination pathway, predominantly occurring via *meta* dechlorination, was observed: 2,3,4-TrCA → 2,4-DCA and 2,3-DCA (low levels). Similarly, for the other trichlorinated anilines tested, dechlorination at the *meta* and *para* positions led to the removal of a Cl substituent form adjacently positioned Cl atoms, except for 2,4,6-TrCA which was transformed predominantly via *ortho* dechlorination (Table 7.1).

Kuhn and Suflita (1989) reported the sequential dechlorination of 2,3,4,5-TeCA at the *para* and *ortho* positions to form 2,3,5-TrCA and then 3,5-DCA, but further dechlorination of 3,5-DCA was not observed under methanogenic conditions in their study. Dechlorination of 3,4-DCA at the *para* position resulted in the formation of 3-CA, and similar to our results, none of the three monochloroaniline isomers was dechlorinated in these assays under methanogenic conditions. Similarly, Struijs and Rogers (1989) observed the reductive dechlorination of dichloroanilines to monochloroanilines in

anoxic pond sediment slurries, but the resulting monochloroanilines were not transformed under the conditions of their study. Enzymatic specificity is an important factor involved in the microbial reductive dechlorination of chlorinated compounds. The transformation pathway is a function of the microbial community composition, acclimation, and enrichment conditions. Culture enrichment resulting in the development of specific dechlorinating species which have different enzymes and/or enzymatic activities may result in different dechlorination pathways.

Methane and carbon dioxide production was monitored in each culture series during the batch dechlorination assays. In all culture series amended with the seventeen CA congeners, methane and carbon dioxide production was very similar to the control culture which was not amended with CAs. Thus, all CA congeners tested at approximately 3 μM did not have any inhibitory effect on the activity of the mixed culture. As an example, the time course data of the cumulative methane produced in the PCA-amended culture series along with the cumulative dechlorination are shown in Figure 7.5. Both methane production and dechlorination are expressed in terms of milli electron equivalents (meeq). These calculations were based on 8 meeq/mmol of methane produced and 2 meeq/mmol of chlorine substituent removed. As mentioned previously, all batch assays were conducted with initial electron donor concentrations higher than the normal enrichment culture feeding level to eliminate any electron donor limitations. According to electron equivalence calculations, the fraction of electron equivalents diverted to dechlorination reactions relative to methanogenesis was very low and ranged from 0.008 to 0.014%. These results are consistent with previous observations where the fraction of electrons used by mixed cultures for the reductive dechlorination of

haloorganic compounds was relatively very small as compared to that in halorespiring pure cultures (Pavlostathis and Prytula, 2000; Prytula and Pavlostathis, 1996; Löffler *et al.*, 1999; Pavlostathis *et al.*, 2003). Although *Dehalococcoides* was detected in the mixed, enrichment culture (Chapter 6), it is not known at present which dechlorination reactions in the observed sequential dechlorination of PCA are mediated by *Dehalococcoides*. The possibility of the existence of other dechlorinating organisms in the mixed culture should not be discounted.

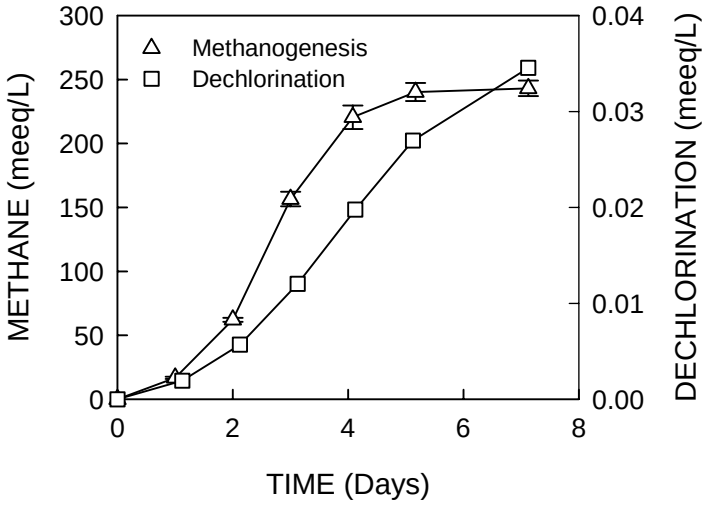


Figure 7.5. Cumulative methane produced and dechlorination equivalents during the batch PCA dechlorination assay. Error bars represent mean values ± 1 SD (meeq = milli-electron equivalents; see text).

7.3.2. Modeling of Individual Chloroanilines Dechlorination

Nonlinear regressions were performed based on equation 7-4 and time course data of all CA congeners which were tested in separate batch culture series. The resulting estimates of k' and K_C as well as t_h are summarized in Table 7.2. These results confirm that the sequential dechlorination rates of CAs decreased with decreasing degree of chlorination. The CA dechlorination rate per unit biomass measured in the present study ranged from 4.66×10^{-4} to 2.1×10^{-3} μmol/mg VSS·d. The eight CA concentration profiles over the incubation period of each culture series and the fit of equation 7-4 to the experimental data are shown in Figures 7.6 and 7.7. 3,5-DCA was not included in the sequential dechlorination model because of the observed low and partial dechlorination as previously discussed. The Michaelis-Menten model described the observed dechlorination kinetics of all CAs tested very well. It has been previously reported that the sequential microbial reductive dechlorination of chlorobenzenes was best described by the Michaelis-Menten model rather than the pseudo-first-order model (Pavlostathis and Prytula, 2000).

As a result of batch experiments conducted with the enrichment culture, the fastest observed dechlorination rates were for 2,3,4,5-TeCA, PCA, and 2,3,5,6-TeCA (Table 7.2). For the tetrachloroanilines, the following dechlorination rate order was observed: 2,3,4,5-TeCA>2,3,5,6-TeCA, corresponding to *para* > *ortho* dechlorination. Among the five trichlorinated aniline isomers tested, the half-lifetimes increased according to the following order: 2,4,5-TrCA<3,4,5-TrCA<2,3,5-TrCA<2,3,4-TrCA<2,4,6-TrCA. The fastest dechlorination among all trichloroanilines was observed for 2,4,5-TrCA. 2,3,4-TrCA and 2,4,6-TrCA were not detected during the sequential

Table 7.2. Dechlorination rates (*k'*), half-velocity coefficients (K_C), and half-lifetimes (t_h), for penta-, tetra-, and tri-chlorinated anilines tested in separate batch culture series[a]

Parent compound	k' (μM/day)[b]	K_C (μM)[b]	r^2	t_h (d)[c]
PCA	1.12 ± 0.4	0.39 ± 0.3	0.975	2.0 (0.9-3.2)
2,3,4,5-TeCA	1.19 ± 0.1	0.30 ± 0.2	0.998	1.5 (1.2-1.6)
2,3,5,6-TeCA	1.06 ± 0.3	0.22 ± 0.1	0.987	1.7 (1.2-2.3)
2,3,4-TrCA	0.89 ± 0.2	1.72 ± 0.6	0.986	3.3 (2.1-4.5)
2,3,5-TrCA	0.50 ± 0.1	0.11 ± 0.1	0.989	3.3 (2.5-4.1)
2,4,5-TrCA	0.85 ± 0.3	0.13 ± 0.2	0.996	2.3 (1.3-3.3)
2,4,6-TrCA	0.25 ± 0.1	0.38 ± 0.1	0.997	8.5 (4.8-12.2)
3,4,5-TrCA	0.61 ± 0.1	0.23 ± 0.2	0.997	2.8 (2.1-3.5)

[a] Experimental conditions: initial CA concentration 3 μM, initial biomass concentration 536 ± 30 mg VSS/L, incubation temperature 22°C, and pH range 6.8 to 7.2. [b] Nonlinear regression best estimate ± standard error ($n \geq 7$). [c] Mean half-lifetimes for C_0 = 3 μM (see equation 4; value range in parenthesis).

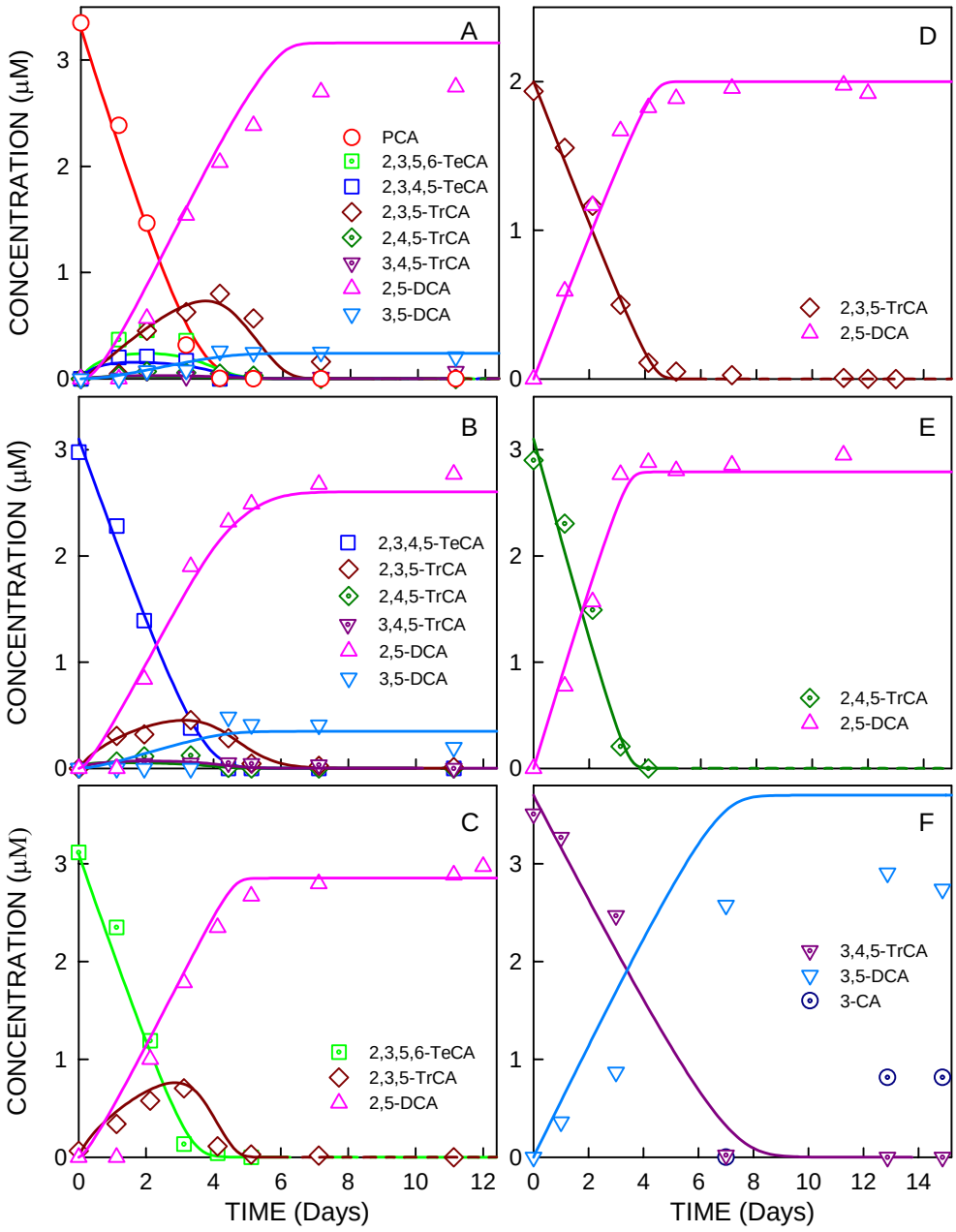


Figure 7.6. Time course of PCA (A), 2,3,4,5-TeCA (B), 2,3,5,6-TeCA (C), 2,3,5-TrCA (D), 2,4,5-TrCA (E), and 3,4,5-TrCA (F) and their dechlorination products during the batch dechlorination assays conducted with each chlorinated aniline tested independently using the PCNB-enriched culture (Lines are model predictions).

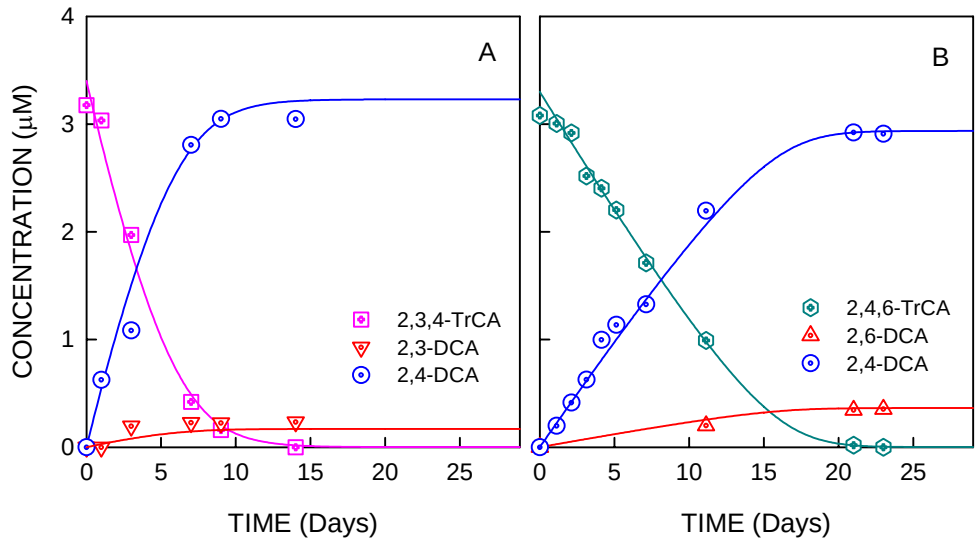


Figure 7.7. Time course of 2,3,4-TrCA (A), 2,4,6-TrCA (B) and their dechlorination products during the batch dechlorination assays conducted with each chlorinated aniline tested independently using the PCNB-enriched culture (Lines are model predictions).

dechlorination of PCA in batch assays, and these compounds had the highest dechlorination half-lifetimes among all chlorinated aniline congeners tested (Table 7.2). It took about 15 and 20 days for the complete depletion of 2,3,4-TrCA and 2,4,6-TrCA, respectively, whereas complete depletion of all other chlorinated aniline congeners that were observed during the sequential PCA dechlorination was observed in about 6 days. These results confirm that the sequential dechlorination rates of CAs decreased with decreasing degree of chlorination.

A good fit of the model to the experimental data was obtained based on a constant biomass concentration during the batch assay (i.e., based on equation 7-3). In an effort to explore the effect of the dechlorinators' growth during the incubation period, an alternative model was developed which included a variable biomass concentration term

(*X*) (equation 7-2). The simulations were performed for two CA isomers (PCA and 2,4,6-TrCA). The predicted rate constants at different initial biomass concentrations are summarized in Table 7.3. In the case of PCA, when the assumed initial dechlorinating biomass concentration was low (0.001 mg VSS/L) the model did not fit the experimental data (Figure 7.8A). With higher initial biomass concentrations (0.03 and 0.3 mg VSS/L), the model fit the experimental data (Figure 7.8B and 7.8C). In the case of 2,4,6-TrCA, when the assumed initial dechlorinating biomass concentration was very low (0.001 mg VSS/L) the model did not fit the experimental data (Figure 7.8D). With higher initial biomass concentration (0.3 mg VSS/L), the model fit the experimental data, but significant differences were not observed between the results obtained based on the model with and without the biomass term (Figure 7.8E). In addition, the predicted *k'* values were practically the same at different initial biomass concentrations. In the case of 2,4,6-TrCA, in order to fit the expanded model to the experimental data, a low biomass concentration (0.01 mg VSS/L) was required, because of either low initial biomass concentration in the enrichment culture to dechlorinate this specific CA, or more likely because of acclimation to 2,4,6-TrCA, because this CA was not detected during the sequential dechlorination of PCA in the batch assay. Based on these results and observations, and because of uncertainties relative to the exact concentration of dechlorinating species, use of the model with an assumed constant biomass concentration (i.e., equation 7-4) to simulate the dechlorination of all CAs tested was justified.

The electron-donating amino group leads to electronic and steric effects, which in turn affect the reductive dechlorination of CAs by making the ring more reactive to electrophilic attack, especially at the *para* and *ortho* position (Susarla *et al*., 1996; Kuhn

Table 7.3. Alternative model runs accounting for dechlorinators' growth[a]

Compound	Figure	X_o (mg VSS/L)	k (μM/mg VSS·d)	$\overline{X}$ (mg VSS/L)	$k' = k\overline{X}$ (μM/d)	Model Fit
PCA[b]	7.8A	0.001	200	0.015	3	Not good
		0.0162	48	0.0275	**1.32**	
	7.8B	0.0262	36	0.0325	**1.17**	**Good**
		0.1	12	0.11	**1.32**	
	7.8C	0.3	4	0.3	**1.20**	
2,4,6-TrCA[b]	7.8D	0.001	60	0.12	0.72	Not good
	7.8E	0.01	16.3	0.017	**0.28**	
		0.08	3	0.08	**0.24**	**Good**

[a] Based on $Y = 0.008$ mg VSS/μmol; $k_d = 0.024$ d^{-1} (from Yu *et al*., 2005, citing Fennell and Gossett, 1998 and Maymo-Gatell *et al*., 1997)(X_o is the initial biomass concentration; $\overline{X}$ is the average biomass concentration during the incubation time). [b] $K_c = 0.39$ μM for PCA and $K_c = 0.38$ μM for 2,4,6-TrCA.

and Suflita, 1989). During the sequential dechlorination of PCA, the observed relative dechlorination rates of CAs can be simplified based on the Cl substituents' location on the aniline ring. In the present study, an increase in the dechlorination rates was observed with increasing number of *para* and *ortho* Cl substituents. For the case of polychlorinated CAs such as PCA and TeCAs, *para* dechlorination was the predominant dechlorination pathway followed by *ortho* dechlorination. However, for the less chlorinated aniline congeners, such as TrCAs and DCAs, *para* and *meta* dechlorination, resulting in the removal of an adjacent Cl substituent, was favorable.

All batch assays reported here were conducted at an initial CA concentration of 3 μM (except monochloroanilines). The effect of the initial PCNB concentration on its biotransformation by the same mixed, enrichment culture was tested (Chapter 6). Based

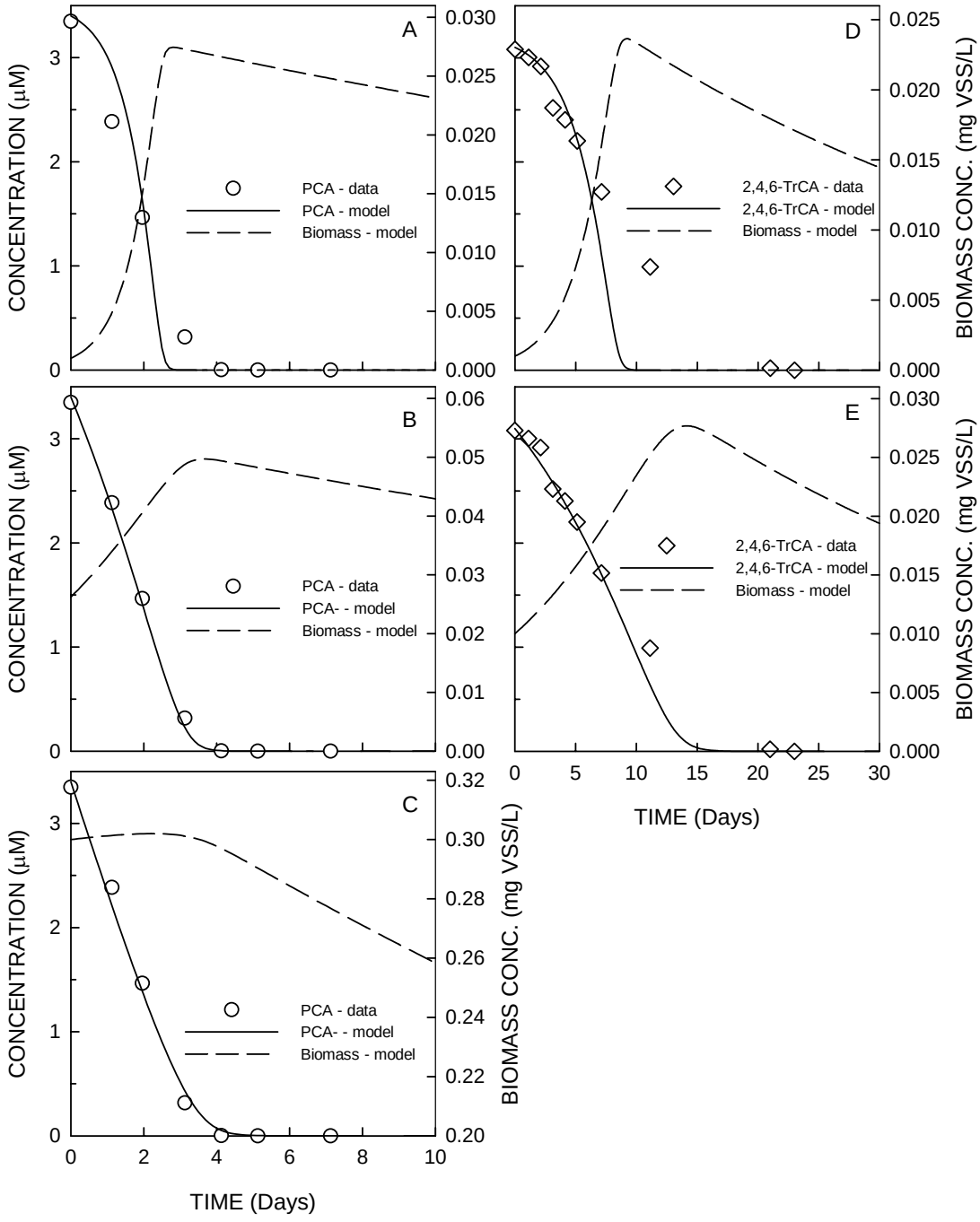


Figure 7.8. Alternative model fit to experimental data accounting for dechlorinators' growth for PCA (A, B, and C) and 2,4,6-TrCA (D and E)(Conditions as shown in Table 7.7).

on these data, the effect of PCA concentration on its initial dechlorination rate was assessed and the results are shown in Figure 7.9. Nonlinear regression based on equation 7-3 evaluated at initial conditions and the experimental data shown in Figure 7.9, resulted in the following values (mean ± standard error): k' = 1.62 ± 0.06 μM/d; K_c = 1.2 ± 0.2 μM; r^2 = 0.998. Data at an initial PCA concentration of 40 μM were not used because of the observed dechlorination inhibition at this concentration (Chapter 6). As expected, the dechlorination rate values (k') increased with increasing initial PCA concentration. Thus, the dechlorination rates reported in Table 7.2 are rates attained at relatively low initial CA concentrations.

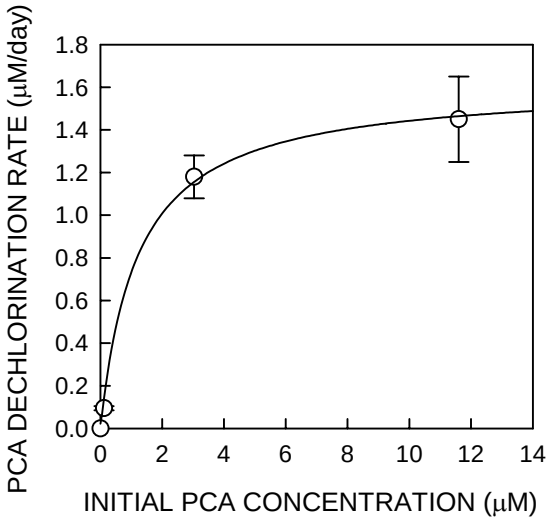


Figure 7.9. Initial PCA dechlorination rates achieved by the PCNB-enriched, mixed culture as a function of initial PCA concentration. Error bars represent mean values ± 1 SD (Line is model prediction).

Susarla *et al.* (1997) reported pseudo-first-order dechlorination rates for PCA, 2,3,4,5-TeCA, 3,4,5-TrCA, and 3,5-DCA in estuarine sediment microcosms under sulfidogenic conditions, as follows: 0.017, 0.012, 0.005, and 0.005 day^{-1}. These dechlorination rates correspond to half-lifetimes of 41, 58, 139, and 139 days, which are one to two orders of magnitude higher than the half-lifetimes measured in the present study (Table 7.2). It should be emphasized that the dechlorination rates depend on the concentration of the specific dechlorinating microorganisms, and in the case of mixed cultures, especially in non-enriched microcosms, these microorganisms may represent a very low fraction of the total biomass. Thus, the difference in dechlorination rates between the present study and those previously reported is attributed to culture enrichment. Caution should be exercised in using kinetic data derived from enrichment cultures for field applications.

7.3.3. Modeling the Sequential Dechlorination of Chloroanilines

Based on the Michaelis-Menten model and independently determined values of k' and K_C for each chloroaniline congener (Table 7.2), a sequential chloroaniline dechlorination model was developed by accounting for the degree of branching and product distribution in each dechlorination step. The sequential dechlorination of PCA was modeled via the following pathway: PCA → 0.44 (2,3,4,5-TeCA) + 0.56 (2,3,5,6-TeCA); 2,3,4,5-TeCA → 0.5 (2,3,5-TrCA) + 0.34 (2,4,5-TrCA) + 0.16 (3,4,5-TrCA); 2,3,5,6-TeCA → 2,3,5-TrCA; 2,3,5-TrCA → 2,5-DCA; 2,4,5-TrCA → 2,5-DCA; 3,4,5-TrCA → 3,5-DCA. The resulting system of the simultaneous ordinary differential equations was solved numerically using MATLAB (MATLAB, 2004). The model

predictions are plotted against the experimental data in Figures 7.6 and 7.7. In the numerical model, in order to simulate the sequential dechlorination of all CAs, the *k'* and K_C values that were calculated from the individual chlorinated aniline dechlorination profiles were used. The model simulations matched very well the experimental data.

Competitive inhibitory effects have been reported during the sequential dechlorination of chlorinated ethenes (Cupples *et al.,* 2004; Yu *et al.,* 2005) and chlorophenols (Magar *et al.,* 1999). However, in these previous studies, the chloroorganic concentrations tested were 2- to 10-fold higher than in the present study. In order to address the possibility of competitive inhibition, a model simulation of the sequential dechlorination of PCA was performed by including competitive inhibition terms as follows:

$$\frac{dC_{PCA}}{dt} = \frac{-k'_{PCA} C_{PCA}}{K_{C,PCA} + C_{PCA}} \qquad (7-5)$$

$$\frac{dC_{TeCA}}{dt} = \frac{-k'_{TeCA} C_{TeCA}}{K_{C,TeCA}(1 + \frac{C_{PCA}}{K_{I,PCA}}) + C_{TeCA}} + \frac{k'_{PCA} C_{PCA}}{K_{C,PCA} + C_{PCA}} \qquad (7-6)$$

$$\frac{dC_{TrCA}}{dt} = \frac{-k'_{TrCA} C_{TrCA}}{K_{C,TrCA}(1 + \frac{C_{PCA}}{K_{I,PCA}} + \frac{C_{TeCA}}{K_{I,TeCA}}) + C_{TrCA}} + \frac{k'_{TeCA} C_{TeCA}}{K_{C,TeCA}(1 + \frac{C_{PCA}}{K_{I,PCA}}) + C_{TeCA}} \qquad (7-7)$$

$$\frac{dC_{DCA}}{dt} = \frac{k'_{TrCA} C_{TrCA}}{K_{C,TrCA}(1 + \frac{C_{PCA}}{K_{I,PCA}} + \frac{C_{TeCA}}{K_{I,TeCA}}) + C_{TrCA}} \qquad (7-8)$$

Individually determined K_c values for CAs were used as the inhibition constants (K_I) for this simulation (Magar *et al.,* 1999; Yu *et al.,* 2005). The model simulation with the inhibition terms included did not fit the experimental data (Figure 7.10). Lower dechlorination rates resulted in significant accumulation of intermediate products. In contrast, the model run without competitive inhibition terms using the independently measured *k'* and K_C values simulated the sequential dechlorination of PCA and other CAs very well (Figure 7.6), showing that competitive inhibition caused by CAs was not in effect at initial CA concentrations of 3 μM.

Yu *et al.* (2005) reported that during the reductive dechlorination of chlorinated ethenes by two different mixed cultures, the dechlorination rates of low chlorinated ethenes decreased with increasing concentration of high chlorinated ethenes as a result of inhibition. They also reported that low K_C values correspond to the efficient reductive dechlorination of the highly chlorinated ethenes but caused strong inhibition of the transformation of the less chlorinated products. Cupples *et al.* (2004) showed a competitive inhibition between cis-dichloroethene (c-DCE) and vinyl chloride (VC). Such competitive inhibitory effects have also been observed during the sequential dehlorination of chlorophenols in a fluidized-bed reactor (Magar *et al.,* 1999). However, in all of these previous studies, the contaminant concentrations tested were 2- to 10-fold higher than in the present study. The reported chloroaniline dechlorination rates are based on total biomass concentration. A limitation of the current study is that the actual concentration of dechlorinating species in the mixed culture is not known. The results of this study indicate that stable communities of the dechlorinating culture were maintained during all batch assays.

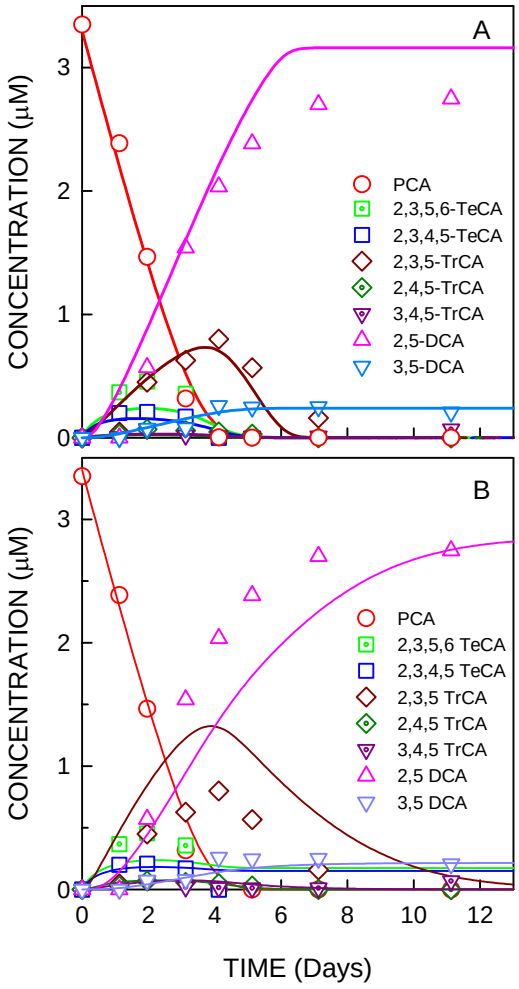


Figure 7.10. Model fit to sequential PCA dechlorination data without (A) and with (B) competitive inhibition terms.

7.3.4. Temperature Effect on PCNB Biotransformation

The effect of temperature on the PCNB (bio)transformation as well as PCA sequential dechlorination was investigated with the mixed enrichment culture at an initial PCNB concentration of 3 μM and a temperature range from 4 to 45°C. Transformation of PCNB to PCA occurred in all cultures incubated in a temperature range of 4 to 45°C. Pseudo-first-order rate constant values for the PCNB to PCA (bio)transformation reported in Table 7.4 show that the transformation rate increased with increasing temperature. Complete transformation was achieved in all cultures in less than 6 hours. It is noteworthy that the PCNB to PCA transformation is a relatively fast process and occurs under both abiotic and biotic reductive conditions (Chapter 5; Klupinski *et al.*, 2004). The rate of abiotic transformation depends on the reducing agent (sulfide) concentration, the presence of certain media components (e.g., Fe^{2+}) as well as biotically generated reductants and/or other factors (Chapter 5). PCNB was abiotically transformed to PCA in autoclaved culture media, which contained 67 mg/L sulfide, 28.1 mg/L Fe^{2+} and 2 μg/L vitamin B_{12}, but at much lower rates as compared to the biotic assays. The pseudo-first order rate constants for the PCNB transformation in autoclaved culture media (0.09 μM PCNB) and in the autoclaved culture controls (3 μM PCNB) at an incubation temperature of 22°C were 0.851 ± 0.004 and 1.7 ± 0.17 d^{-1} (mean ± stand. error), respectively. However, dechlorination of PCA was not observed in any of the abiotic controls (Chapter 5).

PCA sequential dechlorination was observed in cultures incubated in a temperature range of 4 to 35°C (Figure 7.11). PCA dechlorination was not observed in the culture incubated at 45°C. It took about 10 days for the complete sequential

Table 7.4. Rate constants (k_{obs}) for the biotransformation of PCNB to PCA as a function of incubation temperature[a]

Incubation temperature (°C)	k_{obs} (day^{-1})[b]	r^2
4	7.3 ± 1.4	0.977
15	7.4 ± 1.4	0.977
22	8.4 ± 1.7	0.986
35	9.9 ± 1.0	0.999
45	17.7 ± 0.8	0.999

[a] Initial biomass concentration in all culture series equal to 320 ± 10 mg POC/L (mean ± stand. dev.; $n = 3$); initial PCNB concentration 3 μM and pH 6.9 ± 0.1. [b] Nonlinear regression best estimate (mean ± standard error; $n \geq 3$).

dechlorination of PCA at 22°C, whereas complete sequential dechlorination of PCA at 15 and 35°C was observed in about 20 days. PCA dechlorination at 4°C was very slow.

Methane and carbon dioxide production was monitored in each culture series during the batch biotransformation assays. The methane production rate increased with increasing temperature up to 35°C (Figure 7.12). Negligible methane production was observed in the culture incubated at 4°C, whereas methane production was not observed in the culture incubated at 45°C. Although the methane production rate increased with increasing temperature, the extent of methane production was the same at the end of the incubation period. It is noteworthy that, although the methane production rate was the highest at 35°C, the maximum PCA dechlorination rate was observed at 22°C and significantly decreased at 35°C. As it was shown in Chapter 6, although methanogens in the mixed, enrichment culture were completely inhibited by 2-bromoethanesulfonate

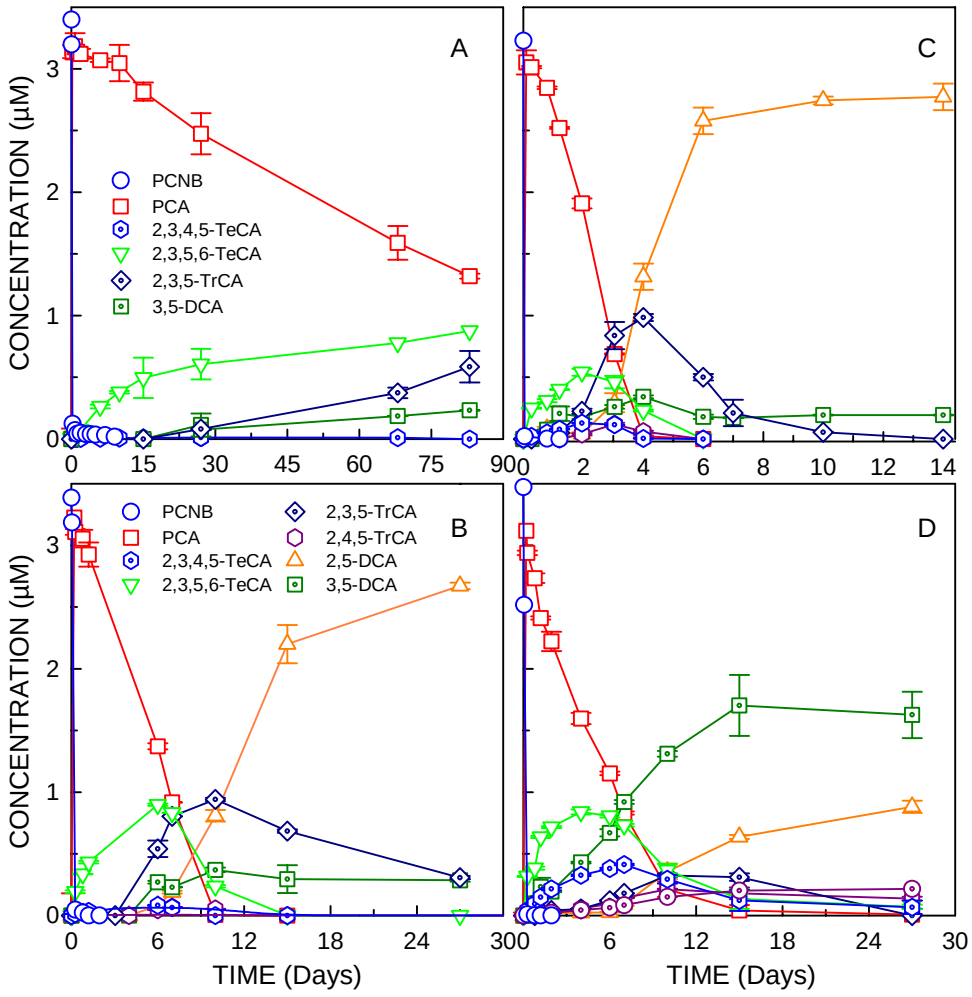


Figure 7.11. Time course of PCNB and its biotransformation products during a batch biotransformation assay conducted at incubation temperatures of 4°C (A), 15°C (B), 22°C (C), and 35°C (D) using the PCNB-enriched culture at a pH 6.9 ± 0.1.

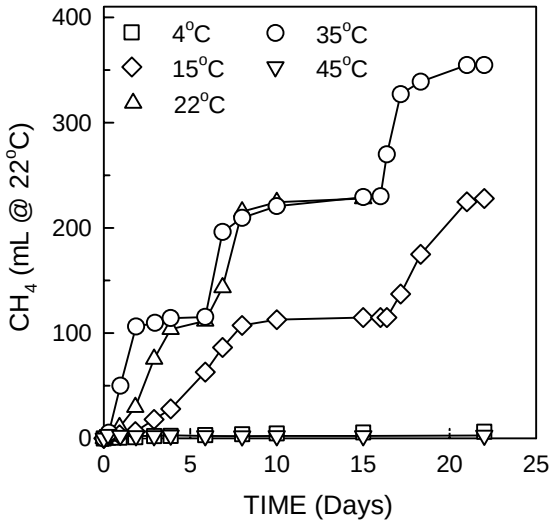


Figure 7.12. Effect of incubation temperature on methane production (the cultures were fed again with only glucose and methanol at the following incubation times: 6 days for the 22°C cultures; 16 days for the 15°C cultures; 6 and 16 days for the 35°C cultures).

(BES), the rate of PCNB transformation to PCA and its dechlorination pathway at 22°C were not affected, leading to the conclusion that methanogens were not directly involved in the sequential dechlorination of PCA.

The dechlorination product distribution at four different incubation temperature values was calculated based on the mole fractions among all dechlorination products measured during the dechlorination of PCA (Table 7.5). The identified intermediates indicate that chlorine atoms were removed from positions *ortho* (2,3,4,5-TeCA, 3,4,5-TrCA, 2,3,5-TrCA, and 3,5-DCA), *meta* (2,3,4-TrCA, 2,4,5-TrCA, 2,5-DCA and 3-CA), and *para* (2,3,5,6-TeCA, 2,3,5-TrCA, 3,5-DCA, and 2,5-DCA). The extent of *ortho* dechlorination of PCA resulting in the formation of 2,3,4,5-TeCA increased with

increasing temperature up to 22°C and then declined as the incubation temperature rose to 35°C. A similar trend was observed for the *meta* dechlorination of 2,3,4,5-TeCA resulting in the formation of 2,4,5-TrCA. However, *para* dechlorination of 2,3,4,5-TeCA resulting in the formation of 2,3,5-TrCA decreased with increasing temperature to 35°C. *Ortho* dechlorination of 2,3,5,6-TeCA resulting in the formation of 2,3,5-TrCA did not change with increasing temperature. The major difference in terms of product distribution as a function of incubation temperature was observed in the case of 2,3,5-TrCA dechlorination. At 22°C, only *meta* dechlorination of 2,3,5-TrCA was observed and resulted in the production of 2,5-DCA. At 4 and 15°C, both *meta* and *ortho* dechlorination of 2,3,5-TrCA resulted in the formation of 2,5-DCA (predominant) and 3,5-DCA, respectively. At 35°C, both *meta* and *ortho* dechlorination of 2,3,5-TrCA were observed, but the predominant product was 3,5-DCA. Production of 3,5-DCA may be indicative of the presence of different dechlorinating species and/or multiple enzymes with different stereospecificity. At a temperature range from 15 to 35°C there was significant branching in terms of products during the dechlorination of 2,3,4,5-TeCA (Table 7.5). At 35°C, dechlorination of 2,3,4,5-TeCA at the *meta* position resulted in the formation of 2,3,4-TrCA which was not observed at incubation temperature values from 4 to 22°C (Table 7.5). At 4°C, 2,3,4,5-TeCA was dechlorinated to 2,3,5-TrCA via *para* dechlorination, which was then converted to 2,5-DCA (predominant) and 3,5-DCA (Table 7.5). These results also indicate that reductive dechlorination of PCA at very low temperatures (e.g., 4°C) is possible, albeit at much lower rates than those attained at higher temperature values (e.g., 15 to 35°C).

Table 7.5. Dechlorination reactions carried out by the PCA-dechlorinating, enrichment culture and product distribution at different incubation temperature values

Compound	Possible products[a]	Position of Cl removal	Observed product distribution[b] (%) at:			
			4°C	15°C	22°C	35°C
PCA	2,3,4,5-TeCA	*ortho*	10	10	35	20
	2,3,5,6-TeCA	***para***	**90**	**90**	**65**	**80**
	2,3,4,6-TeCA	*meta*	0	0	0	0
2,3,4,5-TeCA	2,3,4-TrCA	*meta*	0	0	0	30
	2,3,5-TrCA	***para***	**100**	**60**	**50**	**42**
	2,4,5-TrCA	*meta*	0	40	25	28
	3,4,5-TrCA	*ortho*	0	0	25	0
2,3,5,6-TeCA	**2,3,5-TrCA**	***ortho***	**100**	**100**	**100**	**100**
	2,3,6-TrCA	*meta*	0	0	0	0
2,3,5-TrCA	2,3-DCA	*meta*	0	0	0	0
	2,5-DCA	***meta***	**75**	**75**	**100**	25
	3,5-DCA	***ortho***	25	25	0	**75**
2,4,5-TrCA	2,4-DCA	*meta*	-[c]	0	0	0
	2,5-DCA	***para***	**-**	**100**	**100**	**100**
	3,4-DCA	*ortho*	-	0	0	0
3,4,5-TrCA	3,4-DCA	*meta*	-	-	0	-
	3,5-DCA	***para***	**-**	**-**	**100**	**-**

[a] After one dechlorination step (observed predominant reaction and product shown in boldface type). [b] Relative fractions among observed dechlorination products (molar basis). [c] Reaction not carried out by the enrichment culture during the short batch assays.

The effect of temperature on the PCA dechlorination rate was simulated by using an Arrhenius relationship which accounts for both enzyme activation and deactivation (Bailey and Ollis, 1986).

$$R_d = \frac{\beta T [\exp \frac{-E_a}{RT}]}{1 + [\exp \frac{\Delta S}{R}][\exp \frac{-\Delta H}{RT}]} \quad (7\text{-}9)$$

where, R_d is the PCA dechlorination rate (μM/d); β is the dechlorination proportionality factor (μM/d·K); T is the absolute temperature (K); E_a is the activation energy (kcal/mol), R is the gas constant (=1.987 x 10^{-3} kcal/mol·K); ΔS is the entropy change of enzyme deactivation (kcal/mol·K); ΔH is the enthalpy change of enzyme deactivation (kcal/mol). Non-linear regression of PCA dechlorination rate data obtained with the enriched culture amended with 3 μM PCNB and incubated in a temperature range of 4 to 45°C based on equation 7-9 resulted in the following parameter estimates: β = 1.008 x 10^8 μM/d·K; E_a = 14.2 kcal/mol; ΔS = 0.358 kcal/mol·K; ΔH = 109.5 kcal/mol. Based on these estimates and equation 7-9, the rate of PCA dechlorination increased with increasing temperature up to 29°C and then declined as the temperature rose to 35°C (Figure 7.13).

Zhuang and Pavlostathis (1995) reported the following values for the reductive dechlorination of tetrachloroethene (PCE) by an enriched, mixed culture at an initial PCE concentration of 1.6 mg/L: E_a = 20.61 kcal/mol; ΔS = 0.113 kcal/mol·K; and ΔH = 33.95 kcal/mol. Similarly, Armenante *et al.* (1999) reported E_a values of 13 and 14.7 kcal/mol for the microbial reductive dechlorination of 2,4,6-trichlorophenol and 2,4-dichlorophenol,

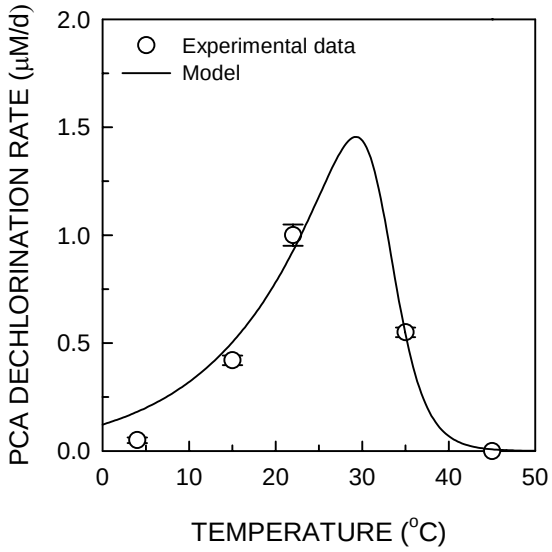


Figure 7.13. PCA dechlorination rate as a function of incubation temperature (The cultures were amended with 3 μM PCA and maintained at a pH 6.9 ± 0.1).

respectively. Thus, the estimated E_a values for the dechlorination of PCA in the present study are comparable to those reported for the microbial reductive dechlorination of chlorinated solvents (Zhuang and Pavlostathis, 1995) and chlorophenols (Armenante *et al.*, 1999). In contrast to the above given E_a values, Aulenta *et al.* (reported E_a values of 0.79 kcal/mol and 0.85 kcal/mol for the formation of vinyl chloride (VC) from PCE and the conversion of VC to ethene, respectively, in a methanogenic biofilm reactor. The low E_a values were attributed to mass transport limitations (diffusion through the biofilm) in the biofilm system.

Based on the Arrhenius equation, the temperature coefficient (Q_{10}), defined as the ratio of the dechlorination rates for a temperature difference of 10°C (within the minimum and optimum temperature range) is as follows:

$$Q_{10} = \frac{k_{T+10}}{k_T} = \exp[\frac{10\ E_a}{R T_1 T_2}] \qquad (7\text{-}10)$$

where, k is the PCA dechlorination rate (μM/d); E_a is the activation energy (kcal/mol); R is the gas constant (=1.987 x 10^{-3} kcal/mol·K); T is the absolute temperature (K). The Q_{10} values for PCA dechlorination ranged from 2.25 to 2.46 for a temperature range of 4 to 29°C. Based on these data, a 2.36 ± 0.15 fold increase/decrease of the PCA dechlorination rate is expected for a 10°C temperature increase/decrease. This Q_{10} value shows that for a temperature decrease below 22°C in subsurface natural systems, a significant decrease in the PCA dechlorination rate is expected.

Similar to our results, microbial reductive dechlorination of trichloroethene (TCE) to cis-dichloroethene (cis-DCE) and VC has been reported at 4°C in anoxic microcosms prepared with cold temperature-adapted aquifer and river sediments from Alaska (Bradley *et al.*, 2005). Backman *et al.* (2004) showed that *Arthrobacter chlorophenolicus* A6 was able to degrade large amounts of 4-chlorophenol in soil at 5°C. The ability of PCNB biotransforming cells to maintain metabolic activity at low temperatures may aid in their survival in sediments and soils especially in cold climates or seasons.

Wu *et al.* (1996, 1997) reported significant differences in the extent and pattern of polychlorinated biphenyl (PCB) reductive dechlorination at different incubation temperatures in freshwater sediment samples. They observed four different microbial

dechlorination processes at temperatures ranging from 4 to 66°C in sediment slurries taken from Woods Pond Sediment (Lenox, Mass) (Wu *et al.*, 1997). Wu *et al.* (1996) reported that *ortho*, *meta*, and *para* dechlorination of 2,3,4,6-tetrachlorobiphenyl took place at different temperature ranges in freshwater sediments taken from two different locations and suggested the presence of different dechlorinating enzymes, and microorganisms with different temperature optima for PCB dechlorination in these sediments.

7.3.5. Modeling PCA Dechlorination at Different Incubation Temperatures

The sequential dechlorination pathway and kinetics of PCA to the less chlorinated aniline isomers by the enrichment culture at 4, 15, 22 and 35°C were simulated using a Michaelis-Menten model which accounted for the observed branching and product distribution at each dechlorination step based on data reported in Table 7.5. The resulting system of the simultaneous ordinary differential equations was solved numerically using MATLAB Version 7, ode23 suite which uses a Runge-Kutta solver (MATLAB, 2004). The values of k' and K_C for PCA and all CAs observed at each incubation temperature were estimated using the Berkeley Madonna 8.0.1 software, which uses a multiple parameter optimization procedure (Berkeley Madonna, 2000) and are reported in Table 7.6. The model simulations for the batch, sequential dechlorination of PCA at 4, 15, 22 and 35°C are plotted against the experimental data in Figure 7.14. The simulated progress curves for PCA and all CAs agree well with the experimental data. The longest PCA half-lifetime was observed at 4°C, whereas the fastest PCA dechlorination rate was observed at 22°C (Table 7.6). These results confirm that the sequential dechlorination rates of CAs

Table 7.6. Dechlorination rates (k'), half-velocity coefficients (K_C), and half-lifetimes (t_h), for the dechlorination of CAs at different incubation temperature values[a]

Compound	k' (μM/day)				K_C (μM)				t_h (day)			
	4°C	15°C	22°C	35°C	4°C	15°C	22°C	35°C	4°C	15°C	22°C	35°C
PCA	0.05	0.42	1.0	0.55	2.98	0.40	0.31	0.98	71.3	4.2	1.7	4.0
2,3,4,5-TeCA	0.01	0.18	0.85	0.53	0.95	0.30	0.30	1.28	215.9	9.5	2.0	4.5
2,3,5,6-TeCA	0.034	0.31	0.81	0.21	1.8	0.32	0.22	0.95	80.8	5.6	2.0	10.3
2,3,5-TrCA	0.003	0.26	0.80	0.78	1.16	0.23	0.11	0.71	768.0	6.4	2.0	2.6
2,4,5-TrCA	-[b]	0.30	0.55	0.17	-	0.11	0.23	0.80	-	5.3	3.0	12.1
3,4,5-TrCA	-	-	0.61	-	-	-	0.23	-	-	-	2.7	-

[a] Initial biomass concentration in all culture series equal to 320 ± 10 mg POC/L (mean ± stand. dev.; n = 3); initial PCNB concentration 3 μM and pH 6.9 ± 0.1. [b] Transformation of the corresponding CA congener at the specified incubation temperature was not carried out by the enrichment culture during the batch assay.

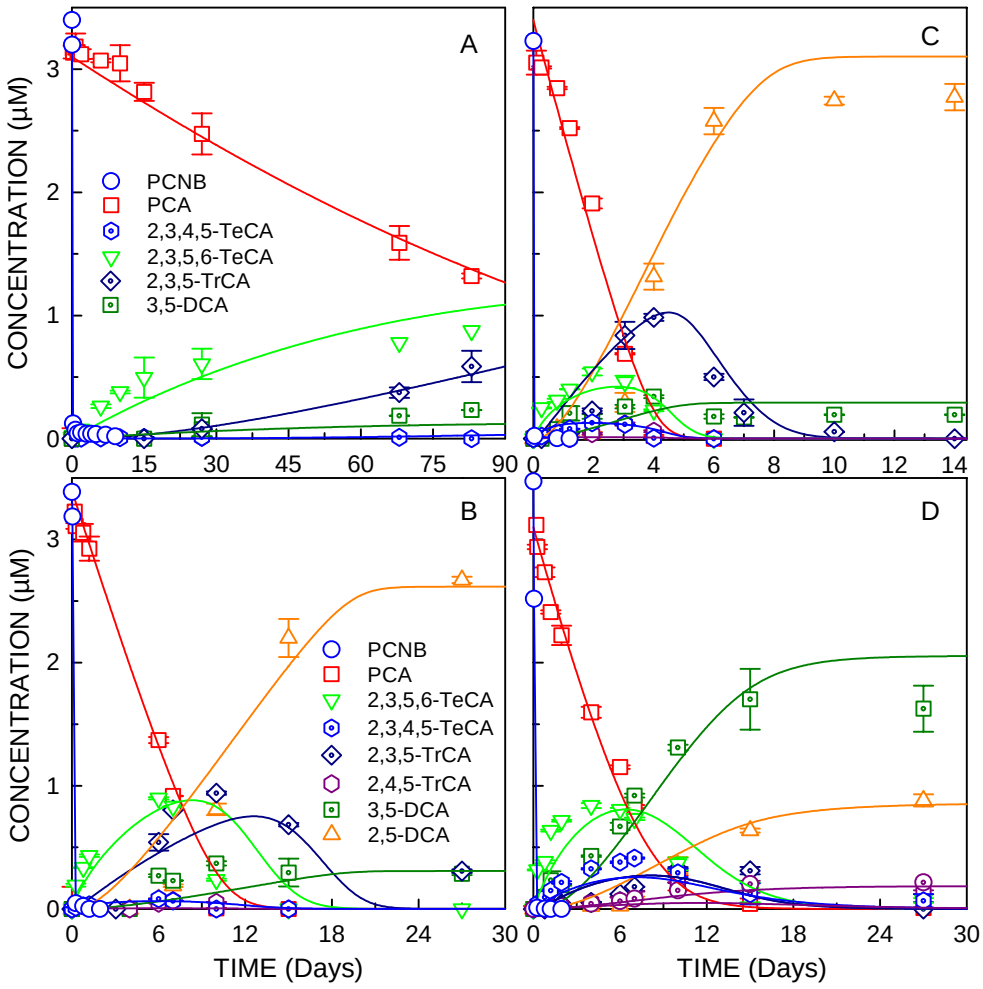


Figure 7.14. Time course of PCNB and its biotransformation products during a batch biotransformation assay conducted at incubation temperature values of 4°C (A), 15°C (B), 22°C (C), and 35°C (D) using the PCNB-enriched culture at a pH 6.9 ± 0.1 (Lines are model predictions).

increase with increasing temperature up to 22°C and then decline as the temperature rises to 35°C. It is noteworthy that according to the Arrhenius model, the maximum PCA dechlorination rate is predicted to occur at 29°C (Figure 7.13).

7.3.6. pH Effect on PCNB Biotransformation

The effect of pH on the PCNB biotransformation as well as PCA sequential dechlorination was investigated with the mixed enrichment culture at an initial PCNB concentration of 3 μM and a pH range from 2.7 to 7.6. The pseudo-first order rate constant values for the PCNB to PCA biotransformation reported in Table 7.7 show that the rate increased with increasing pH. In all cases, complete transformation of PCNB was accomplished within 1 day of incubation, except for the cultures with a pH of 2.7 ± 0.1 and 4.1 ± 0.1 where complete PCNB transformation was achieved in about 40 and 9 days, respectively (Figure 7.15).

Although PCNB transformation to PCA was observed in the cultures incubated at pH 2.7 ± 0.1 and 4.1 ± 0.1, PCA dechlorination did not take place (Figure 7.15A and 7.12B). PCA sequentially dechlorinated to dichlorinated anilines in all cultures with pH values between 6.2 and 7.6 (Figure 7.15). PCA removal rates were progressively slower as the incubation pH decreased. In all cases, complete PCA removal was accomplished within 10 days of incubation, except for the culture at pH 6.2 ± 0.1 which required 40 days. The maximum PCA dechlorination rate was observed at pH 7.6 ± 0.1.

Methane production was not observed in the cultures incubated at pH 2.7 ± 0.1 and 4.1 ± 0.1. The rate of methane production increased with increasing pH up to 7.2 ± 0.1 and decreased slightly at pH 7.6 ± 0.1 (Figure 7.16). The extent of methane

Table 7.7. Rate constants (k_{obs}) for the biotransformation of PCNB to PCA as a function of incubation pH[a]

pH[b]	k_{obs} (day^{-1})[c]	r^2
2.7 ± 0.1	0.05 ± 0.01	0.950
4.1 ± 0.1	1.7 ± 0.2	0.981
6.2 ± 0.1	2.8 ± 0.2	0.999
6.6 ± 0.1	4.2 ± 0.1	0.999
7.2 ± 0.1	10.2 ± 0.1	0.999
7.6 ± 0.1	16.4 ± 0.3	0.999

[a] Initial biomass concentration in all culture series equal to 285 ± 15 mg POC/L (mean ± stand. dev.; $n = 3$); initial PCNB concentration 3 μM and incubation temperature 22°C. [b] pH values throughout the incubation period (mean ± stand. dev.; $n = 3$). [c] Nonlinear regression best estimate (mean ± standard error; $n \geq 3$).

production was the same in all cultures with a pH 6.2 or higher. Although there are methanogens that live in extreme pH environments, the optimum pH reported for methanogenesis is near neutral (Ferry, 1993; Rittmann and McCarty, 2001).

The PCA dechlorination product distribution for all five cultures incubated at different pH values was calculated based on the mole fractions among all dechlorination products measured and found to be the same regardless of incubation pH. During the dechlorination of PCA at a pH range from 6.2 ± 0.1 to 7.6 ± 0.1, 2,3,5,6-TeCA and 2,3,5-TrCA were the predominant intermediates while 2,5-DCA was the predominant end product (Figure 7.15). Therefore, the culture incubation pH did not have any significant effect on the PCA dechlorination pathway (for a pH range 6.2 to 7.6).

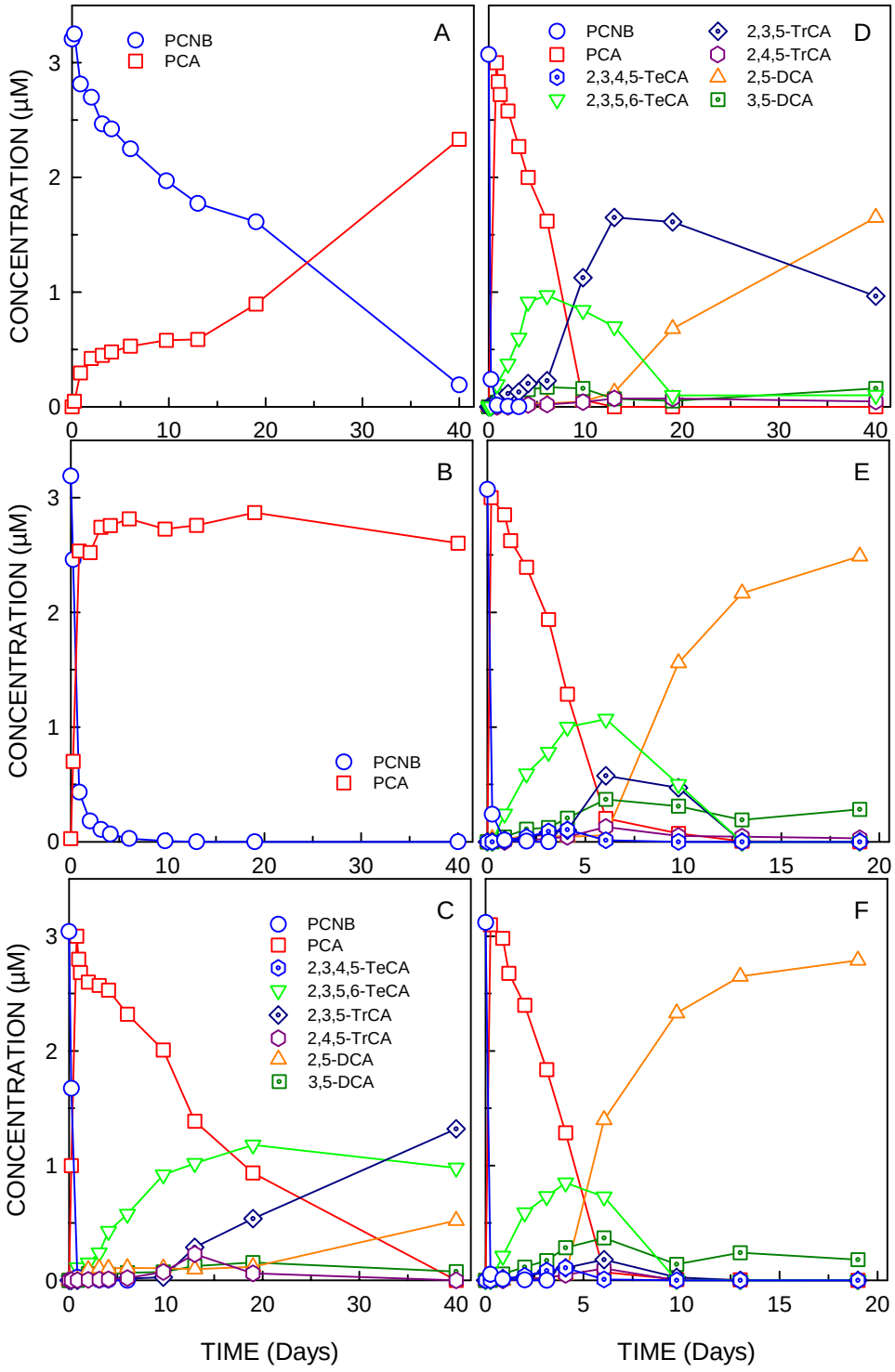


Figure 7.15. Time course of PCNB and its biotransformation products during a batch biotransformation assay conducted at pH 2.7 ± 0.1 (A), 4.1 ± 0.1 (B), 6.2 ± 0.1 (C), 6.6 ± 0.1 (D), 7.2 ± 0.1 (E), and 7.6 ± 0.1 (F) using the PCNB-enriched culture at an incubation temperature of 22°C (Note the difference in the time scale between A, B, C, D and E, F).

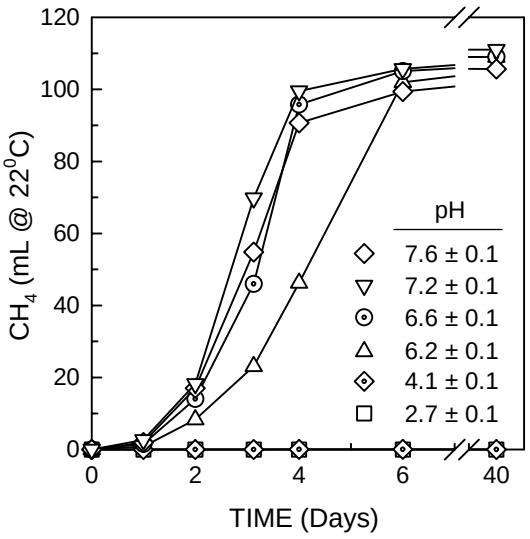


Figure 7.16. Effect of incubation pH on methane production.

Zhuang and Pavlostathis (1995) noted that the maximum reductive dechlorination rate of PCE by an enriched, mixed culture was achieved at an initial pH value of 7. Maximal PCE dechlorination by a Gram-negative anaerobic bacterium (PER-K23) was observed between pH 6.8 and 7.6 (Holliger *et al.*, 1993). Rutgers *et al.* (1998) reported that the optimum, minimum, and maximum pH values for the degradation of pentachlorophenol by *Sphingomonas* sp. Strain P5 were 6.67, 4.14 and 8.79, respectively. The above reports relative to the observed pH range for the microbial reductive dechlorination of various chlorinated compounds agree with the conclusion reached by Fennell and Gossett (2003) who stated that anaerobic systems, which support dechlorination reactions generally have pH values between 6 and 8.

As previously discussed, the PCNB to PCA transformation is a relatively fast process and may occur under abiotic conditions where the rate depends on the reducing agent (sulfide) concentration, the presence of certain media components (e.g., Fe^{2+}) as well as biotically generated reductants. A decrease in pH should lead to decreased reducing capacity as the redox potential will be shifted towards a more positive value. Therefore, the (bio)transformation of PCNB should be less favorable at lower pH values in agreement with our experimental results. Klupinski *et al.* (2004) reported an increase in the PCNB degradation rate constant to PCA as the pH increased at constant goethite and total Fe^{2+} concentrations. They also observed an increase in the sorbed Fe^{2+} concentration with increasing pH, but this increase was not proportional to the observed PCNB transformation rate.

7.3.7. Modeling PCA Dechlorination at Different Incubation pH Values

The sequential dechlorination pathway and kinetics of PCA to the less chlorinated aniline isomers by the enrichment culture at pH values from 6.2 to 7.6 were simulated using a Michaelis-Menten model and the observed branching and product distribution at each dechlorination step reported in Table 7.5. The values of k' and K_C for PCA and all CAs observed at each incubation pH value were estimated using the Berkeley Madonna 8.0.1 software (Berkeley Madonna, 2000). The results for two representative incubation pH values (6.2 ± 0.1 and 7.2 ± 0.1) are shown in Table 7.8. The CA dechlorination rates increased with increasing pH. A three-fold increase was observed in the PCA dechlorination rate when the culture incubation pH increased from 6.2 ± 0.1 to 6.6 ± 0.1, and a four-fold increase was observed when the incubation pH increased from

Table 7.8. Dechlorination rates (k'), half-velocity coefficients (K_C), and half-lifetimes (t_h), for the dechlorination of CAs at two incubation pH values[a]

Compound ↓	k' (μM/day)		K_C (μM)		t_h (day)	
pH[b] →	6.2 ± 0.1	7.2 ± 0.1	6.2 ± 0.1	7.2 ± 0.1	6.2 ± 0.1	7.2 ± 0.1
PCA	0.13	0.51	0.32	0.31	13.6	3.5
2,3,4,5-TeCA	0.45	0.31	0.31	0.22	11.8	5.5
2,3,5,6-TeCA	0.08	0.25	1.94	0.43	36.2	7.4
2,3,5-TrCA	0.02	0.40	0.79	0.48	104.9	4.7
2,4,5-TrCA	0.08	0.20	0.53	0.31	24.0	8.8
3,4,5-TrCA	0.13	0.45	0.47	0.63	14.4	4.4

[a] Initial biomass concentration in all culture series equal to 285 ± 15 mg POC/L (mean ± stand. dev.; n = 3); initial PCNB concentration 3 μM and incubation temperature 22°C. [b] pH value throughout the incubation period (mean ± stand. dev.; n = 3).

6.2 ± 0.1 to 7.6 ± 0.1. The TeCAs and TrCAs dechlorination rates increased four-fold when the pH increased from 6.2 ± 0.1 to 7.2 ± 0.1 and a two-fold increase was observed when the pH increased from 7.2 ± 0.1 to 7.6 ± 0.1. For all pH values tested, the highest dechlorination rates observed were for TeCAs and PCA. The dechlorination rates in all cultures decreased with decreased degree of chlorination. The model simulations for the batch sequential dechlorination of PCA at pH 6.2 ± 0.1 and 7.2 ± 0.1 are plotted against the experimental data in Figure 7.17. The simulated progress curves for PCA and all CAs agree well with the experimental data. It is noteworthy that the pK_a values of PCA and all TrCAs are lower than the tested pH values in this assay (Chapter 2, Table 2.1). Therefore, the observed differences in the dechlorination rates as a function of culture pH are not related to the protonation and deprotonation of the parent compounds.

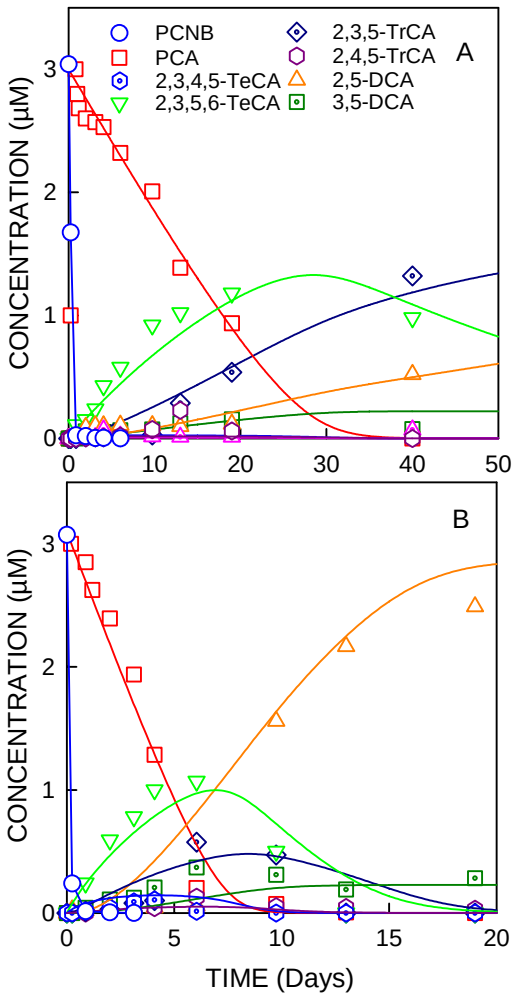


Figure 7.17. Time course of PCNB and its biotransformation products during a batch biotransformation assay conducted at pH 6.2 ± 0.1 (A) and 7.2 ± 0.1 (B) using the PCNB-enriched culture at an incubation temperature of 22°C (Lines are model predictions).

Among all pH values tested, the highest dechlorination rates were observed at pH 7.6 ± 0.1. The longest dechlorination half-lifetimes were at pH 6.2 ± 0.1 (Table 7.8). Similar to our results, Young and Gossett (1997) reported a four-fold and two-fold decrease in the dechlorination of PCE with an enrichment culture that contained *Dehalococcoides* when the pH changed from 7 to 6 and from 7 to 8, respectively.

7.4. Summary

The microbial reductive dechlorination kinetics of PCA, and all other chlorinated anilines, as well as the effect of pH and temperature on the microbial reductive transformation of PCNB were investigated with the mixed, fermentative/methanogenic culture. Batch dechlorination assays were performed for all available chlorinated aniline congeners at an initial concentration of about 3 μM and with an initial biomass concentration of 268 ± 15 mg POC/L. Electron donor and nutrients were provided in excess to eliminate any limitations. The predominant, sequential PCA biotransformation pathway at 22°C and pH=6.9±0.1 was as follows: PCA → 2,3,4,5- and 2,3,5,6-tetrachloroaniline (TeCA) → 2,4,5- and 2,3,5-trichloroaniline (TrCA)→ 2,5-dichloroaniline (DCA) → 3-CA (low levels). Thus, the observed sequential dechlorination pathway proceeded via *para* and *ortho* dechlorination of the highly chlorinated and *para* and *meta* dechlorination of the less chlorinated anilines. The volumetric dechlorination rate of all CAs tested ranged between 0.25 and 1.19 μM/d corresponding to half-lives of 1.5 and 8.5 days. Cultures were incubated at a temperature range from 4 to 45°C at pH 6.9 ± 0.1 and at a pH range from 2.7 ± 0.1 to 7.6 ± 0.1 at 22°C. Significant differences were observed in terms of biotransformation rate, extent,

and products as a function of temperature. Incubation at different pH values resulted in differences in biotransformation rate and extent, but not in terms of products formed. PCNB (3 μM) was transformed to PCA in all culture series. However, sequential dechlorination of PCA was only observed at a temperature range from 4 to 35°C and at a pH range from 6.2 ± 0.1 to 7.6 ± 0.1. The highest PCA dechlorination rate was observed at 22°C and at pH 7.6 ± 0.1. The dechlorination of each parent compound and the production and disappearance of chlorinated aniline intermediates were simulated using a branched chain Michaelis-Menten kinetic model. Model simulations using independently estimated kinetic parameter values matched very well with the experimental data. The effect of temperature on the PCA dechlorination rate was modeled using an Arrhenius relationship which accounts for both enzyme activation and deactivation.

CHAPTER 8

BIOTRANSFORMATION OF PENTACHLORONITROBENZENE UNDER IRON-REDUCING CONDITIONS

8.1. Introduction

Microbial reductive dechlorination is the predominant degradation pathway of chlorinated compounds under anaerobic conditions (Genthner *et al*., 1989; Gibson and Suflita, 1986; Mohn and Tiedje, 1992). The biotransformation of many chlorinated organic compounds under methanogenic conditions is well documented (Kuhn and Suflita, 1989; Masunaga *et al*., 1996; Prytula and Pavlostathis, 1996; Pavlostathis *et al.*, 2003). Relatively recent studies have demonstrated the significant role of iron and manganese reducing bacteria in the cycling of organic matter and the biotransformation of organic contaminants (Lovley, 1991). Ferric iron is by mass the most abundant alternative electron acceptor in anaerobic soil and sediment environments (Cornell and Schwertmann, 1996) and occurs mostly in the form of poorly bioavailable, insoluble oxides at neutral pH. Humics could serve as electron shuttles in the microbial reduction of insoluble Fe^{3+} compounds (He and Sanford, 2003; Lovley and Blunt-Harris, 1999), as well as electron-transfer mediators between Fe^{2+} and halogenated organic compounds (Haderlein and Schwarzenbach, 1995). Another way to enhance the bioavailability of insoluble Fe^{3+} is to increase its solubility with chelators, such as ethylene diamine tetraacetic acid (EDTA)(Lovley and Woodward, 1996). The role of dissimilatory iron-

reducing bacteria in the reduction of nitrobenzenes has been elucidated (Heijman *et al*., 1995; Klausen *et al*., 1995). This transformation involves two coupled reactions, one biotic, which is mediated by the iron-reducing bacteria, and one abiotic, where reduced iron [Fe^{2+}] serves as the intermediate electron donor and nitrobenzenes serve as electron acceptors. In view of the fact that several electron acceptors are always present in the environment, the biotransformation potential of PCNB under different electron accepting conditions should be taken into account. However, little is known about the effect of iron reduction on PCNB biotransformation.

The specific objectives of the research reported in this chapter were to assess: a) the influence of iron reduction on the reductive biotransformation of PCNB in the mixed, fermentative/methanogenic enrichment culture; b) the effect of alternative iron types (completely bioavailable and less bioavailable) on PCA dechlorination; and c) the effect of a humics analog on iron reduction and the sequential reductive dechlorination of pentachloroaniline (PCA), which results from the reductive (bio)transformation of PCNB.

8.2. Materials and Methods

8.2.1. PCNB Biotransformation Assay

It is well known that acetate is one of the preferred electron sources for both methanogens and iron reducers (He and Sanford, 2003; Roden and Wetzel, 2003; van Bodegom *et al*., 2004). In Chapter 6 it was also shown that both acetate and methanol could serve as electron donors for the biotransformation of PCNB. Therefore, in the following assays, in addition to methanol, acetate was used as an electron donor. The

biotransformation of PCNB (0.5 μM) under methanogenic conditions by the fermentative/methanogenic enrichment culture was investigated using acetate (300 mg/L), yeast extract (20 mg/L), and methanol (250 mg/L) as electron donor.

The effect of iron reduction on the PCNB biotransformation was investigated with the same enrichment culture by adding Fe(III)citrate (25 mM) or Fe(III)EDTA (25 mM) as a completely bioavailable iron source. To maintain prolonged iron reducing conditions, the cultures were amended with Fe(III)citrate or Fe(III)EDTA when the red color, characteristic of Fe(III)citrate and Fe(III)EDTA, started to disappear. Acetate (300 mg/L), methanol (250 mg/L), and PCNB (ca. 0.3 μM in methanol) were introduced twice, Fe(III)EDTA was introduced to the culture three times during the 3 months incubation period, and Fe(III)citrate was introduced to the culture twice during the 19 days incubation period. The initial biomass concentration in these cultures was 315±40 mg POC/L. The assays were conducted in 160 ml serum bottles which were sealed with Teflon-lined septa and flushed with nitrogen gas. The cultures were incubated in a 22ºC constant temperature room and the serum bottles were manually shaken once a day. Liquid and gas samples were periodically taken to monitor chlorinated compounds, pH, volatile fatty acids (VFAs), and gas composition.

8.2.2. PCA Dechlorination Assay

PCA dechlorination was assessed directly in the Fe(III)citrate culture after complete iron reduction. Because of the relatively fast transformation of PCNB to PCA under both methanogenic and iron reducing conditions, in subsequent batch experiments, PCA dechlorination rather than PCNB biotransformation was investigated. Batch PCA

dechlorination assays were performed with Fe(III)EDTA as a completely bioavailable iron source. The impact of Fe^{3+} on the reductive dechlorination of PCA was investigated with the sediment-free, mixed, enrichment culture. Fe(III)EDTA (25 mM) and FeOOH (25 mM) were used as the source of iron in order to assess the rate and extent of PCA dechlorination and iron reduction. The preparation of FeOOH source was explained in detail in Chapter 3. The assay was conducted in 160 mL serum bottles that were sealed with Teflon-lined septa and flushed with nitrogen gas. The initial biomass concentration in all cultures in this assay was 310±20 mg POC/L. The cultures were amended with 0.5 µM PCA dissolved in methanol (250 mg/L), acetate (300 mg/L), and yeast extract (20 mg/L). Possible inhibitory effects of EDTA (25 mM) and sodium (50 mM) resulting from the Na_2EDTA were also tested. AQDS (200 µM), a humic acid model compound was added to select iron-amended cultures in order to increase the availability and thus the reduction rate of ferric iron. Another control was set up to test for any possible effect of AQDS on the reductive dechlorination of PCA in the absence of iron. Two additional controls were also setup to test for PCA dechlorination in the presence of only autoclaved culture media and 25 mM ferrous iron ($FeCl_2 \cdot 4H_2O$). All serum bottles were incubated in a 22ºC constant temperature room and were manually shaken once a day.

8.3. Results and Discussion

8.3.1. PCNB Biotransformation

Batch assays were performed with the sediment-free enrichment culture under both methanogenic and iron reducing conditions. Under methanogenic conditions, as previously shown in Chapter 5, PCNB was transformed to PCA, which was then

sequentially dechlorinated as follows: PCA → 2,3,4,5- and 2,3,5,6-tetrachloroaniline (TeCA) → 2,4,5- and 2,3,5-trichloroaniline (TrCA) → 2,5- and 3,5-dichloroaniline (DCA) → 3-chloroaniline (CA)(low levels). The predominant end product was 2,5-DCA. In the culture amended with Fe(III)citrate, a linear increase in the cumulative methane production (8.3 mL CH_4/d; r^2= 0.971) was observed during Fe(III)citrate reduction (Figure 8.1A). After 19 days of incubation, when all of the ferric iron was reduced, this culture was allowed to proceed under fermentative/methanogenic conditions. Consequently, the methane production rate increased significantly (23.3 mL CH_4/d; r^2 = 0.970)(Figure 8.1A). During the latter incubation period, citrate fermentation resulted in the production and significant accumulation of acetate, which is the main fermentation product of citrate (Walther *et al*., 1977). Under iron reducing conditions, PCNB was transformed to PCA with biotransformation rates comparable to those obtained under methanogenic conditions. However, further dechlorination of PCA was not observed in the culture amended with excess bioavailable iron [Fe(III)citrate] for an incubation period of 19 days (Figure 8.2A). When the Fe(III)citrate was completely reduced, very fast sequential PCA dechlorination to dichlorinated anilines (mainly 2,5-DCA) was observed.

In the culture amended with Fe(III)EDTA, methane production was not observed during the incubation period under active iron reducing conditions. Methane production started after 90 days of incubation when all of the ferric iron was reduced (Figure 8.1B). Under iron reducing conditions, PCNB was transformed to PCA at biotransformation rates comparable to those obtained under methanogenic conditions. Complete transformation of PCNB to PCA in the sediment-free culture occurred in less than 6

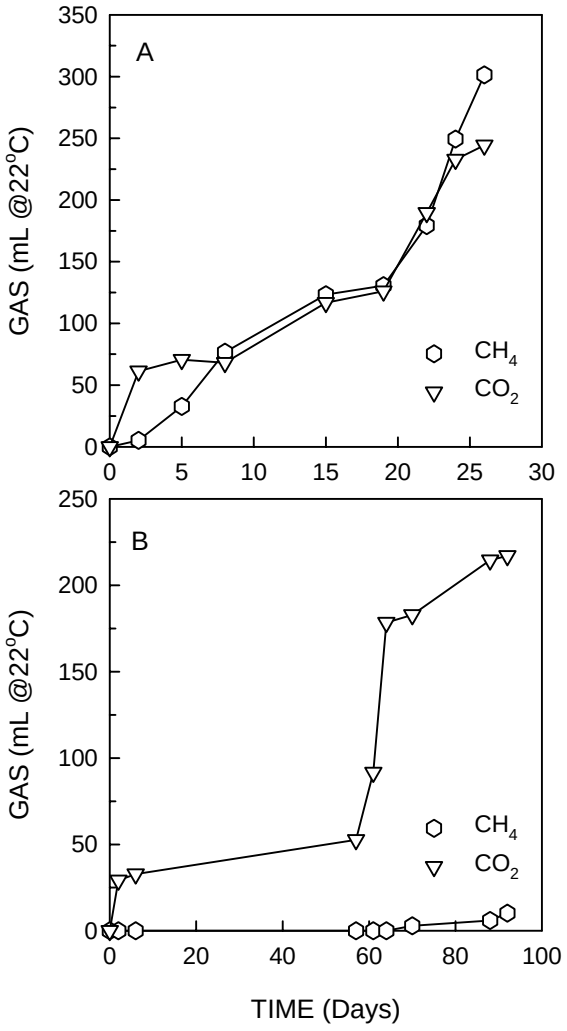


Figure 8.1. Cumulative methane and carbon dioxide production in the cultures amended with Fe(III)citrate (A) or Fe(III)EDTA (B) as the ferric iron source (sediment-free culture).

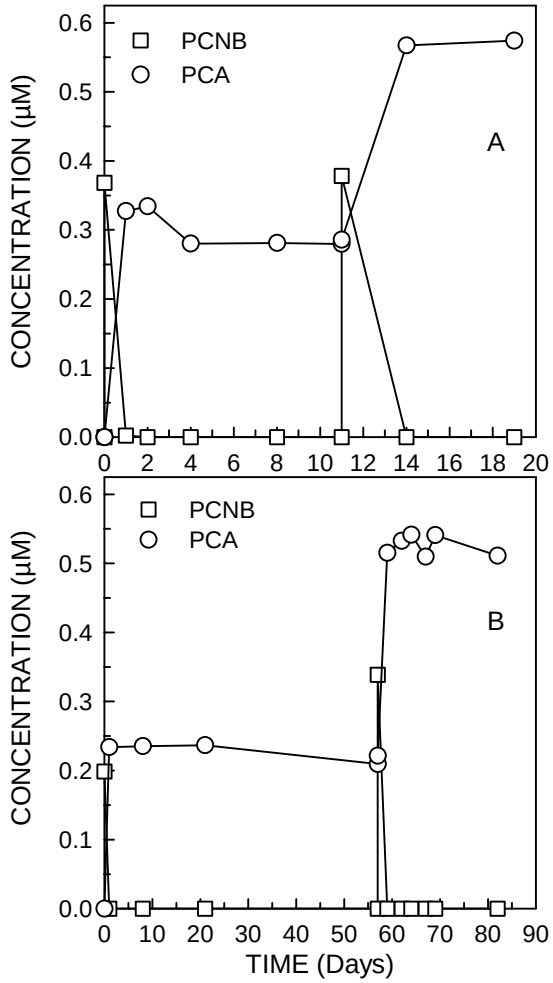


Figure 8.2. PCNB biotransformation under iron reducing conditions using Fe(III)citrate (A) or Fe(III)EDTA (B) as the ferric iron source (sediment-free culture; Fe(III)citrate, Fe(III)EDTA, and PCNB were added twice, three times, and twice, respectively).

hours under both methanogenic and iron reducing conditions using Fe(III)EDTA. Further dechlorination of PCA was not observed in the culture amended with excess bioavailable iron for an incubation period of more than 3 months (Figure 8.2B). Thus, both methanogenesis and PCA dechlorination were inhibited while active iron reduction was maintained in the Fe(III)EDTA-amended enrichment culture.

The enrichment culture developed from a contaminated estuarine sediment achieved a very high transformation rate of PCNB to PCA under either fermentative/methanogenic or iron reducing conditions. As mentioned in Chapter 5, several culture media components (i.e., sulfide, Fe^{2+}, vitamin B_{12}), as well as culture products have the potential to abiotically mediate the reductive transformation of PCNB to PCA. According to abiotic control experiments conducted with autoclaved culture media, deionized water amended with sulfide (67 mg/L), and autoclaved fermentative/methanogenic culture, the pseudo-first-order rate constants for the transformation of PCNB to PCA at an initial PCNB concentration of about 0.1 μM were 0.851 ± 0.004, 0.107 ± 0.008, and 40.8 ± 3.7 d^{-1} (mean ± standard error), respectively (Chapter 5). In the present study, the pseudo-first order rate constant for the transformation of PCNB to PCA (39.6 ± 0.1 d^{-1}) by the active methanogenic culture at an initial PCNB concentration of 0.09 μM, was very similar to the rate obtained with the autoclaved culture controls. These results indicate that, in addition to the culture media components (i.e., sulfide, Fe^{2+}, vitamin B_{12}), biotically-derived reductants and/or other factors facilitated the observed transformation of PCNB to PCA.

8.3.2. PCA Dechlorination and Iron Reduction

The pH and ORP values in the iron-free, control culture were 7.0 ± 0.3 and -170 ± 5 mV, respectively (mean ± standard deviation; n = 3). In the cultures amended with Fe(III)EDTA or FeOOH, the initial and final ORP values were +138 ± 2, -120 ± 4 mV, and -60 ± 14, -120 ± 70 mV, respectively. The pH values were similar to those of the control culture in all Fe(III)EDTA-amended cultures. However, at the end of the 60-day incubation period, the pH increased from 7.2 ± 0.1 to 8.1 ± 0.4 in the FeOOH amended cultures. PCA was dechlorinated to 3,5-DCA and 2,5-DCA and all acetate was consumed in the control culture under methanogenic conditions in 10 days of incubation without any delay (Figure 8.3). However, dechlorination of PCA was not observed in cultures amended with either Fe(III)EDTA or Fe(III)EDTA+AQDS until 30 days of incubation when more than 90% of the added Fe^{3+} was reduced.

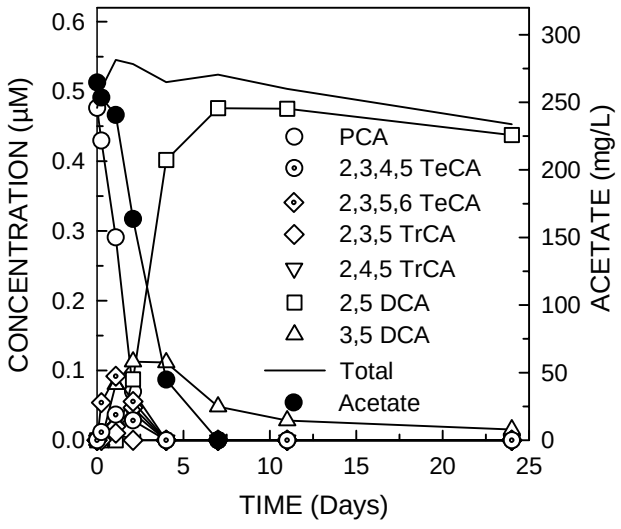


Figure 8.3. PCA dechlorination and acetate consumption under methanogenic conditions (sediment- and Fe^{3+}-free culture).

The PCA dechlorination pathway was similar to that of the control culture after 30 days of incubation in both the Fe(III)EDTA- and Fe(III)EDTA+AQDS-amended cultures (Figure 8.4A and 8.4B). PCA dechlorination was not observed during 60 days of incubation in the culture amended with Na_2EDTA (Figure 8.5A). Thus, EDTA had an inhibitory effect on the dechlorinating culture. PCA dechlorination was not observed in the presence of only culture media or culture media amended with 25 mM ferrous iron (Figure 8.5B and 8.5C). In contrast, PCA dechlorination proceeded as in the control culture without any inhibitory effect in the culture which was amended with 50 mM Na^+ (Figure 8.6A).

Dechlorination of PCA started after 2 days of a lag period in the culture amended with FeOOH, but the dechlorination rates were lower than in the control culture Figure 8.3 and 8.4D). Dechlorination of PCA was slower in the FeOOH+AQDS amended culture compared to the culture amended with only FeOOH (Figure 8.4D). AQDS did not have any inhibitory or stimulatory effect on the dechlorination of PCA in the absence of FeOOH (Figure 8.6B).

Gas production by all cultures during the incubation period is shown in Figure 8.7. AQDS did not have any inhibitory or stimulatory effect on the dechlorinating, mixed fermentative/methanogenic culture in terms of methane production. A slight inhibition in terms of methane production was observed in the culture amended with NaCl. Very low methane production was observed during the 60 days of incubation in the control culture amended with Na_2EDTA. In the cultures amended with FeOOH, methane production was observed after a lag period of 2 days similar to the dechlorination activity of these cultures. Very low methane production was observed in the cultures amended with

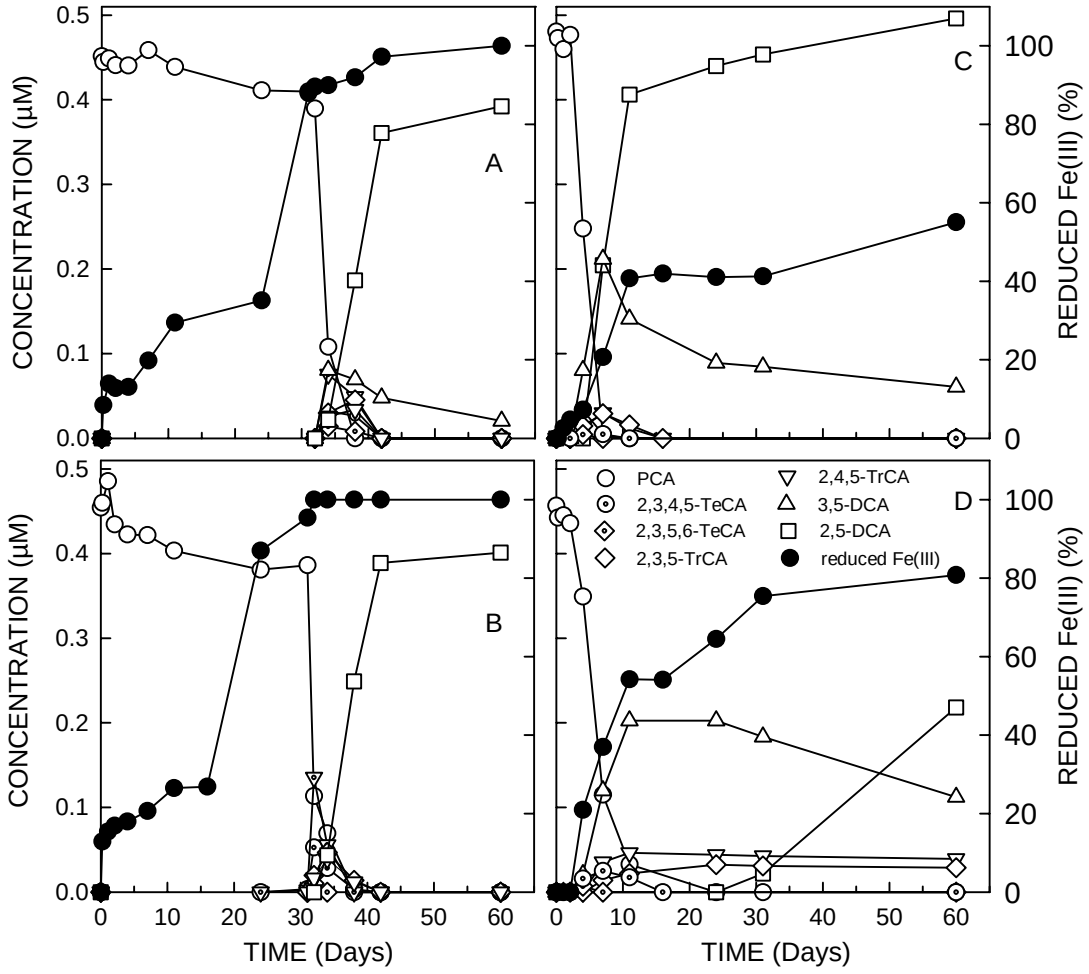


Figure 8.4. Iron reduction and PCA dechlorination in cultures amended with Fe(III)EDTA (A), Fe(III)EDTA+AQDS (B), FeOOH (C), and FeOOH+AQDS (D).

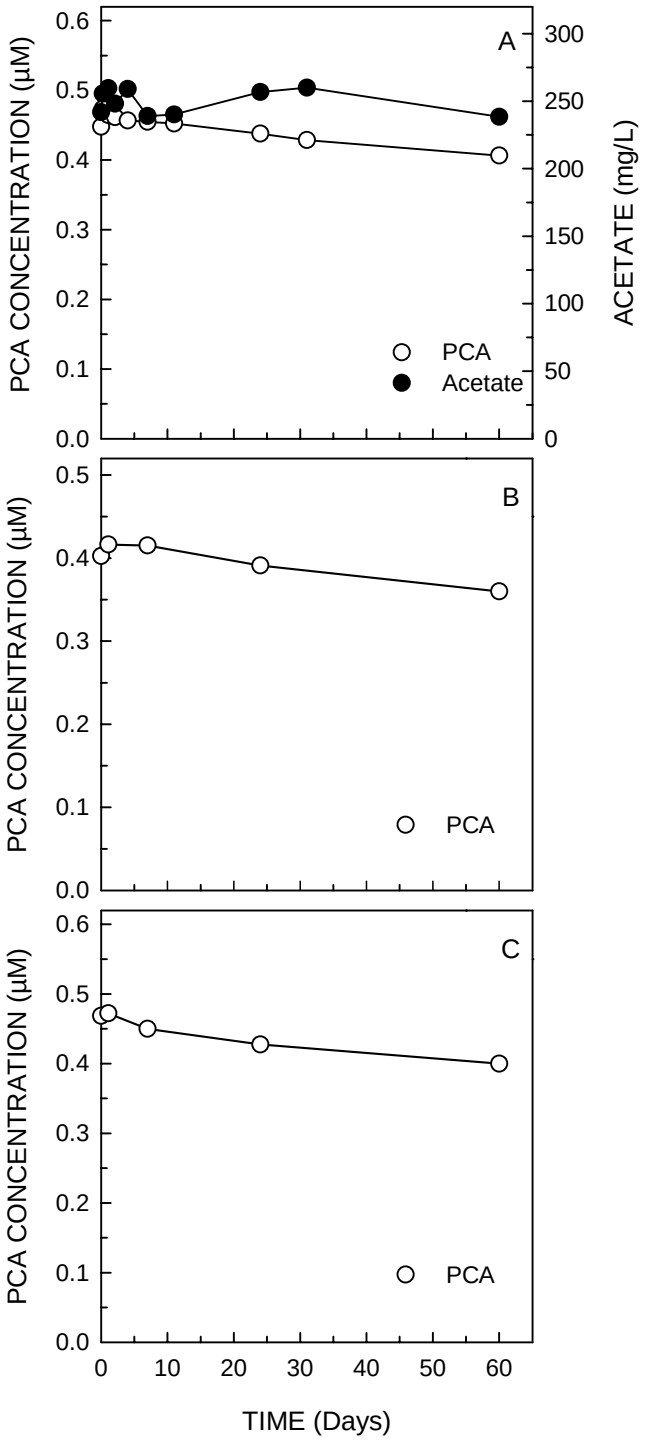


Figure 8.5. PCA dechlorination in cultures amended with Na_2EDTA (A), anaerobic autoclaved culture media (B), anaerobic autoclaved culture media amended with Fe^{2+} (25 mM)(C).

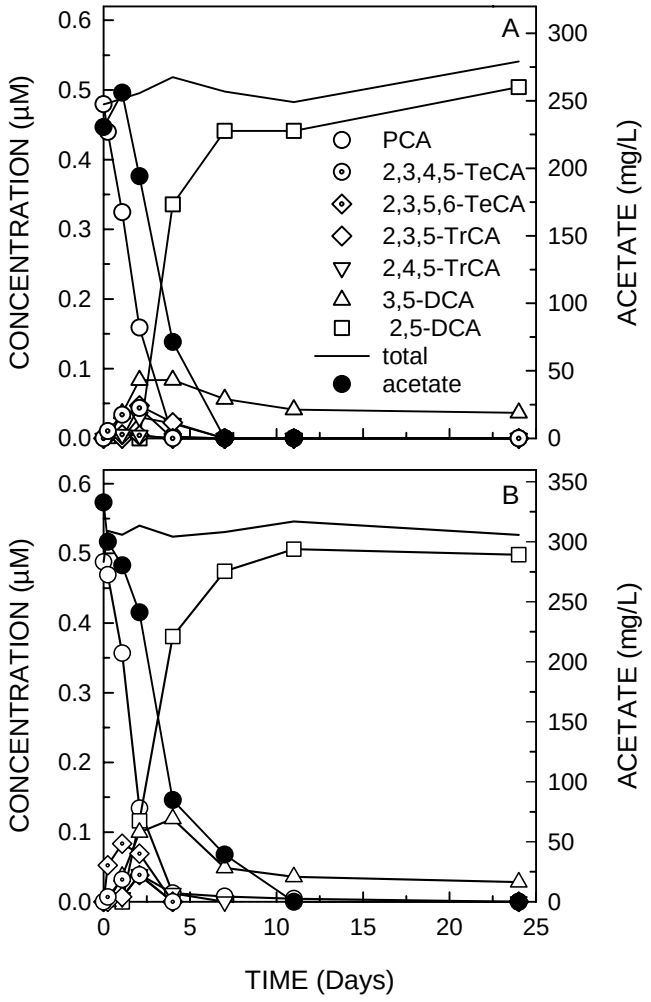


Figure 8.6. PCA dechlorination products and acetate consumption under methanogenic conditions in the culture amended with 50 mM Na^+ (A) and AQDS (B)(sediment- and Fe^{3+}-free culture).

Fe(III)EDTA until 30 days of incubation, but significant methane production and PCA dechlorination was observed after 30 days of incubation when more than 90% of Fe^{3+} was reduced. Methane production was lower in the culture amended with both FeOOH and AQDS compared to that of the culture amended with only FeOOH.

AQDS addition increased the Fe^{3+} reduction rate to some degree in the Fe(III)EDTA-amended culture, but the Fe^{3+} reduction rate and extent were considerably increased in the FeOOH-amended culture (Figure 8.8). Complete iron reduction was observed in the Fe(III)EDTA and AQDS amended cultures after 30 days of incubation. On the other hand, complete iron reduction was observed in the culture without AQDS amendment after 60 days of incubation. Iron reduction was not observed for the first 2 days of incubation in the culture amended with FeOOH+AQDS. However, after 10 days of incubation, the extent of Fe^{3+} reduction in the AQDS-amended culture was significantly higher than in the culture without AQDS amendment (Figure 8.8B).

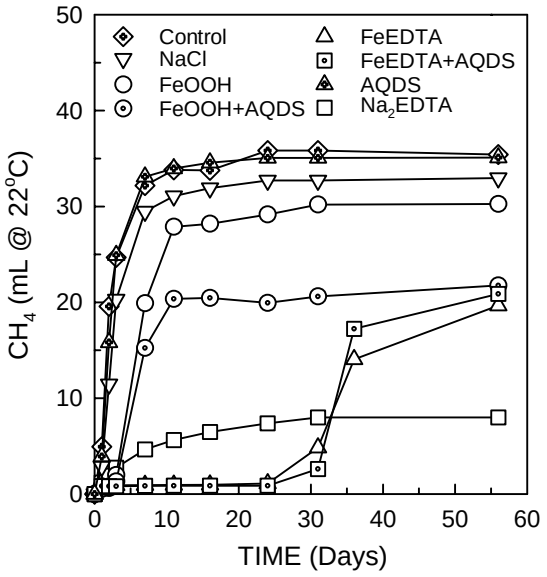


Figure 8.7. Methane production in all PCA-amended cultures.

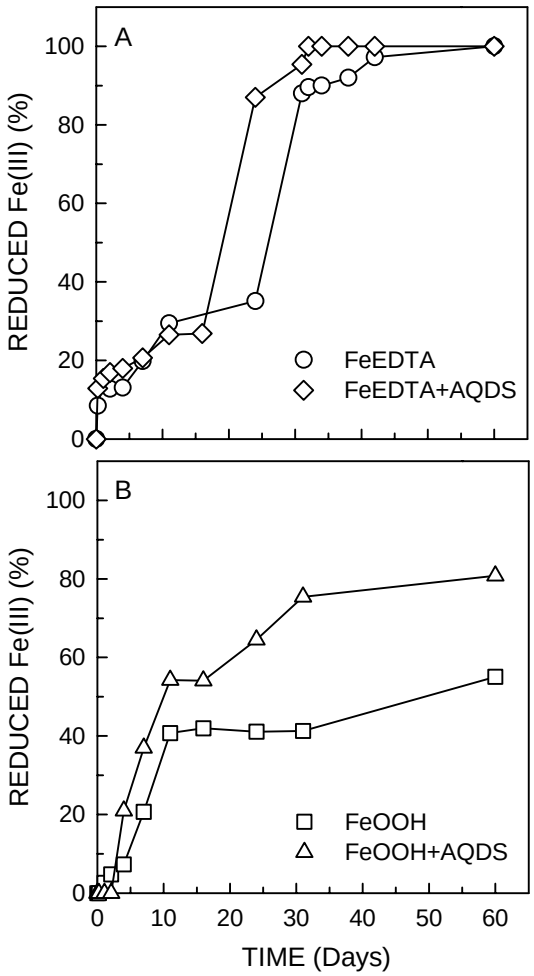


Figure 8.8. Iron reduction in the cultures amended with Fe(III)EDTA (A) or FeOOH (B).

Incomplete iron reduction was observed in all cultures amended with FeOOH (Figure 8.8B) in spite the fact that both the Fe(III)EDTA- and FeOOH-amended cultures received the same amount of electron donors.

An electron equivalents (eeq) balance was calculated at the end of the incubation period based on the electron donor eeq added and by accounting for each process, except for microbial growth. Methane production, iron reduction, acetate and methanol consumption and dechlorination were expressed in terms of eeq as follows: 8 eeq/mol of methane produced, 1 eeq/mol of ferric iron reduced, 8 eeq/mol of acetate and 6 eeq/mol of methanol consumed, and 2 eeq/mol of chlorine substituent removed. In all cultures, the total eeq accounted for amounted to between 96 and 97% of the electron donor eeq added at the beginning of the assay (Figure 8.9). Using bioenergetic principles (Rittmann and McCarty, 2001), the theoretical biomass production was calculated for each culture and amounted to between 4 and 11% of the total electron donor eeq added to each culture. Therefore, the amount of eeq unaccounted for (3-4%; see above) can be assumed to represent microbial growth. On the basis of these calculations for the PCA-amended, Fe^{3+}-free control culture, the fraction of eeq of the added carbon source (acetate and methanol) accounted for in the main products was as follows: biomass growth, 0.03; and methane production, 0.97. The fraction of eeq diverted to the sequential dechlorination of PCA to dichloroanilines was very small (0.0033 %) and this fraction is not shown in Figure 8.9. According to these calculations, under Fe(III)EDTA reducing conditions, about 30% of eeq were used for iron reduction and about 66% were used for methanogenesis. The addition of AQDS did not influence the fraction of eeq used for iron reduction and methanogenesis when the iron source was completely bioavailable. On the

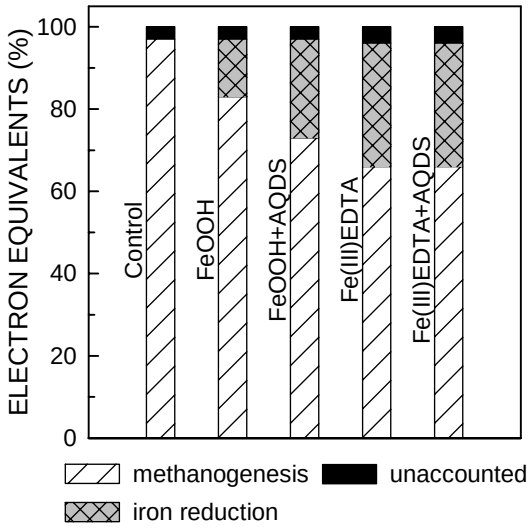


Figure 8.9. Electron equivalents balance in the cultures under iron reducing and methanogenic conditions (end of incubation period; see text).

other hand, in the cultures amended with FeOOH, the fraction of eeq used for iron reduction decreased from 30% to 14% and that for methanogenesis increased from 66% to 82%. Addition of AQDS to the FeOOH-amended culture further increased the fraction of eeq used for iron reduction (24%) and decreased that for methanogenesis (73%).

Sequential dechlorination of PCA to di- and mono-chlorinated anilines took place under fermentative/methanogenic conditions. However, when the culture was amended with a completely bioavailable iron source (i.e., Fe(III)EDTA or Fe(III)citrate), PCA dechlorination did not take place until all of the Fe^{3+} was reduced. In natural anoxic environments, several biogeochemical redox processes take place sequentially or in parallel. As previously discussed in Chapter 2, the free energy released during the transformation of PCNB to PCA, as well as the sequential dechlorination of chlorinated

anilines, is higher than that associated with metabolic processes such as sulfate reduction or methanogenesis. Although dechlorination reactions are often more thermodynamically favorable than anaerobic metabolic processes such as sulfate reduction or methanogenesis, dechlorination in many cases takes place at the same time with other metabolic processes. It should be pointed out that the bioavailability of the electron acceptors plays a significant role in terms of the free energy that can be obtained from the various redox reactions. As mentioned previously, ferric iron is by mass the most abundant electron acceptor in anaerobic soil and sediment environments (Cornell and Schwertmann, 1996). In natural environments, poorly crystalline hydrous ferric oxide has been suggested as the principal form of ferric iron (Lovley and Phillips, 1986; 1988). Besides the surface-bound iron species, dissolved iron species are also ubiquitous components in contaminated sites and may play an important role in redox processes in subsurface environments (Lovley, 1997; Tuccillo *et al*., 1999). On the other hand, most of the iron chelates typically used in laboratory experiments do not occur as such in the natural environment and exhibit redox potentials quite different from those of ferric oxides found in nature (Straub *et al*., 2001). Previously reported redox potentials of various redox couples relevant to iron metabolism at pH 7 and 25°C are as follows (Susarla *et al*., 1997; Wilson, 1978): Fe(III)NTA/Fe(II)NTA, +385 mV; Fe(III)citrate/Fe(II)citrate, +372 mV; Fe(III)EDTA/Fe(II)EDTA, +96 mV; γ-FeOOH (lepidocrocite)/Fe^{2+}, -88 mV; α-FeOOH (goethite)/Fe^{2+}, -274 mV; α-Fe_2O_3 (hematite)/Fe^{2+}, -287; Fe_3O_4 (magnetite)/Fe^{2+}, -314 mV. These values indicate that the energy gained from chelated iron forms is significantly higher than that from the ferric oxides. Based on published reduction potentials of key redox couples of Fe^{3+}/Fe^{2+} and

organic compounds (Haderlein and Schwarzenbach, 1995), polychlorinated organic compounds can be reduced by a number of Fe^{2+}-bearing compounds. Chlorinated methanes [e.g., carbon tetrachloride (CT) and chloroform] were abiotically dechlorinated in anaerobic systems containing fine-grain iron metal (Matheson and Tratnyek, 1994). Also, CT was reduced by Fe^{2+}-containing clays and oxides (Kriegman-King and Reinhard, 1992; 1994). Among the alternative electron acceptors, nitrate is an important one and results from both natural and anthropogenic activities. As a result of the extensive use of nitrate as an agricultural fertilizer (Spalding and Exner, 1993), it is possible to find environments contaminated with both nitrate and PCNB. Therefore, it is important to understand the influence of nitrate on the biotransformation of PCNB. This topic will be addressed in Chapter 9.

In the cultures amended with Fe(III)EDTA, the iron reduction rate and extent were considerably higher than in the culture amended with FeOOH (Figure 8.8). It has been shown that synthetic chelators such as EDTA and nitrilotriacetic acid (NTA) play an important role in metal/radionuclide speciation, solubility, and mobility in surface waters and groundwater (Thomas *et al*., 1998; Urrutia *et al*., 1999). It is noteworthy that, although EDTA is very resistant to biodegradation in the environment and conventional aerobic biological systems (Bolton *et al*., 1993; Madsen and Alexander, 1985; Oviedo and Rodriguez, 2003; Pavlostathis and Morrison, 1994), some studies have shown that EDTA can be degraded under aerobic conditions by specially enriched bacterial cultures (Bucheli-Witschel and Egli, 2001; Chen *et al*., 2005; Nörtemann, 1999) and in wastewater treatment plants chronically receiving EDTA-containing effluents (van Ginkel *et al*., 1999; Kaluza *et al*., 1998; Nörtemann, 1999). Weilenmann *et al*. (2004) and

Willett and Rittmann (2003) observed that, once EDTA becomes complexed with Fe^{3+}, its biodegradation stops under aerobic conditions. However, there is very limited information on the biodegradation of EDTA under anoxic/anaerobic conditions and in most of these studies EDTA was reported as not biodegradable (Bucheli-Witschel and Egli, 2001). Allard *et al*. (1996) assessed the potential biodegradation and sediment/water partition of ^{14}C-labelled EDTA in a freshwater sediment putatively contaminated with EDTA and concluded that aerobic microbial processes were not effective in mineralizing EDTA (i.e., absence of $^{14}CO_2$). In addition, due to the low partition of EDTA into the sediment phase, anaerobic biodegradation was assumed negligible. A recent study reported Fe(III)EDTA degradation by sulfate reducing bacteria in an upflow anaerobic sludge blanket (UASB) reactor where Fe(III)EDTA was used as the sole carbon source (Tsuneda *et al*., 2003). Therefore, in the present study, more likely biodegradation of EDTA did not take place during the relatively short incubation periods and in the presence of easily biodegradable carbon sources (i.e., acetate, methanol).

In one of the control cultures amended with 25 mM Na_2EDTA, culture activity was not observed in terms of gas production, acetate consumption or PCA dechlorination during 60 days of incubation. Therefore, EDTA had an inhibitory effect on the dechlorinating and methanogenic populations. In contrast, the presence of iron in the Fe(III)EDTA-amended cultures eliminated the inhibitory effect of EDTA, more likely because of binding with Fe^{3+} as well as Fe^{2+} resulting from iron reduction. Straub *et al*. (2001) suggested that the inhibitory effect of EDTA could be due to its interaction with divalent cations, such as Ca^{2+} or Mg^{2+}, causing an imbalance in the supply of these ions. Such interactions may be detrimental, especially for Gram-negative bacteria, which

depend on such cations in their outer membranes. In addition to EDTA toxicity, a slight inhibition in terms of methane production was also observed in the culture amended with 50 mM NaCl, but dechlorination of PCA was not affected. In a previous study, sodium concentrations ranging from 150 to 240 mM were moderately inhibitory and 347 mM were strongly inhibitory to methanogens at mesophilic temperatures (McCarty, 1964), which can explain the slight inhibition of methanogenesis in the culture amended with 50 mM NaCl in the present study (Figure 8.7).

Amendment of the cultures with Fe(III)EDTA resulted in increased ORP, and methanogenesis was not observed until more than 90% of the Fe(III)EDTA was reduced (Figure 8.7). At the end of the incubation period the ORP of these cultures decreased due to the exhaustion of Fe^{3+}. In contrast, although Fe(III)citrate provided bioavailable iron, simultaneous methanogenesis and iron reduction took place. Fetzer and Conrad (1993) found no inhibition of redox potential on methanogenesis at ORP values below +420 mV. Therefore, inhibition of methanogenesis due to the redox potential is unlikely in all of the cultures used in the present study. Notably, it was observed that methane production was lower in the culture amended with both FeOOH and AQDS compared with that of the culture amended with only FeOOH, which is directly related to the bioavailability of iron. Similar to our results, He and Sanford (2003) reported inhibition of 2-chlorophenol dechlorination by *Anaeromyxobacter dehalogenans* strain 2CP-C when the iron source was soluble (ferric pyrophosphate), whereas insoluble FeOOH did not have a significant effect on dechlorination. van Bodegom *et al*. (2004) reported direct inhibition of methanogenesis in the presence of ferric iron, and found that methanogens grown on H_2/CO_2 were more sensitive to ferric iron than methanogens grown on acetate.

Electron equivalence calculations (Figure 8.9) showed that when the iron source was completely bioavailable (i.e., Fe(III)EDTA), iron reduction was favored over methanogenesis and dechlorination. Nevertheless, when the iron source was less bioavailable (i.e., FeOOH), methanogenesis, iron reduction, as well as dechlorination took place simultaneously. Addition of AQDS to the culture amended with FeOOH increased the iron reduction rate and extent and decreased the rate and extent of both methanogenesis and dechlorination. Similar to our findings, Lovley *et al*. (1996) reported the acceleration of the reduction of amorphous Fe(III) oxyhydroxide at a very low concentration of AQDS (100 μM). The humic acid analog AQDS, which serves as an electron shuttle, provides the means for Fe^{3+} reducers to access insoluble Fe^{3+} compounds (Lovley *et al*., 1996). Complete iron reduction was not observed in cultures amended with FeOOH (Figure 8.8B). Similarly, it has been shown that even the most available iron oxide -- amorphous hydrous ferric oxide -- cannot be totally reduced microbially (Lovley and Phillips, 1986; Roden and Zachara, 1996).

As previously discussed, PCA is the first product of PCNB abiotic and biotic transformation under anoxic/anaerobic conditions (Chapter 5; Klupinski *et al*., 2004), and the sequential dechlorination of PCA was observed under fermentative/methanogenic conditions. However, under iron reducing conditions, especially when the iron source was completely bioavailable (i.e., Fe(III)EDTA or Fe(III)citrate), PCA dechlorination did not take place. As discussed in Chapter 2, PCA has a lower aqueous solubility (0.025 mg/L at 25°C) and a higher tendency to partition into natural organic matter (log K_{ow} = 5.08) compared to PCNB (0.44 mg/L at 25°C; log K_{ow} = 4.64). Because of its higher tendency to partition into natural organic matter, PCA has a higher bioconcentration

factor (1000) compared to that of PCNB (750)(U.S. EPA, 2003) and is expected to bioaccumulate in the food chain. Therefore, PCA may become more toxic because of its higher potential for bioaccumulation in the food chain, especially in environments where PCA dechlorination either does not take place or proceeds at a very low rate. Formation and persistence of PCA in anoxic natural environments, where dechlorination of PCA does not take place, may pose a potential long-term environmental risk.

8.4. Summary

The effect of iron reduction on the microbial reductive transformation of PCNB, was investigated with a mixed, methanogenic culture enriched from a contaminated sediment. Fe(III)EDTA, Fe(III)citrate (completely bioavailable) and FeOOH (less bioavailable) were used as the iron source. PCNB was transformed to PCA, but dechlorination of PCA and methanogenesis were not observed in cultures amended with Fe(III)EDTA until all of the added Fe^{3+} was reduced to Fe^{2+}. Although PCA dechlorination did not take place, low rate methanogenesis was observed simultaneously with iron reduction in the culture amended with Fe(III)citrate. In contrast, both methanogenesis and PCA dechlorination took place at the same time with iron reduction in the same mixed, methanogenic culture amended with FeOOH, but at a lower rate as compared to the Fe^{3+}-free control culture. Addition of anthraquinone 2,6-disulfonate (AQDS) to the culture amended with FeOOH resulted in a higher iron reduction rate, as compared to cultures devoid of AQDS, and a lower rate of both PCA dechlorination and methanogenesis. Therefore, the reductive dechlorination of PCA is adversely impacted under conditions favoring high iron reduction rates. The interaction of these potentially

competing processes (i.e., iron reduction, methanogenesis, and dechlorination) can significantly influence the environmental fate of PCNB and its transformation products, especially in soil and sediments. The results of this research have significant implications relative to the fate and biotransformation of PCNB and PCA under iron reducing conditions encountered in most anaerobic soil and sediment environments.

CHAPTER 9

NITRATE REDUCTION AND PENTACHLORONITROBENZENE BIOTRANSFORMATION

9.1. Introduction

The biotransformation potential and kinetics of PCNB under methanogenic and iron reducing conditions is well documented (Chapters 5, 6, 7, and 8). Aside from carbonate (methanogenesis) a wide variety of alternative electron acceptors can also be used by microorganisms. Nitrate and sulfate are alternative electron acceptors commonly considered for anaerobic subsurface processes (i.e., nitrate and sulfate reduction) and bioremediation applications.

Nitrate is a common electron acceptor in the environment, resulting from many agricultural and/or industrial activities. In both Europe and the U.S. the nitrate concentration in groundwater samples in agricultural areas has been reported to exceed the threshold value for drinking water recommended by the World Health Organization (50 mg NO_3^-/ L)(Kraft and Stites, 2003; Laegreid *et al.*, 1999; Postle, 1999). Due to its high mobility, nitrate may move easily in anoxic/anaerobic soil and sediment environments where microbial reductive transformation of organic contaminants, including PCNB, takes place. Relatively few studies exist on the effect of nitrate reduction on the reductive biotransformation of hydrophobic organic compounds, even though process interactions may have a detrimental effect on the rate and extent of

contaminant biotransformation. Inhibition of the biotransformation of halogenated organic compounds in the presence of alternative electron acceptors has been previously reported (Milligan and Häggblom 1999; Genthner *et al.*, 1989; Häggblom *et al.*, 1993; Picardal *et al.*, 1995; Yonezawa *et al.*, 1994; Chen *et al.*, 2002). The reasons for the observed dechlorination inhibition has been linked to preferential use of alternative electron acceptors instead of chlorinated compounds, competition for electron donors, and direct inhibition of enzymes involved in the reductive dechlorination (Dolfing and Beurskens, 1995; Miller *et al.*, 1997; Magnuson *et al.*, 1998; Gerritse *et al.*, 1999; Luijten *et al.*, 2004). Several researchers have previously reported biotransformation of halogenated organic compounds under denitrifying conditions (Bae *et al*., 2002, 2004; Gerritse *et al*., 1999; Häggblom and Young, 1999; Coshigano *et al*, 1994; Petersen *et al*., 1994; Sherwood *et al*., 1996; Freedman *et al*., 2004). Although there are denitrifying microorganisms that can degrade halogenated organics, a decrease in the denitrification activity in the presence of nitroaromatic contaminants has been previously observed (Fuller and Manning, 1998; Siciliano *et al*., 2000). Because of the increasing problem of nitrate pollution worldwide (Prasad, 1999), the occurrence of nitrate- and PCNB-contaminated sites should be expected.

In view of the fact that nitrate is a widespread alternative electron acceptor, especially in agricultural fields, its effect on the reductive biotransformation of PCNB needs to be investigated. To our knowledge, the biotransformation potential of PCNB under nitrate reducing conditions has not been previously investigated. The objectives of the research reported here were to: a) assess the effect of nitrate reduction on the biotransformation of PCNB in a mixed, fermentative/methanogenic culture; b) to assess

the biotransformation potential of PCNB in a denitrifying culture derived from a contaminated sediment; and c) to investigate possible inhibitory effects of PCNB on enriched denitrifying cultures.

9.2. Materials and Methods

9.2.1. Nitrate Reduction and PCNB Biotransformation Assay

The effect of nitrate reduction on the biotransformation of PCNB was investigated in a batch assay with the mixed, fermentative/methanogenic, PCNB-biotransforming culture. Six culture series were prepared with an initial PCNB concentration of 3 μM and nitrate concentrations of 0, 10, 20, 50, 100, and 200 mg N/L. Two control cultures were prepared with initial nitrate concentrations of 10 and 200 mg N/L, without PCNB. The assay was conducted using triplicate 160 mL serum bottles which were flushed with helium. The initial pH value and biomass concentration in the cultures were 6.95 ± 0.2 and 307 ± 11 mg POC/L (mean ± standard deviation; n = 8), respectively. All cultures were amended with the same initial glucose (333 mg/L), yeast extract (17 mg/L), and PCNB (3 μM) dissolved in methanol (1535 mg/L). The same amount of glucose, yeast extract, and methanol was also added to the PCNB-free control cultures. The amount of the electron donor provided (40 meeq) was in excess of the required eeq for the highest nitrate addition (200 mg NO_3^--N/L; 8.6 meeq). All serum bottles were incubated in a 22ºC constant temperature room and were manually shaken once a day. Liquid and gas samples were periodically taken to monitor chlorinated compounds, nitrate, nitrite, pH, volatile fatty acids (VFAs), and gas composition.

9.2.2. Denitrifying Culture Development

Two denitrifying enrichment cultures were developed from a contaminated estuarine sediment obtained from Bayou d'Inde, a tributary of the Calcasieu River near Lake Charles, LA, USA. The cultures were initiated by diluting 80 g of sediment in 1.5 L of mineral media (Chapter 3, Table 3.2) in helium-flushed, 2 L glass flask reactors, capped with Teflon-lined stoppers. Both cultures were fed at the beginning of each 7-day feeding cycle with glucose, and yeast extract resulting in initial concentrations of 333 and 17 mg/L, respectively. The PCNB-free control culture was fed with pure methanol (53 mg/L) whereas the PCNB-acclimated culture was amended with 0.09 μM PCNB dissolved in methanol (53 mg/L). The nitrate concentration was kept in excess (> 100 mg N/L) in both cultures which were incubated in the dark in a 22°C constant temperature room. After several feeding cycles, culture aliquots (100 mL) were transferred into He-flushed, 2 L glass flask reactors and diluted with 1.5 L mineral media (second generation cultures). Finally, after several weekly feeding cycles, similar culture transfers took place in 1.5 L mineral media (third generation cultures). The retention time of the enrichment cultures was 42 days. The steady-state biomass concentration and pH of the sediment-free, PCNB-acclimated and PCNB-free control cultures were 240 ± 65 and 264 ± 54 mg POC/L, 7.0 ± 0.1 and 7.8 ± 0.2, respectively. The batch assay reported here was performed with the sediment-free, third generation denitrifying cultures.

9.2.3. Effect of PCNB on Denitrifying Cultures

The effect of PCNB on the denitrifying enrichment cultures was investigated in an assay using 160 mL serum bottles which were sealed with Teflon-lined septa and flushed

with helium gas. Two culture series (PCNB-acclimated and PCNB-free control) were amended with an initial PCNB concentration of 13 μM dissolved in methanol (PCNB-acclimated and PCNB-amended control). Another control culture was also set up with the PCNB-free culture without any PCNB amendment (PCNB-free control). The initial amount of carbon sources in all serum bottles was as follows: glucose, 666 mg/L; yeast extract, 34 mg/L; and methanol, 1535 mg/L. The chemical oxygen demand (COD) of the glucose and methanol added to each serum bottle was 710 and 2300 mg/L, respectively, and the initial nitrate concentration was 60 mg N/L. The initial biomass concentration was 230 ± 35 mg POC/L (mean ± stand. dev.; n = 3). All serum bottles were incubated in a 22ºC constant temperature room in the dark and mixing was provided with a rotating tumbler (4 rpm). Liquid and gas samples were periodically taken to monitor chlorinated compounds, pH, nitrate, nitrite, volatile fatty acids (VFAs), and gas composition.

9.3. Results and Discussion

9.3.1. Effect of Initial Nitrate Concentration on PCNB Biotransformation

The effect of nitrate reduction on the PCNB biotransformation was investigated with the mixed enrichment culture at an initial PCNB concentration of 3 μM, which is not inhibitory to the PCNB-biotransforming fermentative/methanogenic culture. The pH values at the end of the incubation period in the 3 μM PCNB and nitrate amended cultures (initial nitrate concentration in parenthesis, mg N/L) were as follows: 7.08 (0), 7.13 (10), 7.17 (20), 7.45 (50), 7.51 (100), and 7.80 (200). The final pH values in the two PCNB-free control cultures amended with an initial nitrate concentration of 10 and 200 mg N/L were 6.91 and 7.51, respectively. The final pH values show that denitrification

resulted in a significant pH increase as a result of alkalinity production (i.e., H^+ removal from the media) as expected.

The biomass concentration at the end of the incubation period in the 3 μM PCNB and nitrate amended cultures (initial nitrate concentration in parentheses, mg N/L) were as follows (mean ± stand. dev.; *n*=3): 423 ± 23 (0), 420 ± 20 (10), 426 ± 20 (20), 252 ± 9 (50), 235 ± 2 (100), and 308 ± 11 (200). The final biomass concentration in the two PCNB-free control cultures amended with an initial nitrate concentration of 10 and 200 mg N/L were 428 ± 6 and 311 ± 9, respectively. Significant biomass production was observed at the end of the 34 days of incubation in the cultures amended with low initial nitrate concentrations (≤ 20 mg N/L). The final biomass concentration was significantly lower in the cultures amended with high initial nitrate concentrations (≥ 50 mg N/L) more likely due to biomass decay at the end of the long incubation period (96 days).

The rates of PCNB transformation to PCA in all nitrate-amended cultures were very similar to that in the nitrate-free control culture. More than 90% of PCNB was transformed to PCA in all culture series in less than 2 hours. The pseudo-first-order rate constant for the transformation of PCNB to PCA was 2.29 ± 0.3 d^{-1} (mean ± stand. dev.; *n* = 6) in cultures with an initial PCNB concentration of 3 μM. Thus, initial nitrate concentrations up to 200 mg N/L did not affect the rate of PCNB transformation to PCA. According to abiotic control experiments conducted with autoclaved culture media and autoclaved fermentative/methanogenic culture, the pseudo-first-order rate constants for the transformation of PCNB to PCA at initial PCNB concentrations of about 0.1 μM and 3 μM were 0.851 ± 0.004 and 1.7 ± 0.17 d^{-1} (mean ± standard error), respectively (Chapter 5). The culture media contained 67 mg/L sulfide, 28.1 mg/L (503 μM) Fe^{2+}, and

2 µg/L (1.5 nM) vitamin B_{12}, which were also the same in this assay. In the present assay, the pseudo-first-order rate constant for the transformation of PCNB to PCA (2.29 ± 0.3 d^{-1}) was very similar to the rate obtained in the autoclaved culture controls. These results indicate that, in addition to the culture media components (i.e., sulfide, Fe^{2+}, vitamin B_{12}), biotically-derived reductants and/or other factors facilitated the observed transformation of PCNB to PCA, and the presence of nitrate did not affect this transformation. However, it should be emphasized that dechlorination of PCA did not occur in any of the abiotic controls (Chapter 5).

PCA was dechlorinated to 3,5-DCA and 2,5-DCA in the control culture under fermentative/methanogenic conditions in 15 days of incubation without any delay (Figure 9.1A). PCA sequentially dechlorinated to DCAs (mainly 2,5-DCA) in the culture amended with 10 mg N/L (Figure 9.1B). The dechlorination pattern observed in this culture was very similar to the dechlorination pattern in the nitrate-free control culture. Accumulation of TrCAs (predominantly 2,3,5- and 2,4,5-TrCA) was observed in the culture amended with 20 mg N/L at the end of 96 days of incubation (Figure 9.1C). Partial dechlorination of PCA resulted in the production of predominantly 2,3,5,6-TeCA at the beginning of the incubation in the culture amended with 50 mg N/L (Figure 9.1D). PCA dechlorination stopped in this culture after 5 days of incubation when most of the NO_3^- was reduced to NO_2^- (Figure 9.1D and 9.2D). PCA dechlorination resumed at 16 days of incubation when all the NO_2^- was removed, but stopped again at 50 days of incubation because of the accumulation of intermediate denitrification products (i.e., N_2O) as discussed below. Very slow and partial PCA dechlorination to 2,3,5,6-TeCA was observed only during the first 2 days of incubation in the cultures amended with 100 and

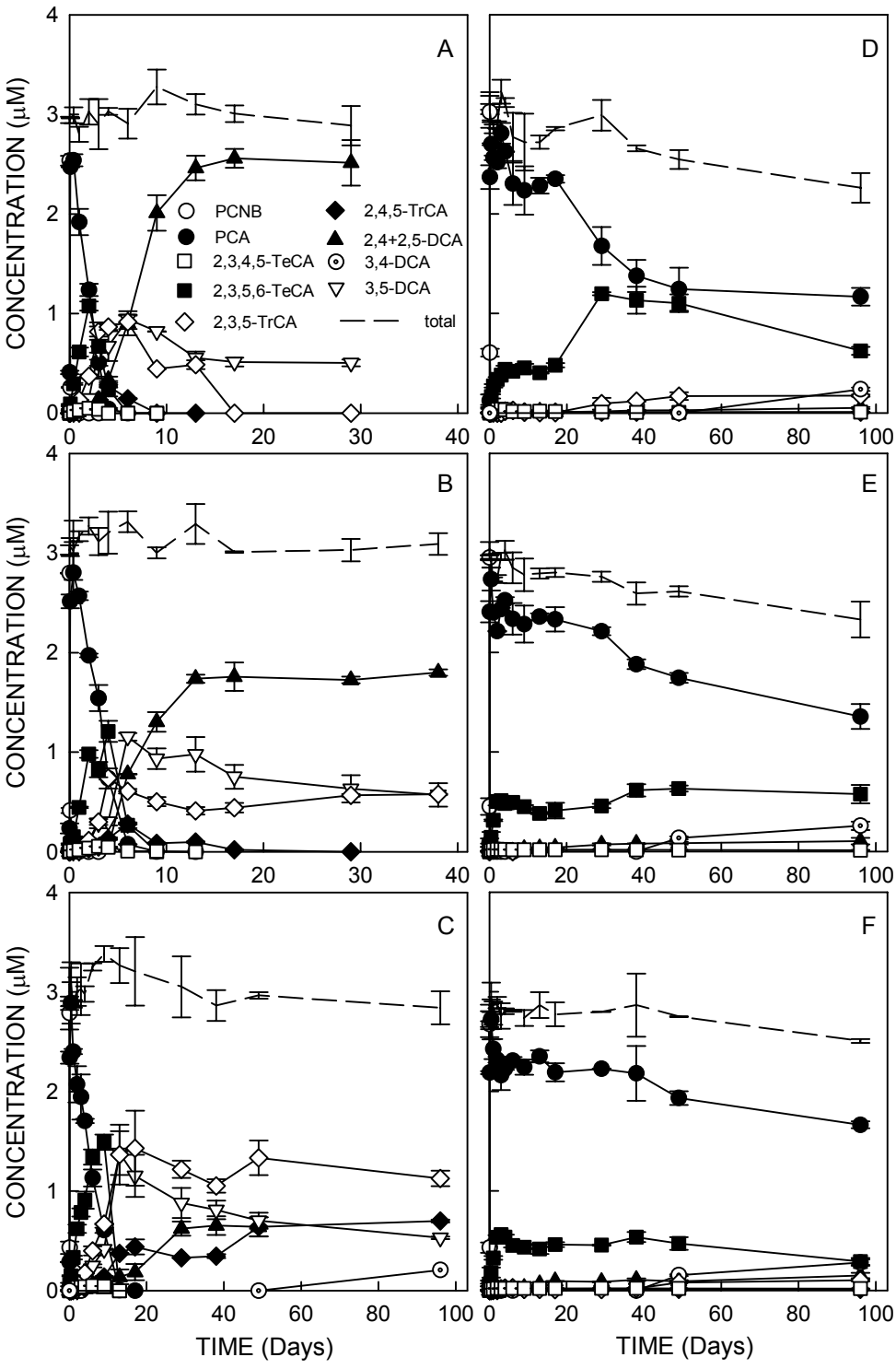


Figure 9.1. Effect of initial nitrate concentration on the PCA sequential dechlorination rate and product distribution (Initial nitrate concentration, mg N/L: 0 (A), 10 (B), 20 (C), 50 (D), 100 (E), and 200 (F))(Error bars represent mean values ± one standard deviation).

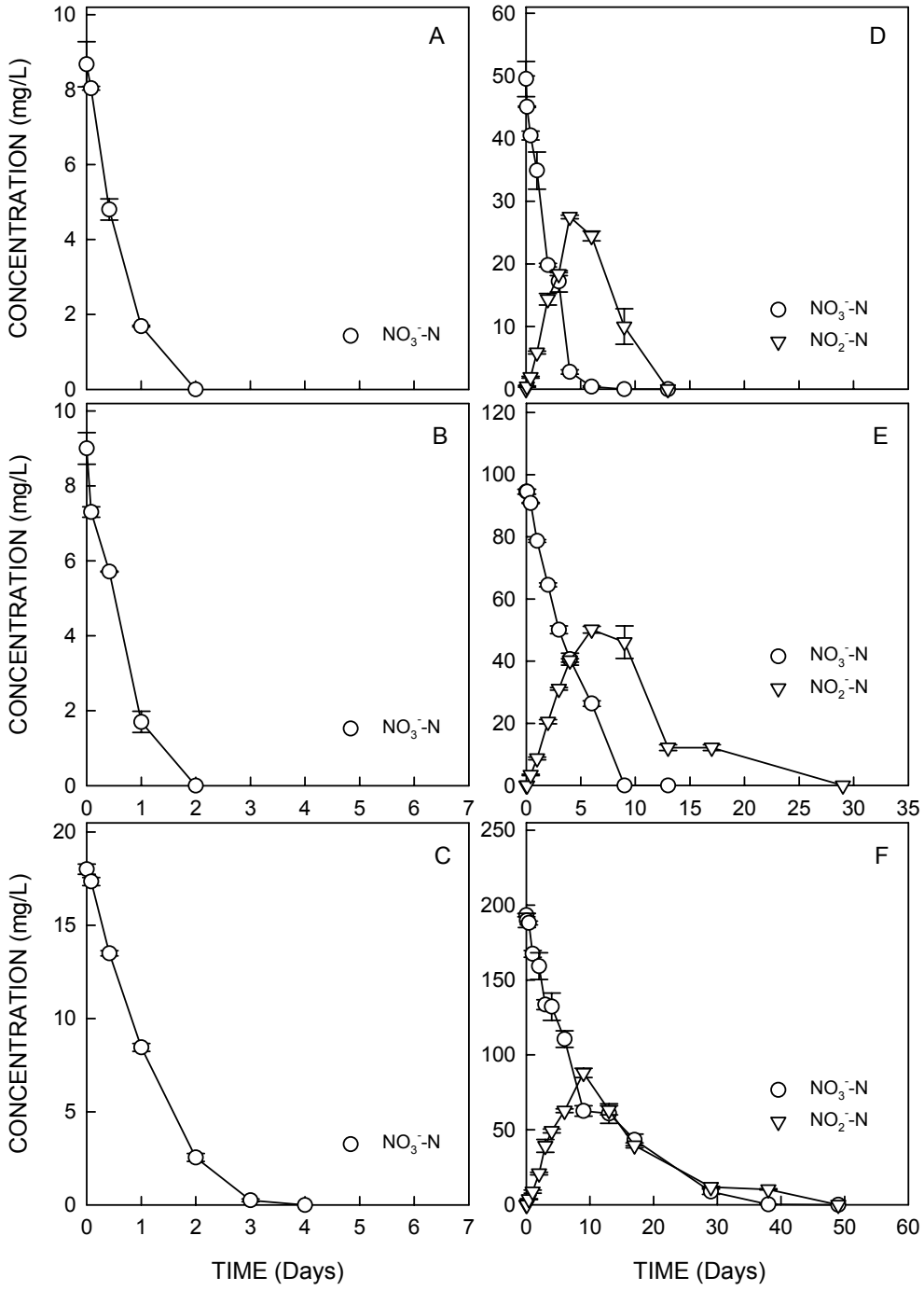


Figure 9.2. Comparison of nitrate reduction, nitrite production and reduction in the PCNB-free, 10 mg NO_3^--N/L amended culture (A), 3 µM PCNB and nitrate-amended cultures with initial nitrate (mg N/L): 10 (B), 20 (C), 50 (D), 100 (E), and 200 (F)(Error bars represent mean values ± one standard deviation).

200 mg N/L (Figure 9.1E and 9.1F) as a result of significant nitrite production (Figure 9.2E and 9.2F).

Figure 9.2 shows the nitrate and nitrite concentration profiles in the cultures amended with 10 to 200 mg N/L. Nitrate reduction took place in all cultures without any lag period. Nitrate was completely removed in 2 days in the cultures amended with 10 mg NO_3^--N/L, with and without PCNB. Thus, the presence of 3 μM PCNB did not affect the nitrate reduction rate (Figure 9.2A and 9.2B). As the initial nitrate concentration increased, a progressively longer incubation time was required for the complete removal of nitrate. Nitrite was not detected in the cultures amended with 10 and 20 mg NO_3^--N/L. However, significant nitrite accumulation was observed in the cultures amended with 50 mg N/L and higher. The pseudo-first-order and initial volumetric rate constants for the nitrate removal as a function of initial nitrate concentration are shown in Table 9.1. It was also confirmed that the presence of 3 μM PCNB did not affect the nitrate reduction rate at initial nitrate concentrations of 10 and 200 mg N/L (Table 9.1). The initial volumetric nitrate removal rates were used to evaluate the nitrate reduction kinetics in the enrichment culture. The initial volumetric nitrate removal rate (V_0) increased monotonically but non-linearly with increasing initial nitrate concentration (Table 9.1 and Figure 9.3). Based on a non-linear regression of the experimental data according to equation 9-1, the following parameter values were obtained (mean ± standard error): k' = 18.1 ± 0.2 mg N/L·d and K_S = 16.9 ± 0.8 mg N/L (r^2 = 0.999).

$$V_0 = \frac{k'C_0}{K_S + C_0} \tag{9-1}$$

Table 9.1. Pseudo-first-order rate constants (k_{obs}) and initial volumetric rate Constants (V_0) for the nitrate removal in the mixed, sediment-free culture as a function of initial nitrate concentration

Initial NO_3^- Concentration (mg N/L)	Initial PCNB Concentration (μM)	k_{obs} (day^{-1})[a]	r^2	V_0 (mg NO_3^--N/L·d)[a]	r^2
10	-	1.59 ± 0.13	0.994	9.42 ± 0.12	0.999
10	3	1.44 ± 0.25	0.975	7.00 ± 0.80	0.881
20	3	0.88 ± 0.07	0.992	9.66 ± 0.36	0.996
50	3	0.45 ± 0.05	0.979	13.29 ± 2.05	0.929
100	3	0.24 ± 0.02	0.983	15.65 ± 1.42	0.991
200	3	0.099 ± 0.005	0.992	16.74 ± 2.01	0.989
200	-	0.076 ± 0.006	0.979	16.77 ± 2.83	0.982

[a] Mean ± standard error (n ≥ 4).

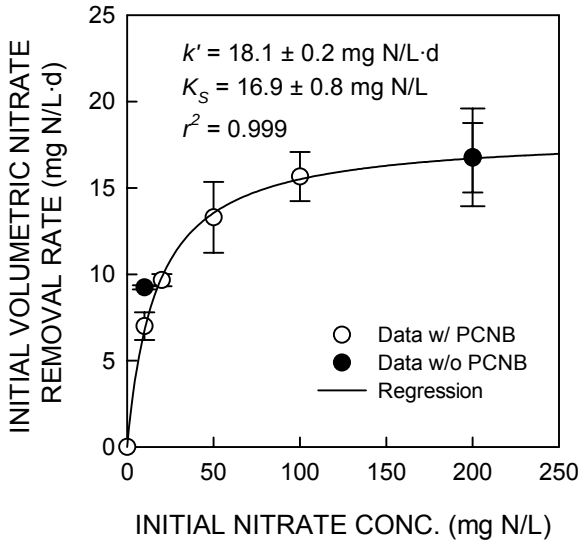


Figure 9.3. Non-linear fit of initial volumetric nitrate reduction rate data as a function of initial nitrate concentration (Error bars represent mean values ± one standard deviation) (Line is model prediction for cultures amended with PCNB).

where: V_0 is the initial volumetric nitrate removal rate (mg N/L·d); k' is the maximum nitrate removal rate (mg N/L·d); C_0 is the initial nitrate concentration (mg N/L); K_S is the half-velocity coefficient (mg N/L).

Cumulative methane production in the eight culture series over the 96 days of incubation is shown in Figure 9.4A. The maximum methane production rate was observed in the nitrate-free control culture. Methane production started after 2 days of a lag period in the culture amended with 10 mg NO_3^--N/L. The methane production rate and extent was the same in the cultures amended with 10 mg/L NO_3^--N, with and without PCNB. Methane production started after 3 days of a lag period in the culture amended with 20 mg/L NO_3^--N. These results confirm that the presence of nitrate up to 20 mg N/L did not inhibit methanogenesis. However, methane production was not observed in the cultures amended with 50, 100, or 200 mg NO_3^--N/L and 3 μM PCNB, as well as in the culture amended with only 200 mg NO_3^--N/L over the 96 days of incubation (Figure 9.4A).

Significant N_2O accumulation was observed in the cultures amended with 50, 100, or 200 mg N/L and 3 μM PCNB, during the incubation period (Figure 9.4C). However, most N_2O was reduced to N_2 after 40 days of incubation in the PCNB-free culture amended with 200 mg N/L (Figure 9.4B and 9.4C). Therefore, at relatively high initial nitrate concentrations, the presence of 3 μM PCNB was inhibitory to denitrifying microorganisms, not for the NO_3^- and/or NO_2^- reduction but for the NO and/or N_2O reduction. Accumulation of NO was not observed in any culture during the incubation. At the end of the incubation period, the following ammonia values (mg NH_4^+-N/L) were measured in the cultures amended with 3 μM PCNB and nitrate (mg N/L in parenthesis):

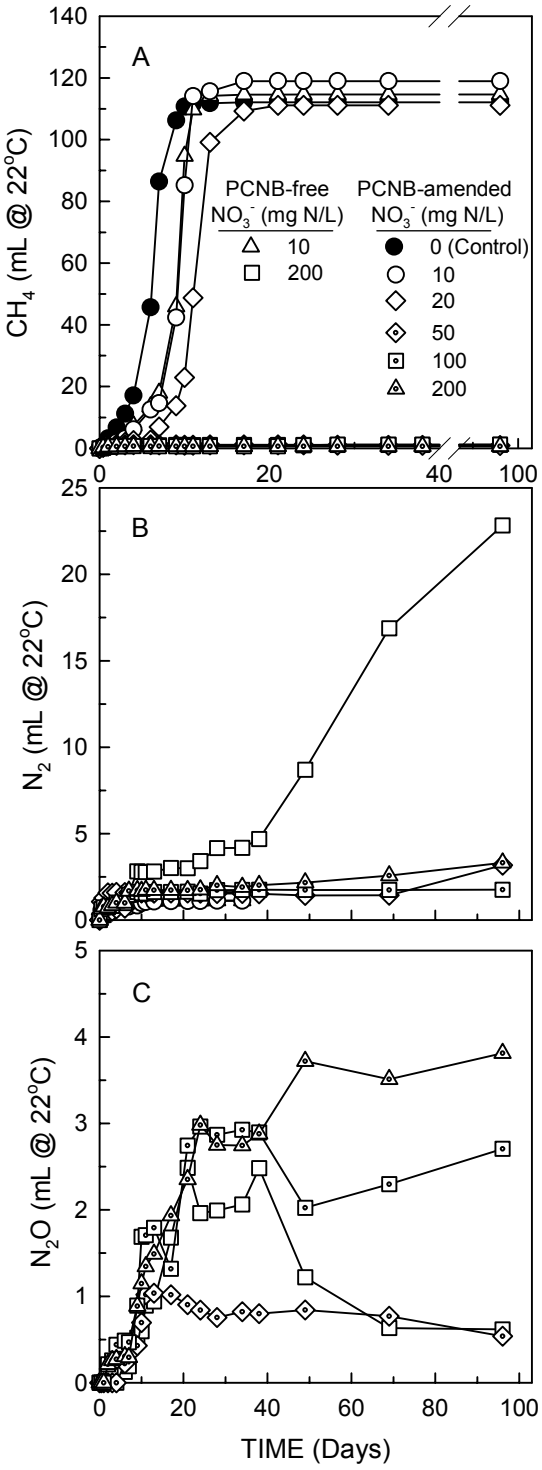


Figure 9.4. Effect of initial nitrate concentration and PCNB on methane production (A), nitrogen production (B), and N_2O production and consumption (C).

36.3 (0), 41.8 (10), 43.2 (20), 92.6 (50), 80.4 (100), 74.3 (200). Thus, an increase in the ammonia concentration was observed with increasing initial nitrate concentration. The ammonia concentration measured in the nitrate-free culture was remaining ammonia contributed by the culture media.

VFAs profiles of all cultures are shown in Figure 9.5. Under fermentative/methanogenic conditions the propionate concentration was slightly higher than the acetate concentration in the control culture, whereas the acetate concentration was higher than the propionate concentration in all nitrate-amended cultures. A slight increase of acetate was observed in the culture amended with 10 mg N/L as compared to the culture under fermentative/methanogenic conditions. The VFAs production and consumption profiles were very similar in the cultures amended with 10 and 20 mg NO_3^--N/L. Inhibition of methanogenesis (Figure 9.4A) resulted in the accumulation of VFAs in the culture amended with 50 mg NO_3^--N/L (Figure 9.5D). It appears that fermenters were not inhibited to the extent methanogens were in the culture amended with 50 mg NO_3^--N/L. After 8 days of incubation, a transient inhibition of fermenters was observed in the culture amended with 100 mg NO_3^--N/L, more likely because of accumulation of intermediate nitrate reduction products (NO_2^-, NO, N_2O)(Figure 9.5E). Although fermentation resumed in this culture after 20 days of incubation, methanogenesis was completely inhibited. VFAs production and consumption was observed during the first 10 days of incubation in the 200 mg NO_3^--N/L amended cultures, but both fermentation and methanogenesis were completely inhibited for the remainder of the incubation period in the cultures amended with and without PCNB (Figure 9.5F)(data not shown for the PCNB-free culture due to its similarity to the PCNB-amended culture). At the end of 96

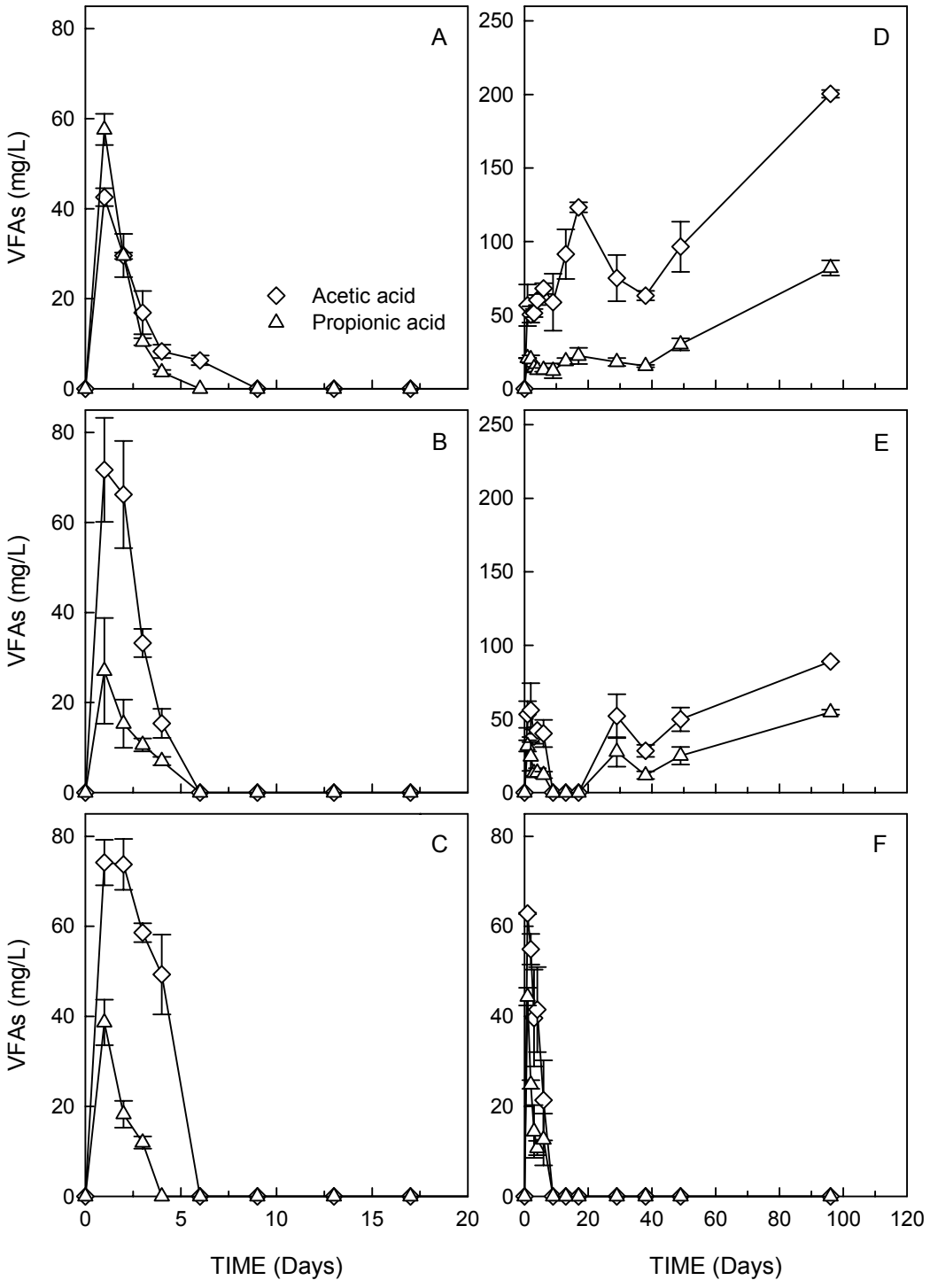


Figure 9.5. Comparison of VFAs production and consumption in the nitrate-free (control) culture (A), and the nitrate-amended cultures with initial nitrate (mg N/L): 10 (B), 20 (C), 50 (D), 100 (E), and 200 (F) (All cultures were amended with 3 μM PCNB)(Error bars represent mean values ± one standard deviation).

days of incubation, 120 ± 9, 150 ± 48, and 100 ± 11 mg/L methanol was measured in the cultures amended with 100 and 200 mg NO_3^--N/L and PCNB, and 200 mg NO_3^--N/L without PCNB, respectively.

Nitrate or the denitrification intermediates NO_2^-, NO and N_2O have been previously reported as potent inhibitors of methanogenesis (Balderston and Payne, 1976; Klüber and Conrad, 1998a; 1998b). The presence of 1.4 mg/L nitrite-N, 14-28 μg/L NO-N, or 0.85 mg/L N_2O-N was sufficient to completely inhibit methanogenesis (Balderston and Payne, 1976; Klüber and Conrad, 1998b; Clarens *et al.*, 1998). Clarens *et al.* (1998) showed that the acetoclastic methanogen *Methanosarcina mazei* was inhibited by NO_2^- and N_2O. They also showed that methane production by this species stopped when the denitrifying species *Pseudomonas stutzeri* was added to this culture which then reduced nitrate to nitrous oxide under the conditions of their study. Chidthaisong and Conrad (2000) reported that glucose-fermenting microbial populations were inhibited by nitrate and/or its denitrification products. NO is a particularly toxic intermediate because of its reactivity with heme and nonheme iron-containing proteins (Ye *et al.*, 1994). In the present assay, after complete nitrate reduction, methanogenesis took place in the cultures amended with up to 20 mg N/L, but methane production was not observed in all cultures amended with 50 mg NO_3^--N/L and higher, where denitrification intermediates (NO_2^- and N_2O) were detected at significant concentrations. Complete inhibition of methanogenesis in the presence of denitrification intermediates (NO_2^- and N_2O) is in agreement with previous studies carried out with methanogenic species (Klüber and Conrad, 1998b; Clarens *et al.*, 1998). On the other hand, as discussed in Chapter 6, although methanogens in the mixed, enrichment culture were completely inhibited by 2-bromoethanesulfonate

(BES), an inhibitor of methanogenesis, the rate of PCNB transformation to PCA and its dechlorination pathway were not affected, indicating that methanogens were not directly involved in the sequential dechlorination of PCA.

It has been reported that nitrate inhibits the reductive dehalogenation activity of anaerobic microbial communities (Milligan and Häggblom, 1999; Genthner *et al.,* 1989; Häggblom *et al.*, 1993; Picardal *et al.*, 1995; Middeldorp *et al.,* 2005). Complete inhibition of the reductive dechlorination of hexachlorobenzene (HCB) was observed in anaerobic sediment microcosms amended with 30 mM nitrate (Chen *et al.*, 2002). Pentachlorophenol dechlorination by a mixed, methanogenic culture was inhibited by the addition of 20 mM nitrate (Chang *et al.*, 1996). Nelson *et al.* (2002) reported that complete removal of nitrate was necessary for the reductive dechlorination of perchloroethene (PCE) even though excess H_2 was provided to the culture to eliminate any H_2 limitation. These researchers also observed inhibition of PCE dechlorination in microcosms amended with high N_2O (100 μM) aqueous concentrations. Similarly, in the present study, sequential dechlorination of PCA was inhibited when the initial nitrate concentration reached levels ≥50 mg N/L, where significant N_2O accumulation was observed.

Sulfide, which was used as a reducing agent in the culture media of the present study (67 mg/L S) has been shown to inhibit N_2O reduction during denitrification (Tam and Knowles, 1979; Sørensen *et al.*, 1980). Schönharting *et al.* (1998) reported that N_2O reduction was significantly inhibited in the presence of sulfide (119 mg/L S) compared to the NO_3^- and NO_2^- reduction. Brunet and Garcia-Gil (1996) suggested that sulfide inhibition of NO and N_2O reductases resulted in the production of ammonia (NH_4^+). On

the basis of experiments conducted with anaerobic freshwater sediments, Brunet and Garcia-Gil (1996) reported that the initial concentration of free sulfide was a factor which determined the type of nitrate reduction. At extremely low concentrations of free S^{2-}, nitrate was reduced via denitrification whereas at higher free S^{2-} concentrations, dissimilatory nitrate reduction to ammonia (DNRA) and incomplete denitrification to gaseous nitrogen oxides took place. Yin *et al.* (2002) reported that 56.4% of the added nitrate was converted to NH_4^+ and organic N in soil samples treated with L-cystein, which is a sulfide-containing reducing agent. Our observations of N_2O accumulation in the cultures amended with an initial nitrate concentration range of 50 to 200 mg N/L are consistent with previously reported studies with respect to the inhibitory effect of sulfide on the denitrification process. In addition, partial ammonia production was observed in these cultures at the end of the incubation.

It has been reported that VFAs stimulate complete reductive dechlorination of chloroethenes to ethene, either as direct substrates for the dechlorinating bacteria or via H_2 formed during VFA degradation (Lu *et al.*, 2002). However, in the present study, although there was significant VFAs production in the cultures amended with 50 to 100 mg NO_3^--N/L, PCA dechlorination did not take place showing that dechlorinating species were completely inhibited more likely because of the production of intermediate denitrification products.

9.3.2. Nitrogen and Electron Balance

A nitrogen mass balance for all nitrate-amended cultures was performed at the end of the incubation period. At the end of a 34-day incubation period, 100% nitrogen

balance closure through the production of N_2 was obtained in all cultures amended with 10 and 20 mg NO_3^--N/L. At the end of a 96-day incubation period, 100% nitrogen balance closure through the production of N_2 (96%) and N_2O (4%) was obtained in the PCNB-free culture amended with 200 mg NO_3^--N/L. In contrast, partial ammonia production (10 to 35% of added NO_3^--N) was observed in the cultures amended with 50, 100, or 200 mg NO_3^--N/L and 3 μM PCNB. At the end of a 96-day incubation period, 100% nitrogen balance closure was achieved in the cultures amended with 50 and 100 mg N/L through the production of N_2 (36-55%), N_2O (10-39%), and NH_4^+ (25-35%). However, only 75% nitrogen balance closure was achieved in the culture amended with 200 mg N/L and 3 μM PCNB through the production of N_2 (34%), N_2O (31%), and NH_4^+ (10%). At the end of a 96-day incubation period, 100% nitrogen balance closure was not achieved in the culture amended with 200 mg NO_3^--N/L and PCNB, whereas 100% nitrogen balance closure was achieved in the culture amended with 200 mg NO_3^--N/L without PCNB. Therefore, in the presence of PCNB, the incomplete nitrogen balance in the culture amended with 200 mg NO_3^--N/L may be attributed to organic nitrogen production which was not measured in the present study.

Electron equivalence calculations were performed at the end of the incubation period based on the electron donor electron equivalents (eeq) added and by accounting for each process. Methane production, nitrate reduction to N_2, nitrate reduction to N_2O, nitrate reduction to NH_4^+, glucose and methanol consumption, acetate, and propionate production were expressed in terms of eeq as follows: 8 eeq/mol of methane produced, 5 eeq/mol of nitrate reduced to N_2, 4 eeq/mol of nitrate reduced to N_2O, 8 eeq/mol of nitrate reduced to NH_4^+, 24 eeq/mol of glucose and 6 eeq/mol of methanol consumed, 8

eeq/mol of acetate, and 14 eeq/mol of propionate produced. The total initial eeq available from methanol and glucose were 34.7 and 5.3, respectively. In these calculations eeq required for biomass production were not taken into account due to the very long incubation period and negligible eeq requirement for biomass production. The electron equivalents balance in all cultures is shown in Table 9.2. At the end of the 34- and 96-day incubation period, 100% eeq closure was achieved in the nitrate-free control culture and the cultures amended with 10 and 20 mg N/L, as well as the culture amended with 50 mg N/L, respectively. The eeq converted to acetate/propionate correspond approximately to 63/30% and 37/25% of the glucose and methanol eeq initially added to the cultures amended with 50 and 100 mg N/L, respectively. At the end of the 96-day incubation period, 67% eeq closure was observed in the cultures amended with 100 mg N/L. Similarly, the total electron balance at the end of the incubation period was approximately 25% of the initially added eeq in the cultures amended with 200 mg N/L. Methanol measurement at the end of incubation period showed that most of the methanol ($\geq$90%) was fermented in the cultures amended with 100 and 200 mg NO_3^--N/L. In addition, although glucose was not monitored in this assay, based on previous assays it is known that glucose was consumed very fast even in the cultures amended with sodium azide (Chapter 6). Thus, it is possible that methanol and glucose were fermented to products other than acetate or propionate (i.e., alcohols, H_2, etc.) which were not measured in the present study. These results are consistent with the observed methanogenesis and fermentation inhibition in the higher nitrate-amended culture series. It is noteworthy that the acetate and propionate eeq in the cultures amended with 50 or 100 mg N/L were higher than the provided glucose eeq. Relative to methanol,

Table 9.2. Electron equivalents balance[a]

Culture Series		N-oxides Reduction (meeq)	Acetate (meeq)	Propionate (meeq)	CH_4 Production (meeq)	Total (meeq)
Initial Nitrate (mg N/L)	Initial PCNB (μM)					
0	3	0	0	0	41	41
10	0	0.4[b]	0	0	39	39.4
10	3	0.4[b]	0	0	39	39.4
20	3	0.8[b]	0	0	36.4	37.2
50	3	2.3[c]	26.6	15.5	0	44.4
100	3	4.4[c]	11.9	10.3	0	26.6
200	3	5.1[c]	0	0	0	5.1
200	0	8.6[b]	0	0	0	8.6

[a] At the end of the incubation period; all cultures were amended with 40 meeq initial electron donor (glucose and methanol). [b] Complete reduction of nitrate was observed. [c] Complete reduction of nitrate was not observed.

methanogens and acetogens may compete for this electron donor. Methanol can be directly converted to methane by methylotrophic methanogens (Touzel *et al.*, 1985). In the presence of sufficient CO_2, methanol can also be converted to acetate by (homo) acetogens (Ljungdahl *et al.*, 1986). Aulenta *et al.*, (2002) reported complete dechlorination of tetrachloroethene to ethene in the presence of methanogenesis and acetogenesis in a methanol-fed anaerobic sediment microcosm, where acetogenesis accounted for about 72% of reducing equivalents from methanol. In the present study, when the methanogens were not active, methanol fermentation resulted in the accumulation of both acetate and propionate in the cultures amended with ≥ 50 mg NO_3^--N/L.

9.3.3. Denitrifying Enrichment Cultures

PCNB biotransformed to PCA in the sediment microcosm (first generation culture) in two days under denitrifying conditions. However, dechlorination or degradation of PCA was not observed for almost one year of incubation in the sediment-free, third generation culture (Figure 9.6). Glucose and methanol were consumed within three days in every feeding cycle. The steady-state PCA concentration of the PCNB-acclimated third generation enrichment culture was 0.35 ± 0.05 μM (mean ± stand. dev.; n = 14). Glucose and methanol consumption as well as the nitrate reduction rate and extent were similar in both the PCNB-amended control and the PCNB-acclimated denitrifying cultures. Figure 9.7 shows a typical gas composition/production profile in the third generation PCNB-free control denitrifying culture during a representative feeding cycle. Although N_2 and CO_2 were the predominant gases, a low level N_2O production and consumption was also observed in every 7-day feeding cycle. Similar gas production profiles were observed in the PCNB-acclimated culture. VFAs accumulation was not observed in both denitrifying cultures at the end of the 7-day feeding cycle.

9.3.4. PCNB Effect on Denitrification

The concentration profiles of PCNB, its biotransformation product PCA and NO_3^- during the batch biotransformation assay conducted with the PCNB-free control and PCNB-acclimated denitrifying enrichment cultures at an initial PCNB concentration of 13 μM are shown in Figure 9.8. The tested PCNB concentration (13 μM) was 37 times higher than the steady state PCA concentration (0.35 ± 0.05 μM) in the PCNB-acclimated culture. PCNB transformation to PCA started after 4 days of a lag period in the PCNB-

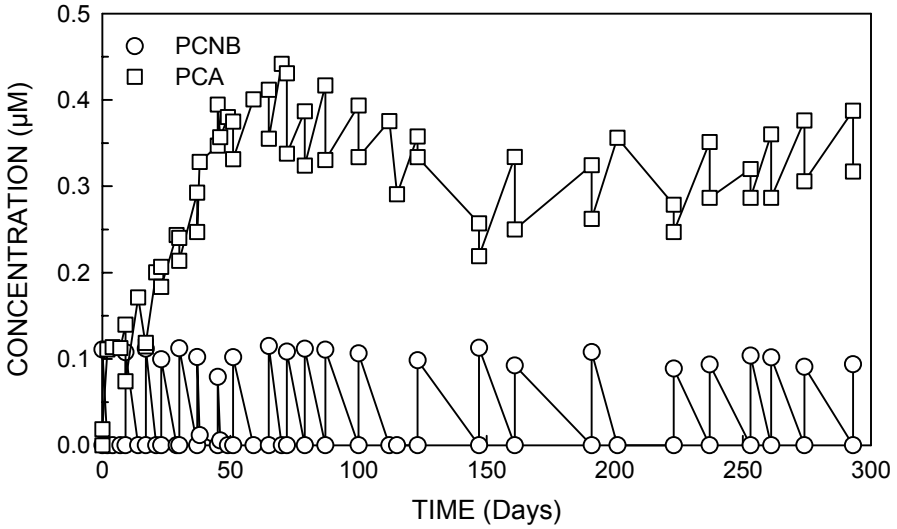


Figure 9.6. PCNB and its transformation product PCA in the PCNB-acclimated, denitrifying culture (multiple feeding cycles; third generation culture).

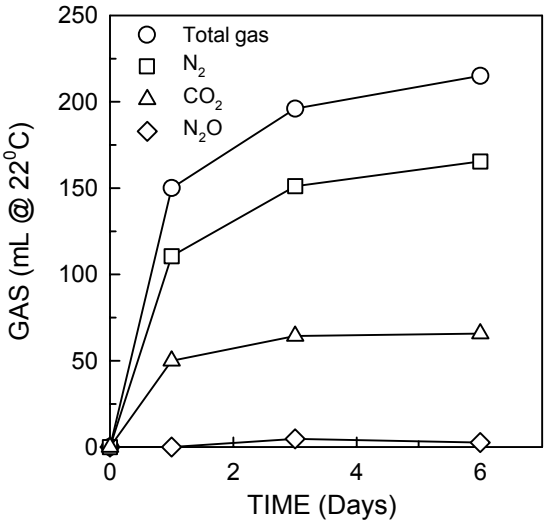


Figure 9.7. Gas production profile by the PCNB-free denitrifying culture during a typical feeding cycle (third generation culture).

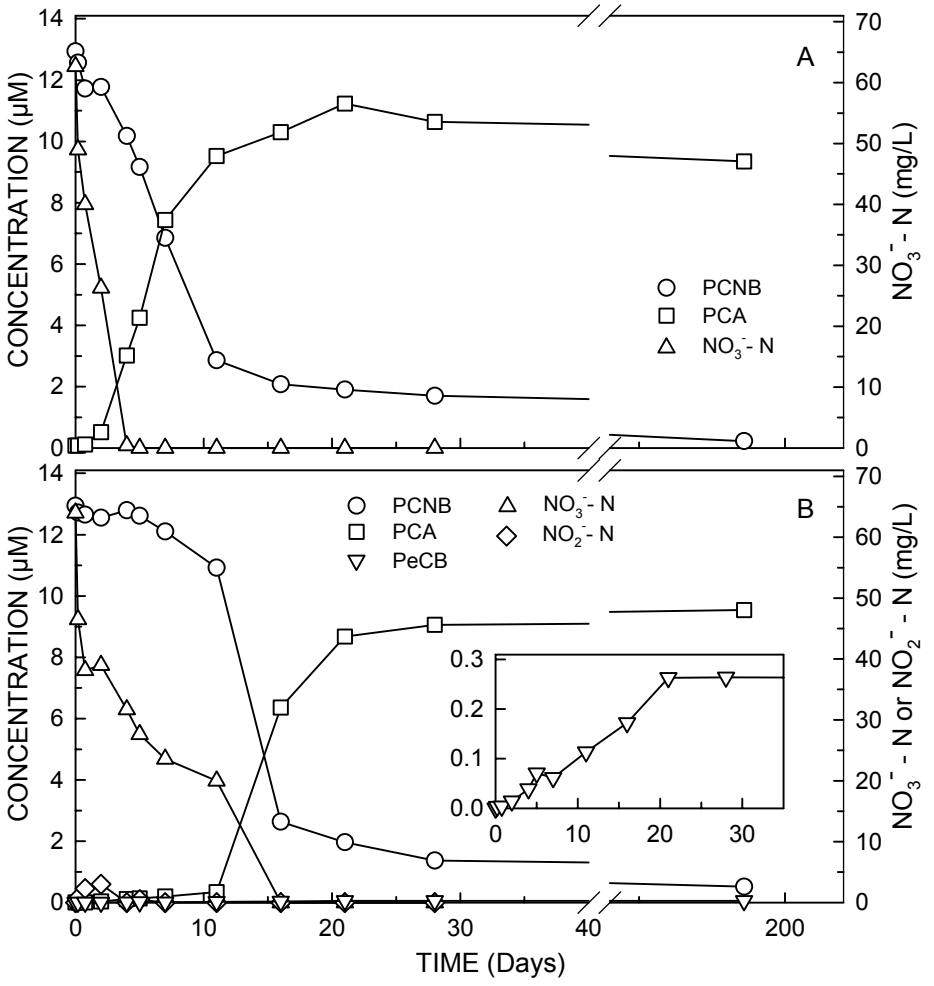


Figure 9.8. Time course of PCNB, its biotransformation product PCA, nitrate and nitrite during a batch biotransformation assay conducted with the PCNB-acclimated (A) and the PCNB-amended control (B) enrichment cultures (Both cultures fed with 13 μM PCNB and 60 mg NO_3^--N/L).

acclimated culture, whereas in the PCNB-amended control culture, PCNB transformation started after 8 days of a lag period. More than 90% of PCNB was transformed to PCA in the PCNB-acclimated and PCNB-amended control cultures within 30 days. Dechlorination of PCA was not observed in either culture over a 190-day incubation period. PCNB biotransformation was not observed in both cultures until the NO_3^- concentration decreased to about 20 mg N/L. The pseudo-first-order rate constants of the PCNB to PCA transformation in the PCNB-acclimated and PCNB-amended control cultures at an initial PCNB concentration of 13 μM PCNB were 0.097 ± 0.009 (r^2 = 0.970) and 0.058 ± 0.013 (r^2 = 0.86) day^{-1}, respectively. The PCNB biotransformation rate in the PCNB-acclimated culture was two times faster than in the PCNB-amended control culture. A low degree of nitro group removal resulting in the formation of pentachlorobenzene (PeCB) was also observed in the PCNB-amended control culture (Figure 9.8B). In both cultures PCNB transformed to PCA only when nitrate reached levels ≤ 20 mg N/L. A previously reported pseudo-first-order rate constant for the transformation of 10 μM PCNB to PCA under fermentative/methanogenic conditions was 5.6 ± 0.4 day^{-1} (mean ± standard error) (Chapter 5). Similar transformation rates were also observed under iron reducing conditions (Chapter 8). Thus, the PCNB biotransformation rate to PCA under nitrate reducing conditions was relatively slow as compared to that under fermentative/methanogenic and iron reducing conditions.

Complete NO_3^- reduction in the PCNB-acclimated culture which was amended with 13 μM PCNB and the PCNB-free control culture occurred in about 4 days, whereas the same transformation required 16 days in the PCNB-amended control culture which was amended with 13 μM PCNB (Figure 9.9). The pseudo-first-order rate constants of

the NO_3^- reduction for the PCNB-acclimated and PCNB-amended control were 0.56 ± 0.07 (r^2 = 0.978) and 0.13 ± 0.02 (r^2 = 0.915) day^{-1}, respectively, whereas the NO_3^- reduction rate was 0.73 ± 0.04 (r^2 = 0.996) day^{-1} in the PCNB-free control culture.

The N_2 production rate and extent were similar in the PCNB-free control culture and the PCNB-acclimated culture amended with 13 μM PCNB (Figure 9.10A and 9.10B). In both cultures, NO_3^- was completely reduced to N_2 in 4 days. Intermediate products of denitrification (e.g., NO, N_2O) were not observed in both cultures. However, a transient inhibition and accumulation of NO and N_2O were observed in the PCNB-amended control denitrifying culture (Figure 9.10C). The CO_2 production rate and extent were very similar in the PCNB-free control culture and the PCNB-acclimated culture amended with 13 μM PCNB (Figure 9.10A and 9.10B). However, lower CO_2 production rate was observed in the PCNB-amended control denitrifying culture as a result of

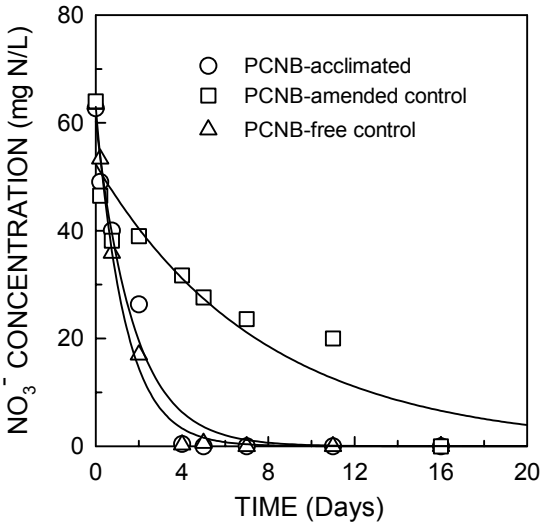


Figure 9.9. Time course of nitrate during a batch biotransformation assay conducted with the PCNB-acclimated culture, the PCNB-free culture, and the PCNB-free control culture (Lines are first-order fits to the nitrate data)(The cultures were fed with 60 mg NO_3^--N/L, and 13 μM PCNB, except the PCNB-free control culture).

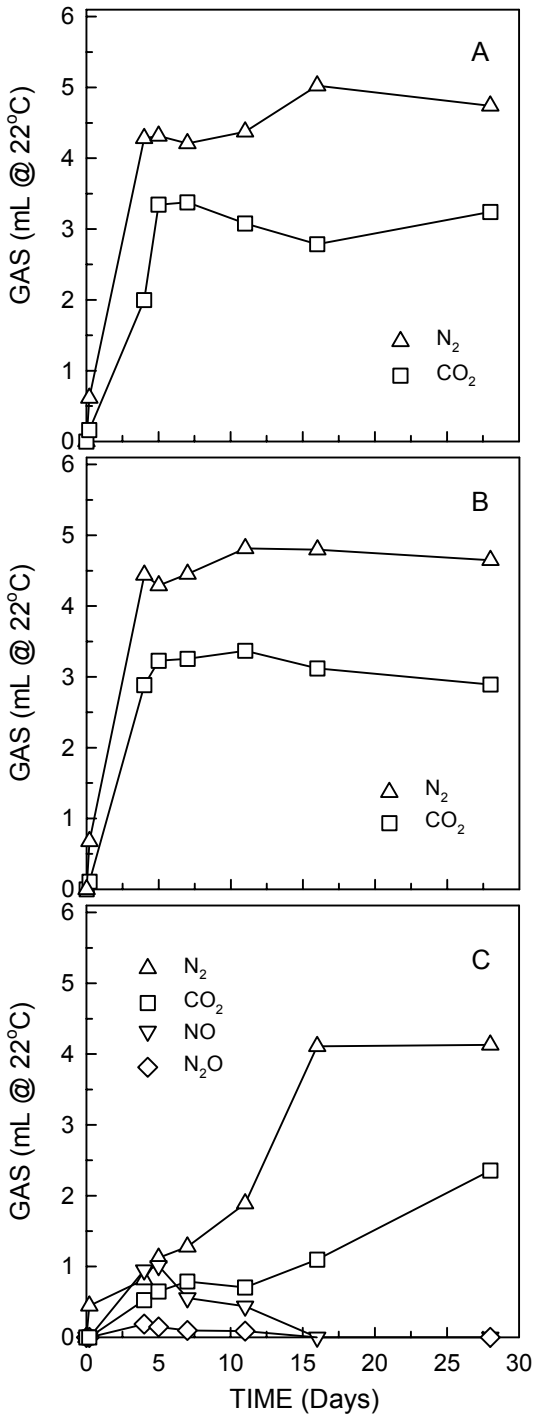


Figure 9.10. Gas production during a batch biotransformation assay conducted with the PCNB-free control culture (A), the PCNB-acclimated culture (B), and the PCNB-amended control culture (C)(The cultures were fed with 60 mg NO_3^--N/L, and 13 μM PCNB, except the PCNB-free control culture).

transient inhibition (Figure 9.10C). After 16 days of incubation, all of the accumulated denitrification intermediates (e.g., NO and N_2O) were reduced to N_2, and the CO_2 production rate increased in the PCNB-amended control denitrifying culture. Similar to our results, Chidthaisong and Conrad (2000) reported inhibition of glucose fermentation by the accumulation of toxic denitrification intermediates (NO_2^-, NO, N_2O). The transient inhibition observed in the PCNB-amended control culture can be explained by the accumulation of NO, which is a highly reactive and non-specifically acting toxic compound for most microorganisms (Mancinelli and McKay, 1983; Zumft, 1993).

VFAs production profiles in the three cultures are shown in Figure 9.11. Accumulation of acetic, propionic, iso butyric and butyric acid was observed in both the PCNB-amended control culture and the PCNB-free control culture, whereas only acetic and propionic acid accumulation was observed in the PCNB-acclimated culture. A relatively low VFAs production was observed in the PCNB-amended control culture within 16 days of incubation. The theoretical COD required to reduce NO_3^- to N_2 was calculated using bioenergetic principles (Rittmann and McCarty, 2001). The total COD required for the complete reduction of 60 mg/L NO_3^--N to N_2 was 170 mg COD/L. At the end of the 190-day incubation period, the amount of total VFAs produced (600 ± 50 mg COD/L) and COD required for the complete utilization of 60 mg/L NO_3^--N matched approximately the amount of glucose added (710 mg COD/L).

On the basis of nitrogen balance calculations, less than 100% nitrogen balance closure was obtained between the 0^{th} and 7^{th} day of incubation, due to NO and N_2O detection limitations (Table 9.3). At the end of a 28-day incubation period, 100% nitrogen balance closure was observed for all three cultures. Nitrogen balance

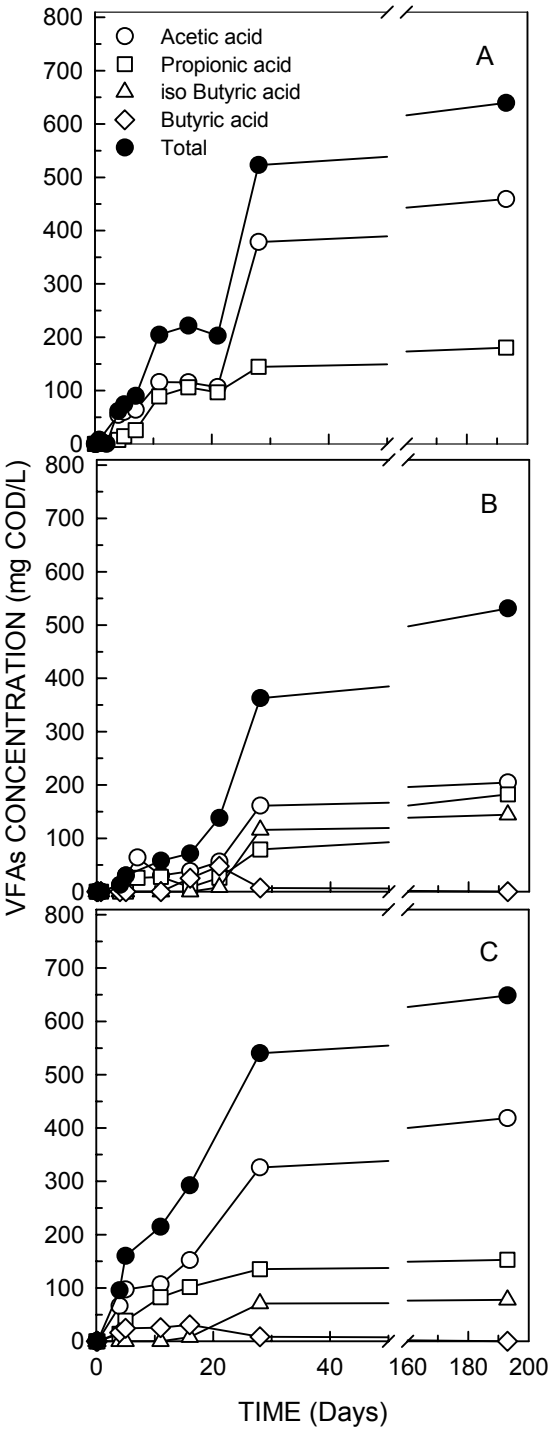


Figure 9.11. Comparison of VFAs production and consumption during a batch biotransformation assay conducted with the PCNB-acclimated culture (A), the PCNB-amended control culture (B), and the PCNB-free control culture (C)(The cultures were fed with 60 mg NO_3^--N/L, and 13 μM PCNB, except the PCNB-free control culture).

Table 9.3. Total nitrogen balance during the incubation period in the denitrifying cultures[a]

Time (Days)	PCNB-free control culture	PCNB-acclimated culture[b]	PCNB-amended control culture[b]
	Total nitrogen balance (%)		
0	105.8	104.5	106.7
0.2	99.9	92.5	85.8
5	77.0	84.9	84.2
7	79.6	96.7	73.2
11	87.9	102.8	80.0
16	107.7	112.0	90.7
28	114.3	104.5	98.6

[a] Initial biomass concentration in all culture series equal to 230 ± 35 mg POC/L (mean ± stand. dev.; $n = 3$); Initial NO_3^- concentration was 60 mg N/L. [b] Cultures were amended with 13 μM PCNB.

calculations showed that nitrate was completely reduced to nitrogen in all three denitrifying cultures. A *Clostridium* sp which dechlorinates chlorinated phenols, was reported to reduce NO_3^- and NO_2^- to ammonium (NH_4^+) (Madsen and Licht, 1992). However, unlike our results, they did not observe any N_2O production during the denitrification.

Several researchers have previously reported biotransformation of halogenated organics by various denitrifying microorganisms. Häggblom *et al*. (1993, 1996) demonstrated that 3- and 4-chlorobenzoate can be degraded by denitrifying enrichment cultures developed from soil and sediments from a variety of environments and geographical locations. Bae *et al*. (2002) examined the degradation potential of 2-chlorophenol (2-CP), 3-CP, 4-CP, 2,4-dichlorophenol (2,4-DCP) and 2,6-DCP under denitrifying conditions. However, they only observed concomitantly nitrate reduction and chlorophenol degradation in the culture amended with 2-CP. Kostyál *et al*. (1997)

reported that dechlorination of di-, tri- and tetrachlorophenols in a pulp mill wastewater by a fluidized-bed biomass was negligible under denitrifying conditions. In the present study, PCA dechlorination was not observed in the enrichment culture under denitrifying conditions during one year of incubation.

The inhibitory effect of nitroaromatic compounds on denitrification was previously reported (Fuller and Manning, 1998; Siciliano *et al*., 2000). Siciliano *et al*. (2000) observed a significant decrease in the denitrification activity in response to increasing 2,4,6-trinitrotoluene (TNT) concentrations. They reported that nitrous oxide reductase was much more sensitive to 2,4,6-trinitrotoluene (TNT) than nitrate reductase, nitrite reductase, and nitric oxide reductase. An investigation in soils chronically exposed to halogenated and nitroaromatic compounds showed that Gram-negative bacteria tend to predominate in these soils due to the presence of an outer lipopolysaccharide on their cell walls (Fuller and Manning, 1998).

Many studies have also indicated that nitrate inhibits the transformation of halogenated organic compounds by anaerobic microbial communities, specifically by inhibiting reductive dehalogenation (Häggblom *et al*., 1993; Genthner *et al*., 1989; Picardal *et al*., 1995; Milligan and Häggblom 1999). Middeldorp *et al*. (2005) reported the immediate inhibition of β-hexachlorocyclohexane (β-HCH) dechlorination when a soil percolation column was amended with 10 mM nitrate. Complete inhibition of the reductive dechlorination of hexachlorobenzene (HCB) was observed in anaerobic sediment microcosms when 30 mM NO_3^- was added to the mixed culture (Chen *et al*., 2002). Milligan and Häggblom (1999) demonstrated the inhibition of anaerobic transformation of the herbicide dicamba (3,6-dichloro-O-anisic acid) by nitrate and

pointed out the environmental implications, especially in agricultural areas where dicamba is used extensively.

Sanford and Tiedje (1997) reported simultaneous dechlorination of mono- and dichlorophenols and denitrification only when the nitrate concentration was below 5 mM. They suggested that although dechlorination and denitrification could take place at the same time at a low nitrate concentration, both processes were mediated by physiologically different populations. However, in the present study, although PCNB biotransformation to PCA was observed at low nitrate concentrations (≤ 20 mg N/L = 1.4 mM), further PCA dechlorination or biodegradation was not observed. Although there are denitrifying cultures that degrade less chlorinated organics under denitrifying conditions, degradation was not observed for all the less chlorinated isomers of chlorophenols (Bae *et al*., 2002; Kostyál *et al*., 1997). In a mixed culture, dechlorination and denitrification processes can take place together by different populations. However, there is no evidence for the dechlorination of highly chlorinated organics under denitrifying conditions. These results indicate that although denitrifying cultures could transform PCNB to PCA, dechlorination of PCA was not mediated by the enriched denitrifying culture.

9.4. Summary

The effect of nitrate reduction on the reductive biotransformation of PCNB, was investigated with a mixed, fermentative/methanogenic culture enriched from a contaminated estuarine sediment. The results of this study indicate that the transformation of PCNB to PCA is a fast process and the presence of nitrate does not affect the transformation rate. However, sequential dechlorination of PCA took place only at low nitrate concentrations (≤ 20 mg N/L). The presence of nitrate at low concentrations (≤ 10

mg N/L) in anaerobic soil and sediments is not expected to negatively affect the sequential dechlorination of PCA to dichlorinated anilines. However, higher nitrate concentrations (≥50 mg N/L) will lead to the accumulation of toxic compounds such as highly chlorinated anilines (i.e., PCA, TeCAs) and reduced nitrogen intermediates (i.e., NO, N_2O).

The effect of PCNB on denitrification was assessed with two denitrifying cultures (PCNB-free control and PCNB-amended) developed from a contaminated estuarine sediment. In the culture repeatedly amended with 0.09 μM PCNB, PCNB was transformed to PCA but further dechlorination or degradation of PCA was not observed for almost one year of incubation. A batch assay was performed with these enrichment cultures to investigate the effect of PCNB on denitrification. PCNB at an initial concentration of 13 μM was transformed to PCA simultaneously with nitrate reduction but only at a low nitrate concentration (≤ 20 mg N/L). PCNB addition at an initial concentration of 13 μM to the control culture developed as PCNB-free under denitrifying conditions resulted in a transient inhibition leading to a transient nitric oxide (NO) and nitrous oxide (N_2O) accumulation. Similarly to the PCNB-acclimated culture, PCNB transformation to PCA started when the nitrate concentration decreased to about 20 mg N/L. A low degree of nitro group removal resulting in the formation of PeCB was also observed in the control culture when amended with 13 μM PCNB. Further transformation or degradation of PCA was not observed in all cultures developed under nitrate reducing conditions. Accumulation of volatile fatty acids (VFAs) was observed in the three batch cultures which were amended with excess electron donor. The presence of nitrate at low concentrations in anaerobic soil and sediments is not expected to negatively affect the

biotransformation of PCNB to PCA, but further dechlorination or degradation of PCA is not mediated under nitrate reducing conditions. The results of this study indicate that the transformation of PCNB to PCA is a relatively fast process and occurs under denitrifying conditions at low nitrate concentrations (≤ 20 mg N/L) whether the culture is acclimated to the PCNB or not. However, reductive dechlorination or biodegradation of PCA was not achieved under denitrifying conditions. As discussed in Chapter 2, PCA is more toxic and hydrophobic than PCNB. Therefore, accumulation of this potentially toxic metabolite may create significant problems in anoxic systems that do not support reductive dechlorination.

CHAPTER 10

SEMI-EMPIRICAL MOLECULAR MODELING OF PENTACHLOROANILINE DECHLORINATION PATHWAY

10.1. Introduction

Several approaches have been used by researchers to describe the observed sequential reductive dechlorination pathways of halogenated organic compounds as it was discussed in Chapter 2. It is known that reductive dechlorination reactions are exergonic under anaerobic conditions using H_2 as the electron donor (Dolfing and Harrison, 1992, 1993). The most commonly observed dehalogenation pathways were mainly found to correlate with the highest free energy release of individual dehalogenation reactions. As discussed in Chapter 2, the overall free energy change can be used as an indicator of the potential that exists for a particular reductive dechlorination reaction to take place and form certain isomer. Susarla *et al*. (1997) reported that under standard conditions, using H_2 as the electron donor, dechlorination of chloroanilines results in available free energy in the range of 123 to 146 kJ/mol of chlorine removed. Based on redox potentials using the H^+/H_2 couple as the electron donor, they noted that the sequential reductive dechlorination of 2,3,4,5-TeCA to 3-CA, observed in their study, followed the route by which reactions with a higher standard redox potential (E_o') are preferred over those reactions with lower redox potentials. However, free energy may not be enough to describe biological reactions where more than one factor may affect such

reactions. In this study, for the case of polychlorinated CAs such as PCA and TeCAs, *para* dechlorination was the predominant dechlorination pathway followed by *ortho* dechlorination (Chapter 7). In the presence of both *ortho* substituents, *para* and/or *ortho* dechlorination was the predominant dechlorination pathway. However, for the less chlorinated aniline congeners, such as TrCAs and DCAs in the absence of one of the *ortho* substituents, *para* and *meta* dechlorination, which leads to the removal of an adjacent Cl substituent, was favorable (Chapter 7). In genaral, the dechlorination rates decreased with decreasing degree of chlorination (Chapter 7).

The objectives of the research reported here were to estimate thermodynamic properties of chlorinated anilines with a semi-empirical molecular orbital method, as well as to compare the theoretically derived distribution of PCA dechlorination products with the experimental results obtained with the mixed, fermentative/methanogenic enrichment culture used in this study. In addition, the experimentally observed dechlorination pathways for chlorinated anilines were compared with previously reported dechlorination pathways for chlorinated benzenes to understand the effect of the amino group on the reductive dechlorination reactions.

10.2. Methods

10.2.1. Molecular Modeling

A semi-empirical molecular orbital estimation program MOPAC (version 6.0) was used to optimize the geometry and carry out the quantum mechanical calculations leading to thermodynamic property estimation of PCNB, PCA, 18 CA congeners, and aniline. Semi-empirical Hamiltonians are implemented, and calculations of vibrational

spectra, thermodynamic quantities, isotopic substitution effects and force constants for molecules, radicals, ions and polymers are combined in MOPAC (Stewart, 1993).

MOPAC was run on a Ultra30 Sun Workstation running in a Solaris 5.6 operating system at the School of Civil and Environmental Engineering of the Georgia Institute of Technology. The input files for each compound consisted of an internal coordinate system. Each molecule was created by defining one atom in space, and then sequentially adding atoms and defining their positions with respect to the previously defined atoms by using approximate bond lengths, bond angles, and dihedral angles. The input geometry should be as close to the optimized geometry as possible; otherwise, MOPAC aborts the optimization process due to its failure to reach the convergence criteria. The approximate molecular configuration of all compounds tested was found by using the three dimensional molecular modeling and analysis program CS Chem3D Pro, version 3.5.2 (CambridgeSoft Corporation, Cambridge, MA). MOPAC was then run to optimize this input geometry. Based on the optimized geometry, MOPAC calculated heat of formation ($\Delta H°_f$) and entropy ($S°$) for each compound.

MOPAC includes three main semi-empirical, self-consistent field (SCF) method subroutines that can be used to optimize molecular structure, molecular orbitals, and to calculate electron densities, charges, heat of formation, and entropy with respect to the molecular geometry. The main semi-empirical, SFC methods in MOPAC that can be used for the geometry optimization and estimation of thermodynamic properties are as follows: Modified Neglect of Diatomic Overlap (MNDO), Austin Model 1 (AM1), and Parametric Method 3 (PM3). It is important to determine which method should be used during the calculation of thermodynamic properties of the selected compounds. In order

to decide which computational method will be used, previously reported heat of formation values calculated by the Benson method for selected chloroanilines (Susarla *et al*., 1997) were compared with the output data of MOPAC obtained by three SCF methods (MNDO, AM1, PM3) (Figure 10.1). The slope and intercept of the AM1 subroutine was the one which is closest to 1 and nearest to 0, respectively. Similarly, Prytula (1998) compared previously reported experimental values of $\Delta H°_f$ for selected chlorobenzenes with three SCF methods (MNDO, AM1, PM3) and reported that the AM1 subroutine gave the closest agreement with experimental values. Therefore, the AM1 subroutine was used for the estimation of $\Delta H°_f$ and S° values in the present study.

ChemDraw Pro, version 4.5 (CambridgeSoft Corporation, Cambridge, MA) and Chem3D Pro, version 3.5.2 were used to visualize and interpret MOPAC output data. The MOPAC output data were transferred into protein data base (pdb) format by a conversion program named Babel (version 1.05d; Dolata Research Group, Department of Chemistry, University of Arizona, Tucson, AZ), which then was opened in Chem3D Pro.

10.2.2. Dechlorination Mechanism

Microbial reductive dechlorination can be described as an electron transfer process where electrons are transferred from an electron donor to the electron acceptor (chlorinated compounds). Microbially-mediated reductive dechlorination requires a source of electrons and protons (Nies and Vogel, 1991). Protons and electrons in combination, such as H_2, can be transferred directly to the aromatic ring of haloaromatic compounds, with the subsequent release of Cl^- and H^+ (Nies and Vogel, 1991).

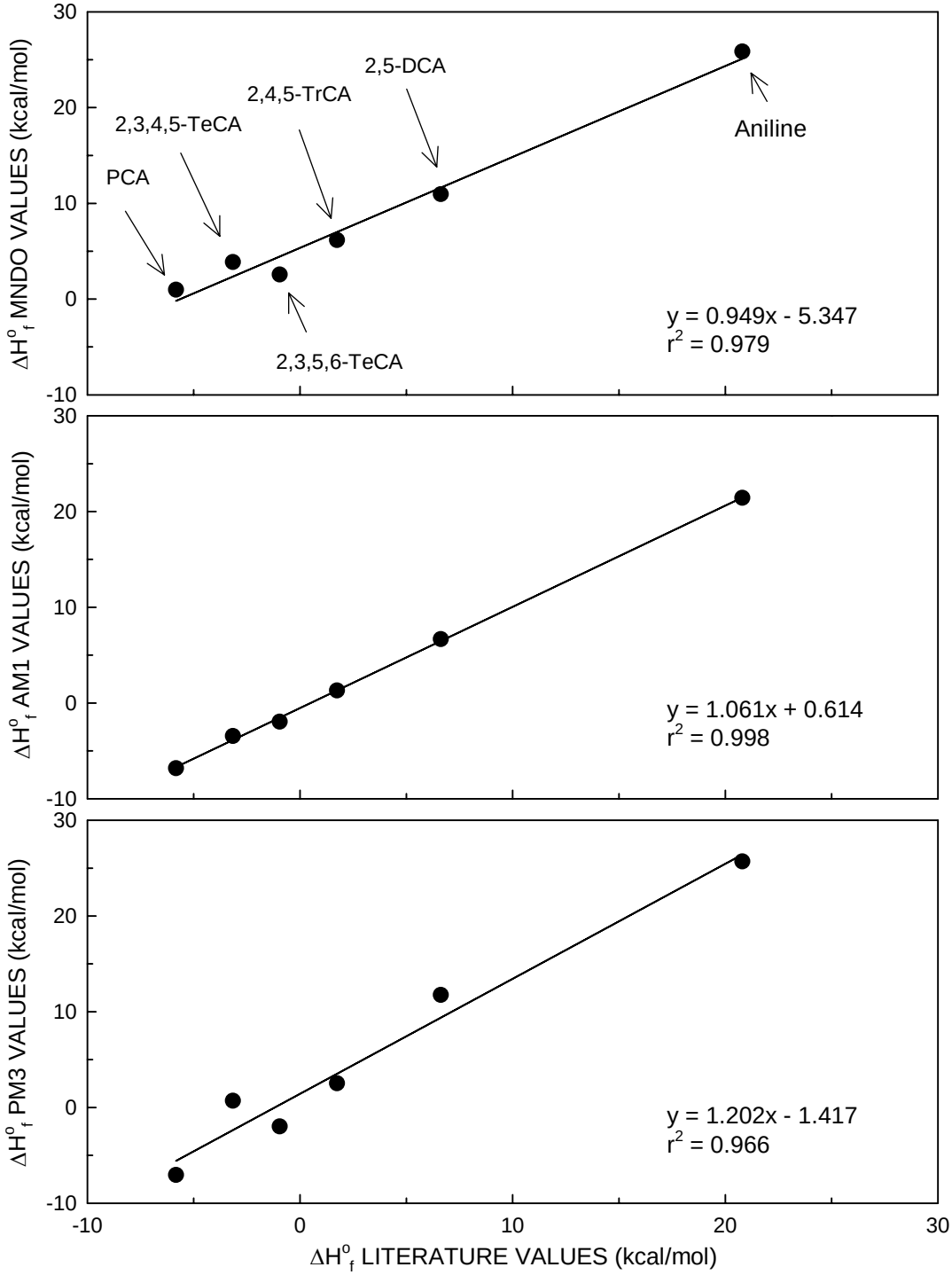


Figure 10.1 MOPAC calculated ΔH^{o}_{f} values compared with literature data reported by Susarla *et al*. (1997)(only ΔH^{o}_{f} for aniline is an experimental value).

According to the one-step reductive dechlorination model, two electrons are simultaneously transferred using H_2 as the electron donor and replace a chlorine-carbon bond with an hydrogen-carbon bond (Figure 10.2). In the two-step reductive dechlorination model, two electrons are transferred one at a time, then the chlorine on the aromatic ring is released as a chloride ion and is replaced by a proton. This mechanism has been proposed previously, and involves the transfer of electrons from H_2 (or another reduced organic or inorganic electron donor) to the organochlorine molecule, which then abstracts a proton from water (Dolfing, 1990; Nies and Vogel, 1991). Based on this model, addition of the two electrons results in the production of a reduced chlorinated molecule with a net double negative charge which then undergoes spontaneous rearrangement followed by the release of a chloride ion to form a less chlorinated and negatively charged aniline intermediate (Figure 10.3). In the final step, this intermediate abstracts a proton from water and a stable, less chlorinated congener is produced.

10.2.3. Thermodynamic Calculations

Using the AM1 subroutine, the optimized molecular geometry was used for the thermodynamic calculations to estimate the heat of formation ($\Delta H°_f$) and entropy ($S°$) of each compound for ideal gas conditions. Entropies were calculated without any statistical consideration of molecular symmetry by setting the ROT=1 in the comment line of the MOPAC input file. The ROT function assigns the symmetry number to the molecule that is being optimized. Symmetry was explicitly accounted for in the relative formation potential calculations as shown in Section 10.2.4.

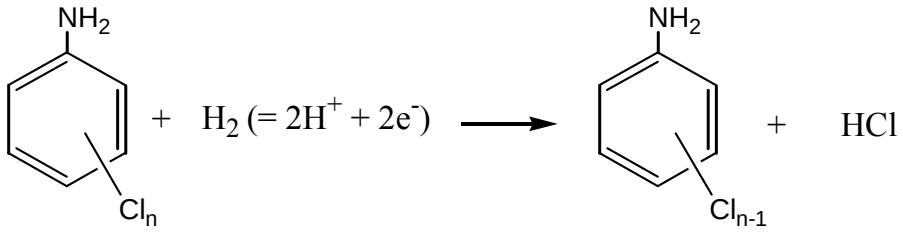


Figure 10.2. Simple reductive dechlorination reaction used in the molecular modeling analysis (One-step two-electrons addition).

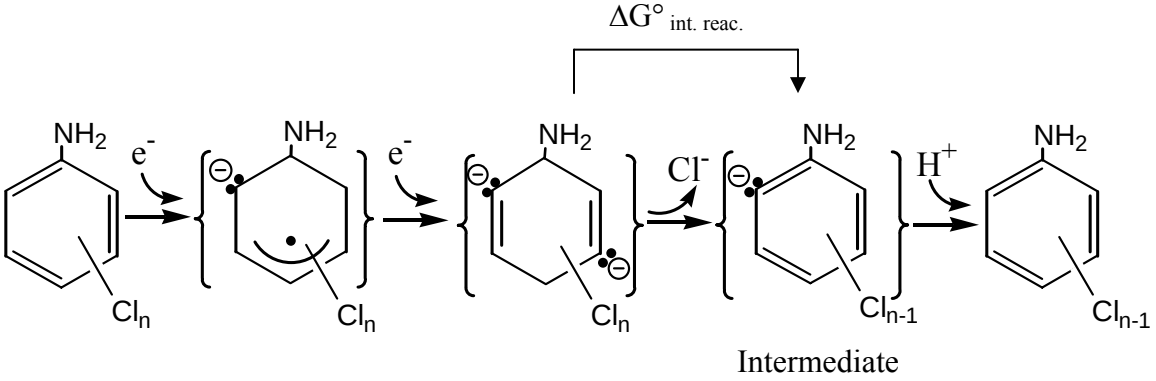


Figure 10.3. Schematic showing the two sequential electron additions during the dechlorination of chlorinated aniline molecules and the formation of intermediates (Two-step two-electrons addition).

Gibbs free energy of formation values ($\Delta G°_f$) were calculated for each of the chlorinated aniline congeners using the following equation.

$$\Delta G_f^o = \Delta H_f^o - T\Delta S^o \qquad (10-1)$$
$$= \Delta H_f^o - T(S^o_{compound} - \sum S^o_{i\ elements})$$

The values of $\Delta S°$ in equation 10-1 were calculated by subtracting the sum of $S^o_{elements}$, i.e., the entropy of each of the constituent atoms (Cl as ½ of Cl_2, H as ½ of H_2, O as ½ of O_2, N as ½ of N_2, and C as graphite, 26.65, 15.60, 24.48, 22.87, and 1.37 cal/mol·K, respectively)(Huang *et al.*, 1996) from the $S^o_{compound}$, i.e., the entropy value of the whole molecule at an absolute temperature (T) of 298 K calculated with MOPAC/AM1.

The overall free energy change of each dechlorination reaction was calculated using the Gibbs free energy of formation values for the reactants and products in the dechlorination reaction as follows.

$$\Delta G^o_{reaction} = \sum \Delta G^o_{f\ products} - \sum \Delta G^o_{f\ \text{reactants}} \qquad (10-2)$$

The reaction used to calculate the Gibbs free energy released from the dechlorination reactions is shown in Figure 10.2. The products were the less chlorinated aniline congeners (or aniline in the case of CA dechlorination) and hydrochloric acid (HCl), and the reactants were the parent chlorinated aniline compound and H_2. The values of the free energy released in the reorganization of the doubly charged ions to form the less chlorinated anions ($\Delta G°_{intermediate}$) were computed using the individual two-

electron reduced species as reactants and the respective negatively charged intermediate molecules and a chloride ion as products (Figure 10.2).

Free energy values in solution ($\Delta G°_{f\,solution}$) for all compounds were calculated from the $\Delta G°_{f\,gas}$ values using vapor pressure and aqueous solubility values according to the procedure described by Dolfing and Harrison (1992) and Huang *et al.* (1996)(Equation 10-3). Solution-phase calculations were not performed for the intermediate molecules because their aqueous solubility and vapor pressure values are not available.

$$\Delta G^{o}_{f\ solution} = \Delta G^{o}_{f\ gas} + RT\ \ln(\frac{P}{P^{o}}) - RT\ln(\gamma_{i}\ \frac{m_{icond}}{m_{i}^{o}}) \qquad (10-3)$$

where: $\Delta G^{o}_{f\,solution}$ is the Gibbs free energy of formation of the ideal solution (kcal/mol); $\Delta G^{o}_{f\,gas}$ is the Gibbs free energy of formation of the ideal gas (kcal/mol); R is the ideal gas constant (1.987 cal/mol·K); T is the absolute temperature (K); P is the vapor pressure (kPa); P^{o} is the standard state pressure (100 kPa); m_{icond} is the molality of the condensed phase solute in water; γ_i is the activity coefficient; m_i^{o} is the standard state molality (1 gmol of solute/kg of water).

10.2.4. Relative Distribution of Dechlorination Product Isomers

In order to predict a possible sequential reductive dechlorination pathway for the chlorinated anilines, it was assumed that dechlorination products with the lowest $\Delta G°_{f}$ values are the ones that are the easiest to be formed. An equilibrium thermodynamics approach was used to develop relative dechlorination product isomer distributions for the

chlorinated anilines with multiple possible dechlorination products. Theoretical equilibrium constants were calculated for each dechlorination reaction using the AM1 estimated $\Delta G°_{reaction}$ values and equation 10-4.

$$K_i = \exp\left(\frac{-\Delta G^o_{reaction}}{RT}\right) \qquad (10-4)$$

where: K_i is the theoretical equilibrium constant for an individual reaction i; R is the ideal gas constant (1.987 cal/mol·K); and T is the absolute temperature (298 K).

When multiple dechlorination products can be formed from a parent compound, the relative formation potential for a given isomer over another is proportional to the ratio of the equilibrium constants for the two reactions as given by equation 10-5.

$$RFP_i = \frac{SF_i\, K_i}{SF_{low} K_{low}} = \frac{SF_i}{SF_{low}} \exp\left[\frac{-(\Delta G^o_{reaction_i} - \Delta G^o_{reaction_{low}})}{RT}\right] \qquad (10-5)$$

where, RFP_i is the relative formation potential of each product isomer relative to the least energetically favorable product (*low*) that can be produced from the same parent compound. The statistical factor (SF_i) is defined as the number of sites on the parent compound at which a Cl substituent can be removed to form the exactly same product. SF_i are statistical factors that show the number of pathways through which each reaction can occur. Using the RFP_i values for each reaction, the relative distribution factors (DF_i) for a particular reaction were calculated using equation 10-6.

$$DF_i = \frac{RFP_i}{\sum_{i=1}^{n} RFPi} \times 100\% \qquad (10-6)$$

The relative percentage of a particular isomer that should be formed from a particular parent compound, based on the thermodynamic and statistical parameters of each possible single-step reductive dechlorination of that parent compound was predicted by calculating the relative distribution factor (*DFi*) for each chlorinated aniline congener.

10.3. Results and Discussion

10.3.1. Thermodynamic Properties of Chlorinated Anilines

Thermodynamic calculations performed using the AM1 subroutine resulted in estimated gas-phase values of heat of formation (ΔH^o_f) and entropy (S^o) for each chlorinated aniline congener. Table 10.1 summarizes the MOPAC/AM1 output and the calculated Gibbs free energy of formation values for PCNB, PCA, CAs, and aniline. The $\Delta H°_{f\,gas}$, S^o_{gas}, and $\Delta G°_{f\,gas}$ values were also calculated for H_2, HCl, H^+, and Cl^-.

The gas-phase free energy of formation, vapor pressure and solubility values for each compound were used to calculate solution-phase free energy of formation and the results are summarized in Table 10.2. The solution-phase adjustment essentially lowers the free energy of formation of the more soluble products with respect to the less soluble ones.

Based on the estimated MOPAC/AM1 Gibbs free energy values, the overall reaction free energy changes for the dechlorination of the chlorinated aniline congeners were calculated using equation 2 for both gas- and solution-phase and results are shown in Table 10.3. As expected, lower reaction free energies (more negative) were observed for highly chlorinated aniline congeners. Statistical factors for each possible

Table 10.1. MOPAC/AM1 thermodynamic output and calculated gas-phase Gibbs free energy of formation values for PCNB, PCA, CAs, and aniline (at 298 K)(Symmetry not included in these calculations)

Compound	$\Delta H°_{f\,gas}$ (kcal/mol)	$S°_{gas}$ (cal/mol·K)	$\Delta G°_{f\,gas}$ (kcal/mol)
PCNB	-5.393	102.190	27.762
PCA	-6.806	98.159	22.208
2,3,4,5-TeCA	-1.952	95.717	24.496
2,3,4,6-TeCA	-3.427	92.576	23.958
2,3,5,6-TeCA	-3.453	93.248	23.731
2,3,4-TrCA	2.345	90.428	27.076
2,3,5-TrCA	1.646	92.028	25.901
2,3,6-TrCA	1.339	88.822	26.549
2,4,5-TrCA	1.306	91.946	25.585
2,4,6-TrCA	0.172	91.219	24.667
3,4,5-TrCA	2.992	90.121	27.815
2,3-DCA	8.095	85.315	31.058
2,4-DCA	6.672	89.369	28.427
2,5-DCA	6.668	85.618	29.540
2,6-DCA	6.565	84.218	29.854
3,4-DCA	8.093	85.744	30.928
3,5-DCA	7.263	87.996	29.426
2-CA	13.668	82.297	34.236
3-CA	14.005	82.415	34.539
4-CA	13.785	81.099	34.711
Aniline	21.415	74.026	41.156
H_2	-5.181	32.123	-5.456
HCl	-24.608	44.616	-25.313
H^+	314.915	0	319.564
Cl^-	37.66	53.290	-45.599

Table 10.2. MOPAC/AM1 thermodynamic output and calculated solution-phase Gibbs free energy of formation values for PCA, CAs, and aniline (at 298 K)

Compound	Aqueous Solubility (mg/L)[a]	Vapor Pressure (kPa)[a]	$\Delta G°_{f\ solution}$ (kcal/mol)
PCA	0.025	0.000000734	20.69
2,3,4,5-TeCA	1.5	0.00003	22.67
2,3,4,6-TeCA	6.8[b]	0.000074[b]	21.77
2,3,5,6-TeCA	2.1	0.000045	21.94
2,3,4-TrCA	30	0.00004	23.55
2,3,5-TrCA	21	0.00004	22.57
2,3,6-TrCA	22[b]	0.00004[b]	23.21
2,4,5-TrCA	18	0.00017	23.22
2,4,6-TrCA	17	0.00029	22.65
3,4,5-TrCA	26	0.0002	25.33
2,3-DCA	471	0.0086	28.97
2,4-DCA	241	0.0009	25.40
2,5-DCA	280	0.00212	26.93
2,6-DCA	352	0.00212	27.11
3,4-DCA	181	0.00212	28.57
3,5-DCA	269	0.00034	25.75
2-CA	3730	0.0333	31.58
3-CA	3730	0.0112	31.24
4-CA	1380	0.0622	33.01
Aniline	35000	0.0799	37.51

[a] Data from Reuther *et al*., 1998
[b] Data from EPIWIN v3.12

dechlorination reaction are also shown in Table 10.3. The statistical factor shows the possibility of formation for each dechlorination product starting with the same parent compound based on the number of C-Cl sites on the parent molecule that can be reduced to form the product isomer without taken into account other controlling factors (e.g., thermodynamic, electronic, steric, etc).

In addition to the reaction free energy calculations for both the gas and solution phase, the free energy required to form intermediate dechlorination products (Figure 10.3) was also calculated and reported in Table 10.4. The reaction free energies for the formation of the dechlorination intermediate products were significantly higher compared to gas- and solution-phase overall reaction free energies. The $\Delta G°_{rec.\ inter.}$ values represent the free energy change between the two-electron reduced parent compound and the intermediate that could be formed from the same parent chlorinated aniline congener (Figure 10.3 and Table 10.4).

Table 10.5 shows the calculated dechlorination product distribution factors (*DFi*) based on the overall reaction Gibbs free energies for both the gas- and solution-phase, and the free energy of dechlorination intermediates. The computed product distribution was compared with the product distribution achieved by the mixed, enrichment culture (Chapter 7). The experimentally observed, predominant, sequential PCA biotransformation pathway was as follows: PCA → 2,3,4,5- and 2,3,5,6-tetrachloroaniline (TeCA) → 2,4,5- and 2,3,5-trichloroaniline (TrCA)→ 2,5-dichloroaniline (DCA) → 3-CA (low levels). Product isomer distributions based on statistical considerations did not match with the observed predominant dechlorination pattern. Product distributions based on the overall reaction free energy changes (which

Table 10.3. MOPAC/AM1 thermodynamic output and calculated gas- and solution-phase Gibbs free energy of reaction for PCA, CAs, and aniline (at 298 K)

Dechlorination Reaction	Statistical Factor	$\Delta G°_{reaction, gas}$ (kcal/mol)	$\Delta G°_{reaction, solution}$ (kcal/mol)
PCA → 2,3,4,5-TeCA	2	-17.56	-17.88
→ 2,3,4,6-TeCA	2	-18.11	-18.78
→ 2,3,5,6-TeCA	1	-18.33	-18.60
2,3,4,5-TeCA → 2,3,4-TrCA	1	-17.28	-18.98
→ 2,3,5-TrCA	1	-18.45	-19.96
→ 2,4,5-TrCA	1	-18.77	-19.31
→ 3,4,5-TrCA	1	-16.54	-17.20
2,3,4,6-TeCA → 2,3,4-TrCA	1	-16.74	-18.08
→ 2,3,6-TrCA	1	-17.27	-18.42
→ 2,4,5-TrCA	1	-18.23	-18.41
→ 2,4,6-TrCA	1	-19.15	-18.98
2,3,5,6-TeCA → 2,3,5-TrCA	2	-17.69	-19.23
→ 2,3,6-TrCA	2	-17.04	-18.59
2,3,4-TrCA → 2,3-DCA	1	-15.88	-14.44
→ 2,4-DCA	1	-18.51	-18.01
→ 3,4-DCA	1	-16.01	-14.83
2,3,5-TrCA → 2,3-DCA	1	-14.70	-13.46
→ 2,5-DCA	1	-16.22	-15.50
→ 3,5-DCA	1	-16.33	-16.67
2,3,6-TrCA → 2,3-DCA	1	-15.35	-14.10
→ 2,5-DCA	1	-16.87	-16.14
→ 2,6-DCA	1	-16.55	-15.96

Table 10.3 (Cont.)

Dechlorination Reaction	Statistical Factor	$\Delta G°_{reaction, gas}$ (kcal/mol)	$\Delta G°_{reaction, solution}$ (kcal/mol)
2,4,5-TrCA → 2,4-DCA	1	-17.02	-17.68
→ 2,5-DCA	1	-15.90	-16.15
→ 3,4-DCA	1	-14.51	-14.50
2,4,6-TrCA → 2,4-DCA	2	-16.10	-17.11
→ 2,6-DCA	1	-14.67	-15.40
3,4,5-TrCA → 3,4-DCA	2	-16.74	-16.61
→ 3,5-DCA	1	-18.25	-19.43
2,3-DCA → 2-CA	1	-16.68	-17.24
→ 3-CA	1	-16.38	-17.59
2,4-DCA → 2-CA	1	-19.04	-13.67
→ 4-CA	1	-18.56	-12.24
2,5-DCA → 2-CA	1	-15.16	-15.20
→ 3-CA	1	-14.86	-15.55
2,6-DCA → 2-CA	2	-15.47	-15.38
3,4-DCA → 3-CA	1	-16.25	-17.19
→ 4-CA	1	-16.07	-15.42
3,5-DCA → 3-CA	2	-14.75	-14.37
2-CA → Aniline	1	-12.94	-13.93
3-CA → Aniline	1	-13.24	-13.59
4-CA → Aniline	1	-13.41	-15.37

Table 10.4. MOPAC/AM1 thermodynamic output and calculated Gibbs free energy of formation values for dechlorination intermediates (at 298 K)(see Figure 10.3)

Dechlorination Reaction	$\Delta G_f^{\circ a}$ (-2 charged) (kcal/mol)	ΔH_f° (kcal/mol)	S° (cal/mol·K)	$\Delta G^\circ_{int.}$ (kcal/mol)	$\Delta G^\circ_{int.\ reac.}$ (kcal/mol)
PCA → 2,3,4,5-TeCA	89.06	7.86	93.55	30.30	-96.42
→ 2,3,4,6-TeCA		6.61	94.11	28.89	-97.84
→ 2,3,5,6-TeCA		9.24	92.92	31.87	-94.86
2,3,4,5-TeCA → 2,3,4-TrCA	100.69	19.60	87.94	40.42	-97.93
→ 2,3,5-TrCA		19.83	91.90	39.47	-98.88
→ 2,4,5-TrCA		16.21	91.40	36.00	-102.35
→ 3,4,5-TrCA		18.91	91.22	38.75	-99.60
2,3,4,6-TeCA → 2,3,4-TrCA	106.88	19.08	87.71	39.97	-104.57
→ 2,3,6-TrCA		20.06	88.37	40.75	-103.79
→ 2,4,5-TrCA		15.57	91.24	35.41	-109.13
→ 2,4,6-TrCA		15.35	91.99	34.97	-109.57
2,3,5,6-TeCA → 2,3,5-TrCA	102.88	16.83	91.36	36.63	-103.91
→ 2,3,6-TrCA		17.41	88.01	38.21	-102.34
2,3,4-TrCA → 2,3-DCA	129.69	33.50	85.38	51.80	-115.55
→ 2,4-DCA		27.78	85.53	46.03	-121.32
→ 3,4-DCA		29.44	85.10	47.82	-119.52
2,3,5-TrCA → 2,3-DCA	122.09	31.48	85.11	49.85	-109.89
→ 2,5-DCA		27.78	85.12	46.33	-113.42
→ 3,5-DCA		28.60	88.96	45.83	-113.91
2,3,6-TrCA → 2,3-DCA	121.46	30.78	84.82	49.24	-109.89
→ 2,5-DCA		27.15	85.06	45.54	-113.59
→ 2,6-DCA		28.83	85.09	47.21	-111.91
2,4,5-TrCA → 2,4-DCA	123.32	28.99	85.60	47.22	-113.76
→ 2,5-DCA		31.31	85.59	49.54	-111.44
→ 3,4-DCA		30.81	85.20	49.16	-111.82
2,4,6-TrCA → 2,4-DCA	123.06	27.62	85.45	45.89	-114.83
→ 2,6-DCA		31.89	84.38	50.48	-110.24
3,4,5-TrCA → 3,4-DCA	130.78	30.15	85.37	48.45	-120.00
→ 3,5-DCA		31.15	88.16	48.62	-127.77

[a] Gibbs free energy of -2 charged parent compound.

Table 10.4 (cont).

Dechlorination Reaction	$\Delta G_f°$[a] (-2 charged) (kcal/mol)	$\Delta H_f°$ (kcal/mol)	S° (cal/mol·K)	$\Delta G°_{int.}$ (kcal/mol)	$\Delta G°_{int.\ reac.}$ (kcal/mol)
2,3-DCA → 2-CA	146.51	12.35	83.55	27.90	-164.21
→ 3-CA		12.25	84.08	27.64	-164.46
2,4-DCA → 2-CA	144.75	12.35	83.55	27.90	-162.45
→ 4-CA		13.46	81.71	29.56	-160.79
2,5-DCA → 2-CA	143.73	12.47	83.40	28.06	-161.27
→ 3-CA		12.37	84.13	27.75	-161.59
2,6-DCA → 2-CA	144.55	12.48	83.61	28.01	-162.14
3,4-DCA → 3-CA	148.95	12.33	84.30	27.66	-166.89
→ 4-CA		14.66	81.71	30.76	-163.79
3,5-DCA → 3-CA	144.11	12.28	84.07	27.67	-162.03
2-CA → Aniline	170.49	29.74	74.86	44.58	-171.51
3-CA → Aniline	169.80	29.77	74.67	44.67	-170.73
4-CA → Aniline	174.63	29.75	74.95	44.56	-175.66

[a] Gibbs free energy of -2 charged parent compound.

Table 10.5. Calculated dechlorination product distribution factor as compared to that observed in the PCA-dechlorinating, enrichment culture

Dechlorination reaction		Calculated Product Distribution (%) based on:				Experimental[c] (%)
		Statistics[a]	Overall Reaction Energy		Intermediate Energy	
			Ideal Gas	Solution[b]		
PCA	→ 2,3,4,5-TeCA	40	19	14	9	44
	→ 2,3,4,6-TeCA	40	47	63	91	0
	→ 2,3,5,6-TeCA	20	34	23	0	56
2,3,4,5-TeCA	→ 2,3,4-TrCA	25	5	12	0	0
	→ 2,3,5-TrCA	25	35	65	0	50
	→ 2,4,5-TrCA	25	59	22	99	34
	→ 3,4,5-TrCA	25	1	1	1	16
2,3,4,6-TeCA	→ 2,3,4-TrCA	25	1	11	0	-[d]
	→ 2,3,6-TrCA	25	3	20	0	-
	→ 2,4,5-TrCA	25	17	19	32	-
	→ 2,4,6-TrCA	25	79	50	68	-
2,3,5,6-TeCA	→ 2,3,5-TrCA	50	75	75	93	100
	→ 2,3,6-TrCA	50	25	25	7	0
2,3,4-TrCA	→ 2,3-DCA	33.33	1	0	0	5
	→ 2,4-DCA	33.33	97	99	95	95
	→ 3,4-DCA	33.33	2	1	5	0
2,3,5-TrCA	→ 2,3-DCA	33.33	3	0	0	0
	→ 2,5-DCA	33.33	44	12	30	100
	→ 3,5-DCA	33.33	53	88	70	0
2,3,6-TrCA	→ 2,3-DCA	33.33	5	2	0	-[d]
	→ 2,5-DCA	33.33	60	56	94	-
	→ 2,6-DCA	33.33	35	42	6	-
2,4,5-TrCA	→ 2,4-DCA	33.33	86	93	95	0
	→ 2,5-DCA	33.33	13	7	2	100
	→ 3,4-DCA	33.33	1	0	3	0
2,4,6-TrCA	→ 2,4-DCA	66.7	96	97	100	89
	→ 2,6-DCA	33.3	4	3	0	11
3,4,5-TrCA	→ 3,4-DCA	66.7	14	2	0	2
	→ 3,5-DCA	33.3	86	98	100	98

[a] Statistical distributions based on the number of different Cl substituents that can be removed from the parent compound to form the product isomer.

[b] Gibbs free energy of reactions calculated using the solution-phase adjusted ΔG°_{f} for each parent and product compound.

[c] Product distribution observed in the PCA-dechlorinating, enrichment culture (Chapter 7).

[d] 2,3,4,6-TeCA and 2,3,6-TrCA were not available and therefore were not tested.

include statistical factors) more closely agree with the experimental results. However, according to the experimental results, production of 2,3,4,6-TeCA was not observed, whereas the product distributions based on the overall reaction free energy changes predicted production of 2,3,4,6-TeCA. The product distribution based on the overall reaction free energy changes resulted in more branching, whereas less branching was observed in assays conducted with the enrichment culture. Although there was relatively higher branching for the highly chlorinated anilines (i.e., PCA, 2,3,4,5-TeCA) product distribution, the branching significantly decreased for the less chlorinated anilines (i.e., 2,3,5-TrCA, 2,4,5-TrCA). The solution-phase corrections did not significantly change the product distribution. The product distribution matched better the experimental data in the case of 2,3,4,5-TeCA dechlorination but worsened in the case of 2,3,5,6-TeCA, 2,3,5-TrCA, 2,4,5-TrCA, and 3,4,5-TrCA (Table 10.5). The solution-phase adjustment essentially lowers the free energy of formation of the more soluble products relative to the less soluble ones. Therefore, isomer distributions are shifted in favor of the production of the more soluble products. Because the product distributions determined based on the solution-phase corrections do not correlate with the experimentally observed dechlorination pathway, product solubility may not be a significant factor controlling the product distribution during microbial reductive dechlorination.

The product distribution based on the free energy of the dechlorination intermediates agrees with the experimentally observed dechlorination pathway for some of the less chlorinated aniline isomers but does not agree in the case of PCA and 2,3,4,5-TeCA (Table 10.5). In the case of PCA, the intermediate with the lowest (i.e., more

negative) $\Delta G^{\circ}_{reac.\ inter.}$ resulted in the formation of 2,3,4,6-TeCA that was not observed in the PCA-dechlorinating enrichment culture.

10.3.2. Electronic Properties of Chlorinated Anilines

In addition to the thermodynamic information, several physical and electronic properties of the chlorinated aniline congeners were estimated using MOPAC/AM1, such as carbon-chlorine bond length, carbon charge, and chlorine charge (Appendix B). These properties were also used to calculate other electronic parameters such as *bond charge* defined as the sum of the net charges on the carbon and the chlorine atom defining the bond, and *charge differential* defined as the subtraction of the net charge of the carbon atom from the net charge of the chlorine atom defining the bond. These results are shown in Table 10.6. The experimentally observed chlorine atoms removed are shown in bold face font in Table 10.6. Based on the experimentally observed dechlorination pathway, chlorine atoms were removed from the most negatively charged carbon atom in all cases except for 2,3,4,5-TeCA, 2,3,4-TrCA, 2,3,5-TrCA, and 2,4,5-TrCA, in which cases dechlorination was observed at the lowest (most positive) charged carbon.

Another electron density correlation was investigated based on the calculated bond charge of all CAs congeners. In this case, the lowest negative bond charge was predicted to be the site most favorable for dechlorination. Similar to the previous correlation, this assumption agreed for all cases except for 2,3,4,5-TeCA, 2,3,4-TrCA, 2,3,5-TrCA, and 2,4,5-TrCA. The charge differential calculations resulted in a positive value representing the magnitude of the charge differential between the carbon and chloride atoms. A higher charge differential might indicate a location where the overlap

Table 10.6. Electronic properties of PCA and chlorinated aniline congeners with multiple possible dechlorination product isomers[a]

		Chlorine Substituent Position on Benzene Ring				
Compound	Property	2	3	4	5	6
PCA	C-Cl Bond Length (Å)	*1.6825*	1.6738	**1.6748**	1.6738	*1.6825*
	Carbon Charge	*-0.1794*	-0.1031	**-0.1697**	-0.1029	*-0.1796*
	Chlorine Charge	*0.1099*	0.1268	**0.1243**	0.1264	*0.1102*
	Bonde Charge	*-0.0695*	0.0237	**-0.0454**	0.0235	*-0.0694*
	Charge Differential	*0.2893*	0.2299	**0.2940**	0.2293	*0.2898*
2,3,4,5-TeCA	C-Cl Bond Length (Å)	1.7002	*1.6967*	**1.6930**	1.6950	-
	Carbon Charge	-0.1371	*-0.007*	**-0.1097**	-0.0066	-
	Chlorine Charge	0.0109	*0.0197*	**0.033**	0.0374	-
	Bonde Charge	-0.1262	*0.0127*	**-0.0767**	0.0308	-
	Charge Differential	0.1480	*0.0267*	**0.1427**	0.0440	-
2,3,4,6-TeCA	C-Cl Bond Length (Å)	1.6968	1.6939	1.6947	-	1.6989
	Carbon Charge	-0.0667	-0.0614	-0.0540	-	-0.0676
	Chlorine Charge	0.0274	0.0360	0.0236	-	0.0096
	Bonde Charge	-0.0393	-0.0254	-0.0304	-	-0.0580
	Charge Differential	0.0941	0.0974	0.0776	-	0.0772
2,3,5,6-TeCA	C-Cl Bond Length (Å)	**1.7012**	1.69652	-	1.6971	**1.7012**
	Carbon Charge	**-0.1576**	-0.0045	-	-0.0049	**-0.1577**
	Chlorine Charge	**0.0070**	0.0201	-	0.0200	**0.0067**
	Bonde Charge	**-0.1506**	0.0156	-	0.0151	**-0.1510**
	Charge Differential	**0.1646**	0.0246	-	0.0249	**0.1644**
2,3,4-TrCA	C-Cl Bond Length (Å)	1.6978	**1.6950**	1.6961	-	-
	Carbon Charge	-0.0694	**-0.0595**	-0.0612	-	-
	Chlorine Charge	0.0202	**0.0293**	0.0142	-	-
	Bonde Charge	-0.0492	**-0.0302**	-0.0470	-	-
	Charge Differential	0.0896	**0.0888**	0.0754	-	-
2,3,5-TrCA	C-Cl Bond Length (Å)	1.6960	**1.6975**	-	1.6996	-
	Carbon Charge	-0.0785	**-0.0522**	-	-0.0515	-
	Chlorine Charge	0.0204	**0.0142**	-	-0.0032	-
	Bonde Charge	-0.0581	**-0.0380**	-	-0.0547	-
	Charge Differential	0.0989	**0.0664**	-	0.0483	-

[a] Values in bold denote the carbon positions where dechlorination was predominantly observed in the PCA-dechlorinating, enrichment culture (Chapter 7).

Table 10.6. (cont.)

		Chlorine Substituent Position on Benzene Ring				
Compound	Property	2	3	4	5	6
2,3,6-TrCA	C-Cl Bond Length (Å)	1.6970	1.6974	-	-	1.6995
	Carbon Charge	-0.0721	-0.0606	-	-	-0.0735
	Chlorine Charge	0.0199	0.0116	-	-	0.0010
	Bonde Charge	-0.0522	-0.0490	-	-	-0.0725
	Charge Differential	0.0920	0.0722	-	-	0.0745
2,4,5-TrCA	C-Cl Bond Length (Å)	1.7030	-	**1.6970**	1.6983	-
	Carbon Charge	-0.1510	-	**-0.1201**	-0.0027	-
	Chlorine Charge	-0.0155	-	**0.0070**	0.0103	-
	Bonde Charge	-0.1665	-	**-0.1131**	0.0076	-
	Charge Differential	0.1355	-	**0.1271**	0.0130	-
2,4,6-TrCA	C-Cl Bond Length (Å)	**1.7044**	-	1.6989	-	**1.7039**
	Carbon Charge	**-0.1464**	-	-0.1170	-	**-0.1465**
	Chlorine Charge	**-0.0142**	-	-0.0069	-	**-0.0137**
	Bonde Charge	**-0.1606**	-	-0.1239	-	**-0.1602**
	Charge Differential	**0.1322**	-	0.1101	-	**0.1328**
3,4,5-TrCA	C-Cl Bond Length (Å)	-	1.6997	**1.6943**	1.6986	-
	Carbon Charge	-	-0.0015	**-0.1252**	-0.0013	-
	Chlorine Charge	-	0.0098	**0.0226**	0.0112	-
	Bonde Charge	-	0.0083	**-0.1026**	0.0099	-
	Charge Differential	-	0.0113	**0.1478**	0.0125	-
2,3-DCA	C-Cl Bond Length (Å)	1.7031	1.6996	-	-	-
	Carbon Charge	-0.1563	-0.004	-	-	-
	Chlorine Charge	-0.008	0.0021	-	-	-
	Bonde Charge	-0.1643	-0.0019	-	-	-
	Charge Differential	0.1483	0.0061	-	-	-
2,4-DCA	C-Cl Bond Length (Å)	1.7058	-	1.7007	-	-
	Carbon Charge	-0.1489	-	-0.1209	-	-
	Chlorine Charge	-0.0236	-	-0.0172	-	-
	Bonde Charge	-0.1725	-	-0.1381	-	-
	Charge Differential	0.1253	-	0.1037	-	-

[a] Values in bold denote the carbon positions where dechlorination was predominantly observed in the PCA-dechlorinating, enrichment culture (Chapter 7).

Table 10.6. (cont.)

		Chlorine Substituent Position on Benzene Ring				
Compound	Property	2	3	4	5	6
2,5-DCA	C-Cl Bond Length (Å)	1.7061	-	-	1.7016	-
	Carbon Charge	-0.1555	-	-	-0.0059	-
	Chlorine Charge	-0.0261	-	-	-0.0152	-
	Bonde Charge	-0.1816	-	-	-0.0211	-
	Charge Differential	0.1294	-	-	-0.0093	-
2,6-DCA	C-Cl Bond Length (Å)	1.706	-	-	-	1.7061
	Carbon Charge	-0.1505	-	-	-	-0.1508
	Chlorine Charge	-0.0244	-	-	-	-0.024
	Bonde Charge	-0.1749	-	-	-	-0.1748
	Charge Differential	0.1261	-	-	-	0.1268
3,4-DCA	C-Cl Bond Length (Å)	-	1.6988	1.6979	-	-
	Carbon Charge	-	-0.0043	-0.1245	-	-
	Chlorine Charge	-	0.0021	-0.0019	-	-
	Bonde Charge	-	-0.0022	-0.1264	-	-
	Charge Differential	-	0.0064	0.1226	-	-
3,5-DCA	C-Cl Bond Length (Å)	-	**1.7031**	-	**1.7024**	-
	Carbon Charge	-	**-0.0003**	-	**-0.0009**	-
	Chlorine Charge	-	**-0.0138**	-	**-0.013**	-
	Bonde Charge	-	**-0.0141**	-	**-0.0139**	-
	Charge Differential	-	**-0.0135**	-	**-0.0121**	-

[a] Values in bold denote the carbon positions where dechlorination was predominantly observed in the PCA-dechlorinating, enrichment culture (Chapter 7).

(electron sharing) between the electron orbitals of the two atoms is lower, thus representing a location where substituent removal is more favorable. The bond strength may be increased (hence bond length decreased) at these locations due to the larger ionic attraction component as a result of the larger charge differential. In general, higher charge differentials correlate with shorter bond lengths. As noted above, however, if this added strength is ionic (electronic attraction) in nature rather than covalent (electron sharing), then these sites may still be more favorable for reduction. When the experimental results are compared with the calculated charge differentials, this assumption agreed for all cases except for 2,3,4,5-TeCA, 2,3,4-TrCA, 2,3,5-TrCA, and 2,4,5-TrCA. It was observed that, for PCA the maximum charge differential which was located at the *para* position resulted in the production of 2,3,5,6-TeCA (56%). The second highest charge differential which was located at the *ortho* position on PCA resulted in the production of 2,3,4,5-TeCA (44%) (Tables 10.5 and 10.6).

Based on electron affinity studies, Arulmozhiraja and Morita (2004) showed that during the dechlorination of polychlorinated dibenzofurans (PCDFs), the ionizing electron is first accepted into the π^* orbitals of the neutral PCDFs and the C-Cl bond length is increased and bend toward the plane of the π system. They suggested that electron attachment weakens the C-Cl bonds and these weak bonds could lead to the dechlorination of PCDFs. Cozza and Woods (1992), using MOPAC/MNDO subroutine, reported a correlation between biologically catalyzed reduction reactions and the carbon atom of the parent compound at which the dechlorination reaction takes place for chlorinated phenols, dihydroxybenzenes, benzoic acids, and chloroanilines. They

observed that in most cases, dechlorination took place on the most negatively charged carbon atoms that were surrounded by the highest electron density.

The energy gap between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) is another electronic parameter that has been used to describe the dechlorination potential of chlorinated compounds. HOMO and LUMO energies of CAs were calculated using MOPAC (Table 10.7). The HOMO energy indicates the ionization potential in a molecule and characterizes the susceptibility of the molecule toward attack by electrophiles. The LUMO energy correlates directly with electronaffinity and characterizes the susceptibility of the molecule toward nucleophilic attack. Heimstad *et al.* (2001) observed that the electronaffinity (i.e., the negative energy of LUMO) of chlorinated bornanes increased with increasing degree of chlorination. However, for the CAs the LUMO energy decreased with decreasing degree of chlorination. The HOMO–LUMO gap was used as an indicator of stability in a molecule (Lynam *et al*., 1998). The increase in the gap makes the molecule more stable toward further reaction. The HOMO–LUMO gap decreases with increasing extent of chlorination (Lynam *et al*., 1998). The HOMO and LUMO energies were also compared with the experimentally observed reductive dechlorination rates of CAs (Chapter 7) and results are shown in Figure 10.4. An increase in the HOMO energy was observed with decreasing degree of chlorination. A correlation was not observed between the experimentally obtained dechlorination rates and HOMO and LUMO energies.

Table 10.7. HOMO and LUMO energies of chlorinated aniline congeners

Compound	HOMO (eV)	LUMO (eV)
PCA	-8.801	-0.653
2,3,4,5-TeCA	-9.138	-0.518
2,3,4,6-TeCA	-8.741	-0.448
2,3,5,6-TeCA	-8.862	-0.507
2,3,4-TrCA	-9.538	-0.334
2,3,5-TrCA	-9.505	-0.363
2,3,6-TrCA	-9.508	-0.353
2,4,5-TrCA	-8.631	-0.199
2,4,6-TrCA	-8.613	-0.180
3,4,5-TrCA	-8.675	-0.135
2,3-DCA	-8.570	0.075
2,4-DCA	-8.467	0.123
2,5-DCA	-8.589	0.029
2,6-DCA	-8.537	0.064
3,4-DCA	-8.499	0.129
3,5-DCA	-8.689	0.052
2-CA	-8.375	0.391
3-CA	-8.459	0.377
4-CA	-8.315	0.469
Aniline	-8.212	0.757

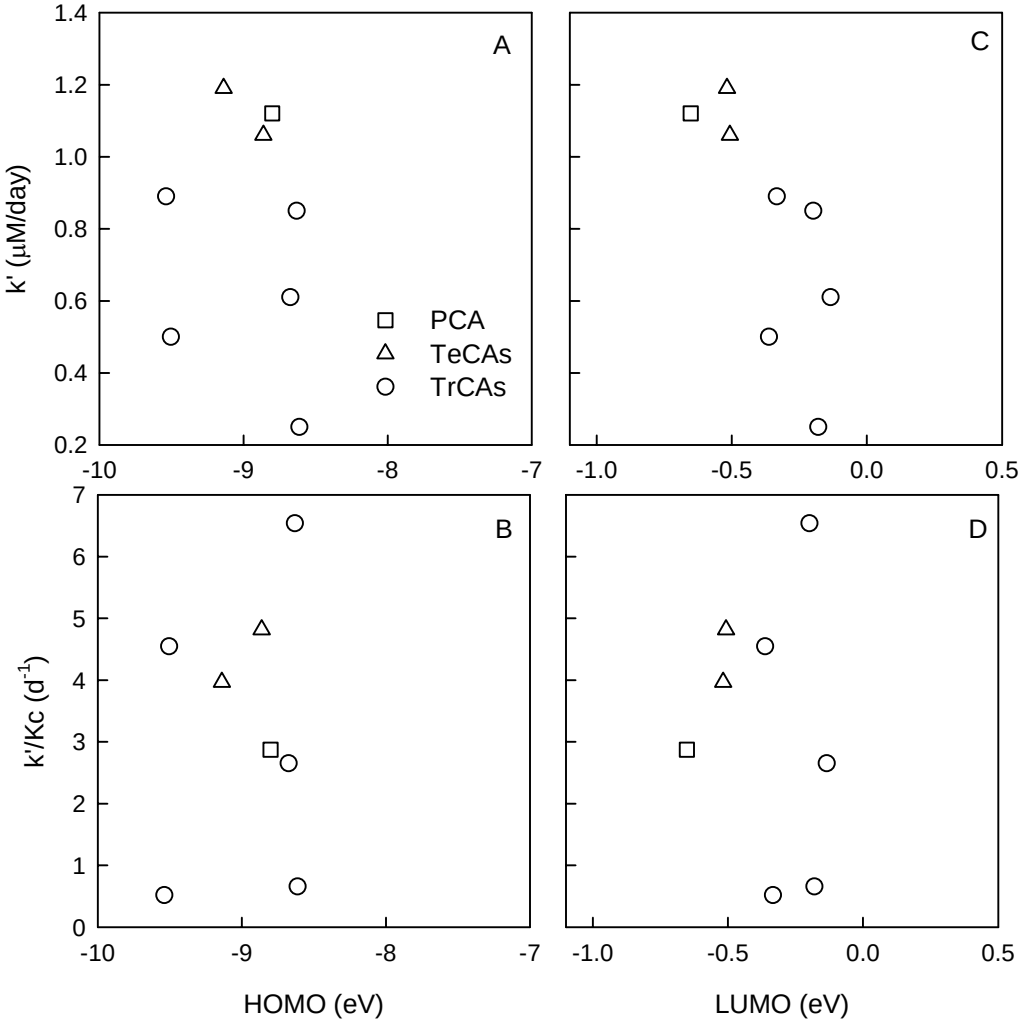


Figure 10.4. Experimentally observed dechlorination rates of CAs vs. HOMO (A and B) and LUMO (C and D) energies.

Enzymatic specificity is another factor involved in the microbial reductive declorination of chlorinated compounds. The transformation pathway is a function of the microbial community composition, acclimation, and enrichment conditions. As it is discussed in Chapter 7, temperature may also play an important role in the reductive declorination pathway of chlorinated compounds. Wu *et al*. (1996, 1997) reported significant differences in the extent and pattern in the microbial reductive dechlorination of a polychlorinated biphenyl (PCB) at different incubation temperatures with freshwater sediment samples. Culture enrichment resulting in the development of specific dechlorinating species which have different enzymes and/or enzymatic activities may result in different dechlorination pathways. For example, cultures acclimated to hexachlorobenzene (HCB) reported to follow the dominant dechlorination pathway which is thermodynamically favorable and form 1,3,5-Trichlorobenzene (TrCB) (Brahushi *et al*., 2004). However, cultures that have been acclimated to 1,2,3,4-TeCB or 1,2,4-TrCB have been shown to dechlorinate HCB via a route that forms 1,2,3,4-TeCB then 1,2,3-TrCB and 1,2-DCB (Middeldorp *et al.,* 1997*;* Ramanand *et al.,* 1993). Susarla *et al.* (1996) reported the following 2,3,4,5-TeCA biotransformation pathway in assays conducted with a contaminated sediment under sulfidogenic conditions: 2,3,4,5-TeCA $\rightarrow$ 3,4,5-TrCA $\rightarrow$ 3,5-DCA $\rightarrow$ 3-CA with traces of 2-CA and 4-CA. In a subsequent study on the reductive dechlorination of chloroanilines by the same contaminated sediment under sulfidogenic conditions, Susarla *et al.* (1997) observed the following reductive dechlorination pathway of 2,3,4,5-TeCA $\rightarrow$ 2,3,5-TrCA$\rightarrow$ 3,5-DCA, $\rightarrow$ 3-CA. Thus, the differences between pathways appear to be controlled by enzyme specificity rather than by thermodynamic potential.

Although the theoretical reaction model based on energetics presented here may be limited in some cases (i.e., enzyme specificity, acclimation period and conditions) it still may be useful in defining the reductive dechlorination pathways under appropriate conditions. All proposed models for the prediction of dechlorination pathways of haloorganic compounds are not sufficient to completely describe the observed dechlorination reactions. Therefore, more investigation is needed to predict biological dechlorination pathways of haloorganic compounds.

10.3.3. Comparison of Chlorinated Anilines and Chlorinated Benzenes Dechlorination Pathways

The dechlorination pathways of chlorinated anilines and chlorobenzenes by mixed, sediment-free enrichment cultures were compared to assess the effect of the amino group on the sequential microbial reductive dechlorination. A previously reported dechlorination pathway for hexachlorobenzene (HCB) in an enrichment culture (Prytula, 1998) was used to compare experimental results obtained with the culture developed in the present study from the same contaminated sediment. The HCB-dechlorinating enrichment culture was fed with glucose, methanol, and yeast extract resulting in initial concentrations of 300, 197, and 30 mg/L, respectively. Incubation was carried out at 22°C. The culture pH ranged between 7.1 and 7.3. The enrichment culture sustained its HCB dechlorination capacity for over 5 years. The experimentally observed, predominant dechlorination reactions were: HCB $\rightarrow$ PeCB $\rightarrow$ 1,2,3,5-TeCB; 1,2,3,4-TeCB $\rightarrow$ 1,2,4-TrCB; 1,2,3,5-TeCB $\rightarrow$ 1,3,5-TrCB; 1,2,3-TrCB $\rightarrow$ 1,3-DCB; and 1,2,4-TrCB $\rightarrow$ 1,4-DCB (Prytula, 1998; Pavlostathis and Prytula, 2000). Prytula (1998) used the

MOPAC/AM1 to estimate the thermodynamic and electronic properties of chlorinated benzene congeners and reaction intermediates and compared these results with the experimentally observed pathway. Prytula (1998) reported that free energy of intermediates described the formation of most of the experimentally observed dechlorination product isomers better than overall reaction free energies. The computed electronic properties of CBs with MOPAC/AM1 are shown in Table 10.8 (Prytula, 1998). He observed a close correlation between the experimentally observed dechlorination pathway and charge distribution of the C-Cl bond sites of parent chlorinated benzene congeners for most but not all of the observed chlorobenzene isomers. The best correlation was observed in terms of the calculated charge differentials except for 1,2,4-TrCB which was not dechlorinated at the site with the highest charge differential (Table 10.8).

The comparison of the experimentally observed sequential dechlorination of CBs and CAs is shown in Figure 10.4. PCA was transformed to 2,3,5,6-TeCA (56%) and 2,3,4,5-TrCA (44%) via *para* and *ortho* dechlorination, whereas PeCB was transformed to 1,2,3,5-TeCB (91%) and 1,2,4,5-TeCB (9%) via *meta* and *para* dechlorination, respectively. 2,3,4,5-TeCA and 1,2,3,4-TeCB were transformed to 2,3,5-TrCA (50%), 2,4,5-TrCA (34%), and 1,2,4-TrCB (100%) via *para* and *meta* dechlorination. The following TrCAs (2,3,4-TrCA, 2,3,5-TrCA and 3,4,5-TrCA, 2,4,5-TrCA) were dechlorinated via *meta* and *para* dechlorination, respectively, whereas 1,2,3-TrCB and 1,2,4-TrCB were dechlorinated via *meta* dechlorination.

CBs and CAs were compared in terms of electronic properties. A similar dechlorination pathway was observed for CBs and CAs in terms of the charge differential

Table 10.8. Electronic properties of five chlorinated benzene congeners with multiple possible dechlorination product isomers (Prytula, 1998)[a]

		Chlorine Substituent Position on Benzene Ring				
Compound	Property	1	2	3	4	5
PeCB	C-Cl Bond Length (Å)	1.6939	1.6914	1.6926	**1.6914**[a]	1.6939
	Carbon Charge	-0.0500	-0.0583	-0.0497	**-0.0583**	-0.0500
	Bond Charge	-0.0187	-0.0116	-0.0025	**-0.0116**	-0.0187
	Charge Differential	0.0813	0.1049	0.0969	**0.1049**	0.0813
1,2,3,4-TeCB	C-Cl Bond Length (Å)	1.6948	1.6925	**1.6925**	1.6948	-
	Carbon Charge	-0.0568	-0.0558	**-0.0558**	-0.0568	-
	Bond Charge	-0.0340	-0.0153	**-0.0153**	-0.0340	-
	Charge Differential	0.0797	0.0964	**0.0964**	0.0797	-
1,2,3,5-TeCB	C-Cl Bond Length (Å)	1.6950	**1.6911**	1.6950	-	1.6965
	Carbon Charge	-0.0477	**-0.0644**	-0.0477	-	-0.0489
	Bond Charge	-0.0221	**-0.0235**	-0.0221	-	-0.0390
	Charge Differential	0.0733	**0.1053**	0.0733	-	0.0589
1,2,3-TrCB	C-Cl Bond Length (Å)	1.6962	**1.6925**	1.6962	-	-
	Carbon Charge	-0.0549	**-0.0618**	-0.0549	-	-
	Bond Charge	-0.0391	**-0.0284**	-0.0391	-	-
	Charge Differential	0.0706	**0.0953**	0.0706	-	-
1,2,4-TrCB	C-Cl Bond Length (Å)	1.6948	**1.6951**	-	1.6977	-
	Carbon Charge	-0.0631	**-0.0540**	-	-0.0558	-
	Bond Charge	-0.0467	**-0.0358**	-	-0.0551	-
	Charge Differential	0.0795	**0.0723**	-	0.0565	-

[a] Values in bold denote the carbon position where dechlorination was predominantly observed in the HCB-acclimated, enrichment culture (Prytula, 1998).

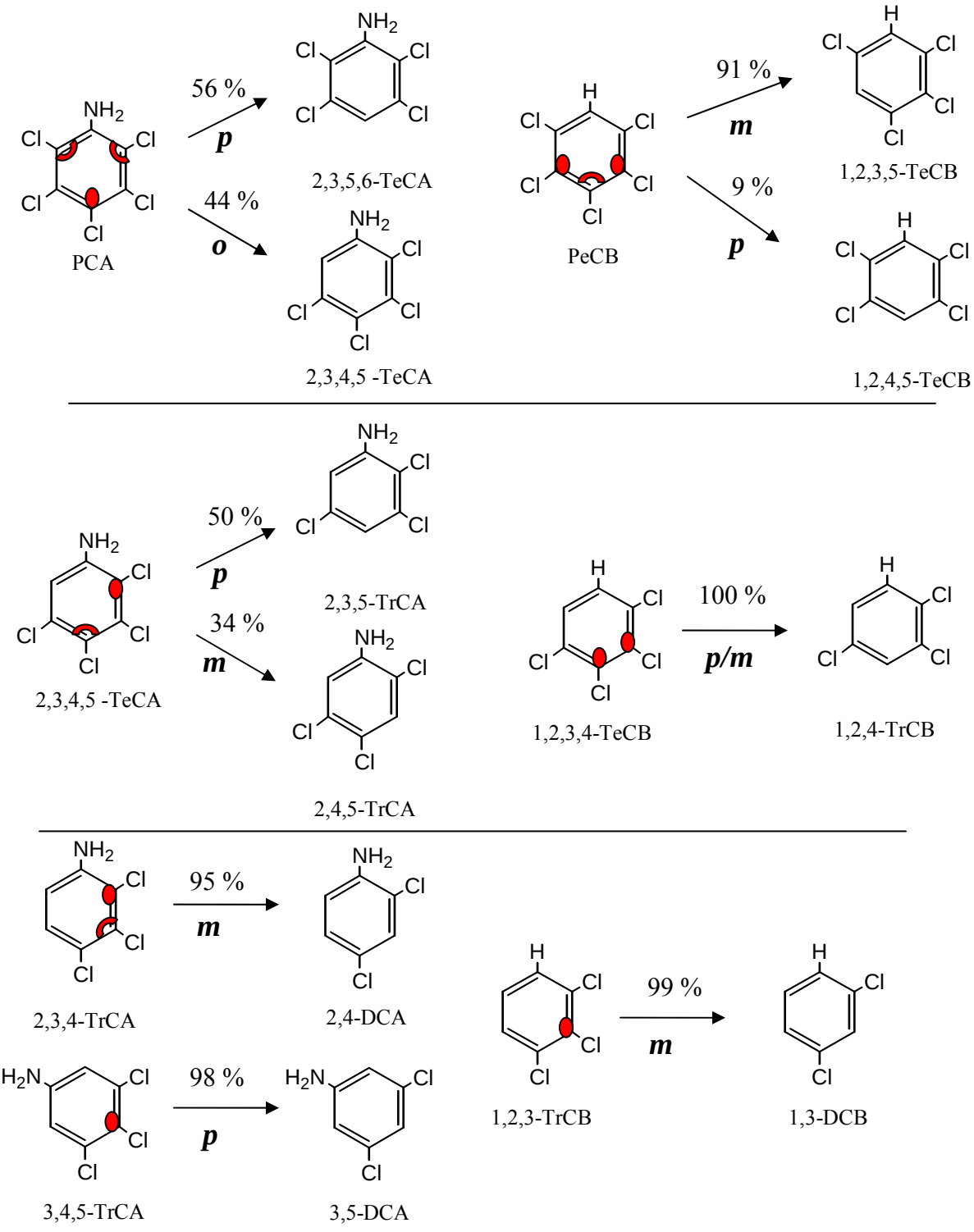


Figure 10.5. Comparison of chloroanilines and chlorobenzenes dechlorination reactions in the sediment-derived enrichment cultures (Chlorobenzenes dechlorination and charge differential source: Prytula, 1998)(Highest charge differential Second highest charge differential)

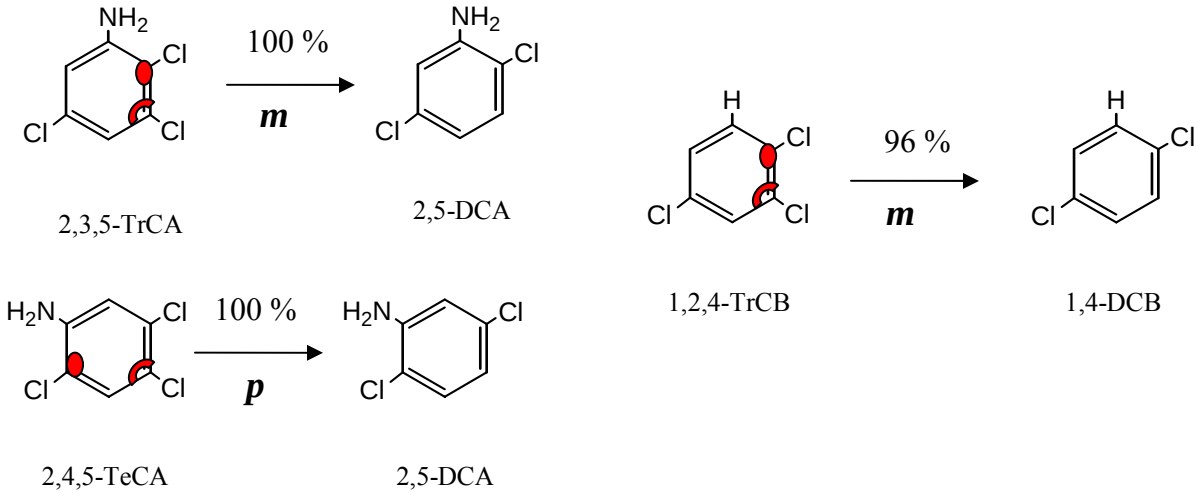


Figure 10.5. (cont.)

values calculated for the carbon-chloride bonds (Figure 10.5). Based on the Cl atoms location on the benzene or aniline ring, the chlorine atoms were removed from the carbon atom that has the highest charge differential or the second highest charge differential. If the highest charge differential for the Cl atom was located adjacent to the amino group, dechlorination took place in the second highest charge differential Cl atom. As discussed in Chapter 2, the chlorine groups are electron-withdrawing substituents which are relatively weak deactivating substituents of the benzene ring. The strong electron-donating property of the amino group affected the sequential dechlorination of chlorinated anilines as oppose to chlorobenzenes. Therefore, the preferential Cl removals in the chlorinated anilines from the second highest charge differential rather than the highest charge differential can be explained by the strong electron-donating property of the amino group.

10.4. Summary

The product isomer distribution during the sequential reductive dechlorination of PCA was examined based on calculated thermodynamic and electronic properties using the semi-empirical molecular modeling method AM1 and then compared to the product distribution achieved by the PCA-dechlorinating, enrichment culture. The dechlorination pathway analysis based on free energy considerations of compounds formed was able to describe the formation of some of the products, especially for the less chlorinated anilines. Correlations between the observed dechlorination pattern and electronic properties of the parent chlorinated aniline congeners were able to explain the formation of most but not all of the observed chloroaniline isomers. The comparison of the experimentally observed sequential reductive dechlorination of CBs and CAs resulted in a similar predicted dechlorination pathways based on the charge differential values calculated for the carbon-chloride bonds. However, thermodynamic and electronic properties of the chlorinated aniline congeners may not be sufficient to describe the sequential microbial reductive dechlorination of chlorinated anilines. Enzymatic specificity, as well as other culture and environmental factors should be considered for the interpretation of observed sequential reductive dehalogenation pathways of haloorganic compounds.

CHAPTER 11

CONCLUSIONS AND RECOMMENDATIONS

The study presented here demonstrates the reductive transformation potential of PCNB to PCA under various environmental conditions. The transformation potential of PCNB to PCA under abiotic conditions indicated that microbial activity is not completely necessary for this transformation process. However, PCA was recalcitrant under abiotic, reductive conditions. The assays conducted in this study showed that microbial activity is necessary for the reductive sequential dechlorination of PCA, which was sequentially dechlorinated to 2,5-DCA in the enrichment culture under fermentative/methanogenic conditions. Glucose, methanol, acetate as well as H_2 were used as electron donors by the enrichment culture and supported PCA dechlorination. The dechlorination kinetics of chlorinated anilines in the enrichment culture were successfully described using a Michaelis-Menten type kinetic model. Variations in the physicochemical and environmental factors (i.e., pH, temperature) significantly affected the reductive (bio)transformation rate and extent of PCNB as well as the sequential dechlorination of PCA. The effect of temperature on the PCA dechlorination rate was modeled using an Arrhenius relationship which accounts for both enzyme activation and deactivation. Although, PCNB transformed to PCA in the presence of alternative electron acceptors (i.e., nitrate, iron), sequential PCA dechlorination was observed only at low nitrate concentrations and in the presence of less bioavailable iron sources.

The following specific conclusions can be drawn based on the results of this study:

1. PCNB was transformed to PCA under both abiotic and biotic conditions, but the transformation rates were significantly higher under biotic conditions. Further investigation with autoclaved culture controls and specific inhibitors showed that the abiotic PCNB transformation to PCA was significantly enhanced in the presence of biotically-derived reductants and/or other factors.
2. Under fermentative/methanogenic conditions, sequential reductive dechlorination of PCA led to the production of mainly dichlorinated anilines via the following biotransformation pathway: PCA → 2,3,4,5- and 2,3,5,6-tetrachloroaniline (TeCA) → 2,4,5- and 2,3,5-trichloroaniline (TrCA)→ 2,5-dichloroaniline (DCA) → 3-CA (low levels). The sequential dechlorination products are more soluble, less hydrophobic, and less toxic as well as are more mobile compared to either PCNB or PCA. The main biotransformation product of PCNB by the mixed, enrichment culture was 2,5-DCA.
3. The biotransformation of PCNB to PCA and its sequential dechlorination pathway were not affected in the presence of the methanogenesis inhibitor 2-bromoethanesulfonate. Therefore, methanogens were not directly responsible for the observed PCA dechlorination in the mixed enrichment culture. Using 16S rRNA gene-targeted primers specific for members of the *Dehalococcoides*, *Dehalobacter*, *Desulfuromonas*, *Geobacter*, and *Anaeromyxobacter* groups, *Dehalococcoides* was detected in the PCNB-biotransforming culture, which may be responsible for the reductive dechlorination of PCA. However, it is not known at present which

dechlorination reactions in the observed sequential dechlorination of PCA are mediated by *Dehalococcoides*.

4. The sequential dechlorination of PCA and other chlorinated aniline congeners was quantitatively described by Michaelis-Menten kinetics. The maximum dechlorination rate (k') of the chlorinated anilines ranged from 0.25 to 1.19 μM/day and the half-saturation coefficient (K_C) ranged from 0.11 to 1.72 μM at an incubation temperature of 22°C and pH 6.9.
5. PCNB was transformed to PCA in a pH range from 2.7 to 7.6 and a temperature range from 4 to 45°C. However, sequential PCA dechlorination was only observed at a temperature range from 4 to 35°C and at a pH range from 6.2 to 7.6. The highest PCA dechlorination rate was observed at 22°C and at pH 7.6. Incubation at different pH values resulted in differences in biotransformation rate and extent, but not in terms of products formed. However, significant differences were observed in terms of biotransformation rate, extent, and products as a function of temperature. Dechlorination of 2,3,5-TrCA resulted in the formation of 2,5-DCA (*meta* dechlorination) at 22°C, whereas 3,5-DCA (*ortho* dechlorination) was formed at 35°C. The activation energy (E_a) for the PCA dechlorination was calculated as 14.2 kcal/mol using an Arrhenius relationship which accounts for both enzyme activation and deactivation.
6. PCNB was transformed to PCA at comparable rates to those under fermentative/methanogenic conditions, but dechlorination of PCA and methanogenesis were not observed in cultures amended with a completely bioavailable iron source (i.e., Fe(III)EDTA). In contrast, both methanogenesis and

PCA dechlorination took place at the same time with iron reduction in the same mixed, fermetative methanogenic enrichment culture amended with a less bioavailable iron source (i.e., FeOOH), but at a lower rate as compared to the Fe^{3+}-free control culture. Addition of anthraquinone 2,6-disulfonate (AQDS) to the culture amended with a less bioavailable iron source (i.e., FeOOH) resulted in a higher iron reduction rate, as compared to cultures devoid of AQDS, and a lower rate of both PCA dechlorination and methanogenesis.

7. PCNB was transformed to PCA under nitrate reducing conditions in the PCNB-biotransforming enrichment culture with transformation rates similar to those observed under fermentative/methanogenic conditions. However, sequential dechlorination of PCA took place only at low nitrate concentrations (≤20 mg N/L). Higher nitrate concentrations (≥50 mg N/L) resulted in the accumulation of toxic compounds such as highly chlorinated anilines (i.e., PCA, TeCAs) and reduced nitrogen intermediates (i.e., NO, N_2O). In a culture developed under nitrate reducing conditions, which was repeatedly amended with 0.09 μM PCNB, PCNB was transformed to PCA but further dechlorination or degradation of PCA was not observed for almost one year of incubation under active nitrate reducing conditions.
8. The thermodynamic and electronic properties of PCA and other chlorinated anilines were computed with a semi empirical model (MOPAC/AM1) and compared to the experimentally observed dechlorination pathways. The dechlorination pathway analysis based on the thermodynamic and electronic properties of chlorinated aniline isomers are able to predict the dechlorination pathway of some but not all of the chloroaniline isomers. Therefore, enzymatic specificity, as well as other culture and

environmental factors should be taken into account for the interpretation of observed sequential reductive dehalogenation pathways.

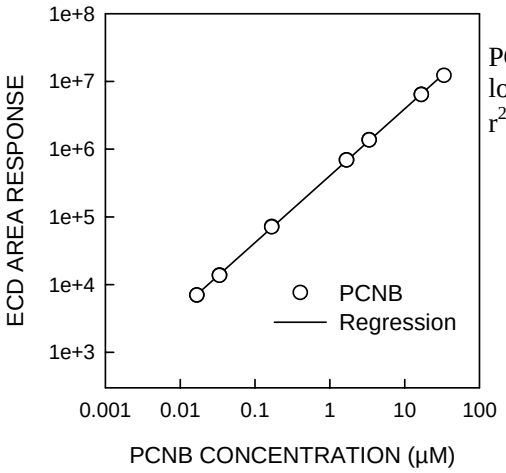
This research showed strong evidence of the presence of PCNB-biotransforming bacterial species which can use chlorinated anilines as terminal electron acceptors. A need for future work relative to the PCNB-biotransforming culture is the isolation of the dechlorinating species. In addition, specific enzymes which mediate the sequential reductive dechlorination of all chlorinated anilines should be identified. Understanding of the physiological characteristics and optimum growth conditions of bacterial strains that transform chlorinated anilines may contribute to the successful application of such bacterial strains in the bioremediation of contaminated sediments and soils.

Although a number of geochemical processes take place in subsurface systems, which affect the fate of contaminants, sorption/desorption and biotransformation are major fate mechanisms for hydrophobic and recalcitrant compounds in natural systems. Sorption and binding of nitroaromatic compounds and their reduction products play an important role in the overall fate of these compounds in subsurface systems. A systematic delineation of the sorption, fate and bioavailability of chloronitroaromatics and chloroanilines in soil systems should be undertaken. Desorption of PCNB from the solid phase (i.e., soil particles) may limit both the rate and extent of reductive biotransformation in natively contaminated (aged) soils and sediments. Further investigation of the bioavailability and transformation of the sediment and/or soil-bound PCNB and/or PCA may result in more accurate estimation of the long-term impact of such contaminants released to the environment.

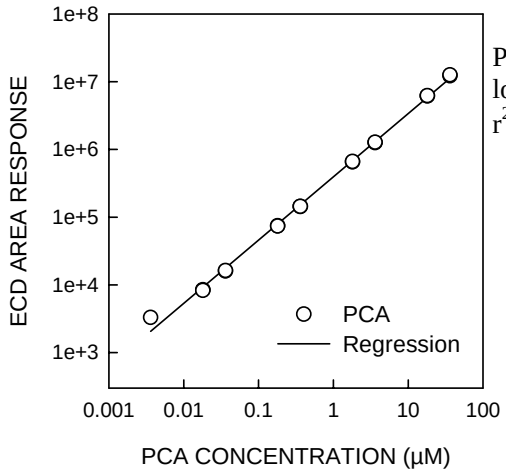
Finally, another area in critical need of further investigation is the biodegradability of the reductive biotransformation products of PCNB under anoxic/aerobic conditions. In certain sites, reductive biotransformation products that are more soluble and thus mobile may migrate into anoxic/aerobic groundwater zones or surface water, where they may be subject to aerobic oxidation processes. Although there is evidence that mono- and di-chlorinated anilines can be degraded under aerobic conditions, this area of research needs further investigation, in particular relative to the factors and mechanisms which control aerobic and/or anoxic biodegradation of the less chlorinated anilines.

APPENDIX A

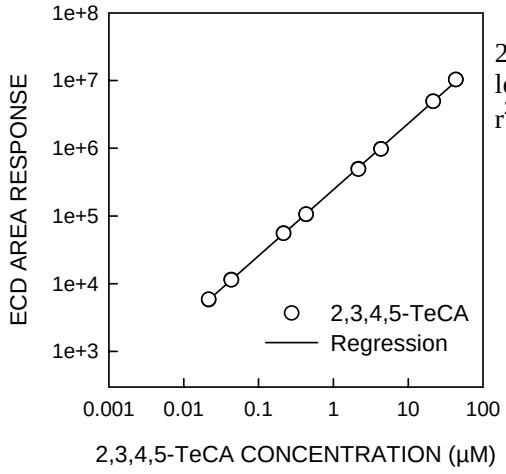
CALIBRATION CURVES



PCNB Calibration:
log (ECD area) = 0.9874 log (µM PCNB) + 5.606
r^2 = 0.999

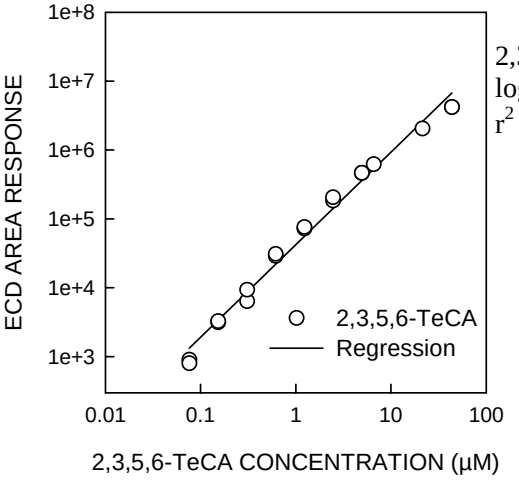


PCA Calibration:
log (ECD area) = 0.9339 log (µM PCA) + 5.595
r^2 = 0.997

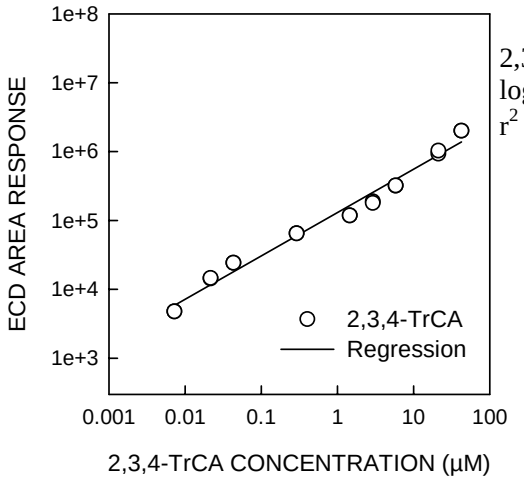


2,3,4,5-TeCA Calibration:
log (ECD area) = 0.9779 log (µM 2,3,4,5-TeCA) + 5.3867
r^2 = 0.999

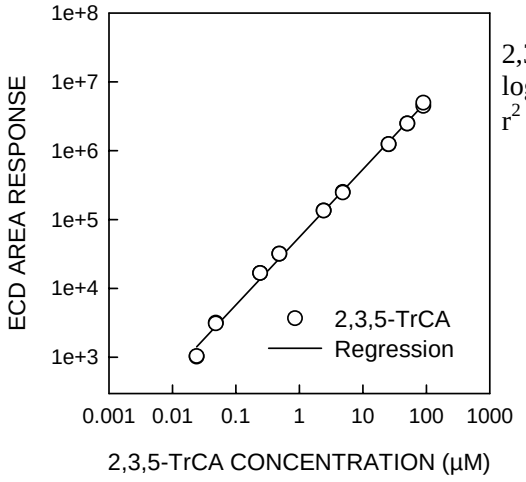
Figure A-1. Sample calibration curves for pentachloronitrobenzene (PCNB), pentachloroaniline (PCA), 2,3,4,5-tetrachloroaniline (2,3,4,5-TeCA).



2,3,5,6-TeCA Calibration:
log (ECD area) = 1.3428 log (µM 2,3,5,6-TeCA) + 4.626
r^2 = 0.986

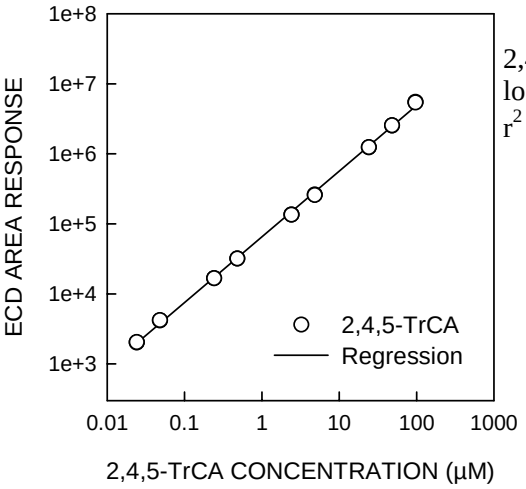


2,3,4-TrCA Calibration:
log (ECD area) = 0.629 log (µM 2,3,4-TrCA) + 5.116
r^2 = 0.981

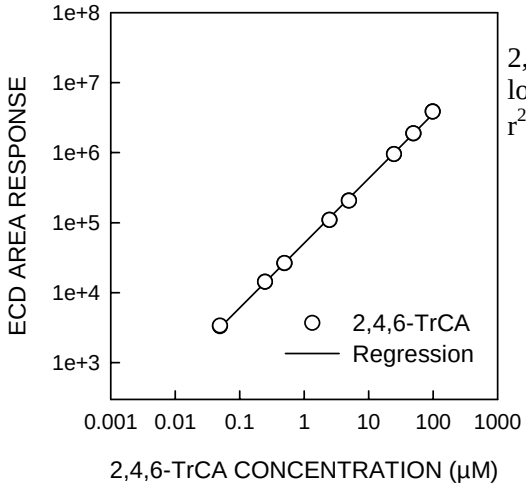


2,3,5-TrCA Calibration:
log (ECD area) = 0.9838 log (µM 2,3,5-TeCA) + 4.744
r^2 = 0.997

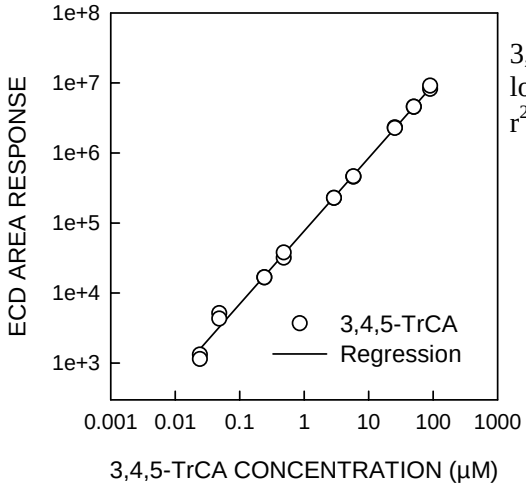
Figure A-2. Sample calibration curves for 2,3,5,6-tetrachloroaniline (2,3,5,6-TeCA), 2,3,4-trichloroaniline (2,3,4-TrCA), 2,3,5-trichloroaniline (2,3,5-TrCA).



2,4,5-TrCA Calibration:
log (ECD area) = 0.9383 log (µM 2,4,5-TrCA) + 4.813
r^2 = 0.999

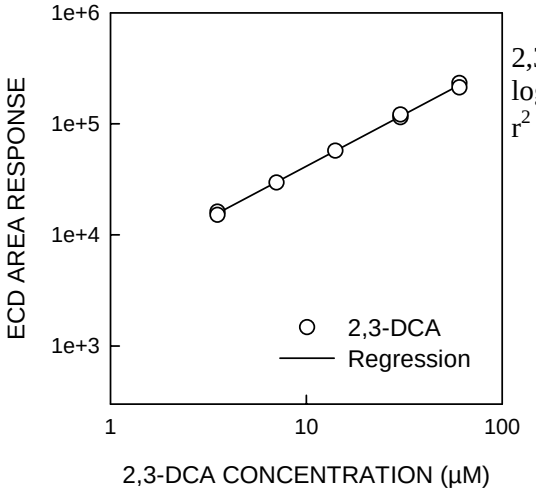


2,4,6-TrCA Calibration:
log (ECD area) = 0.9226 log (µM 2,4,6-TrCA) + 4.707
r^2 = 0.999

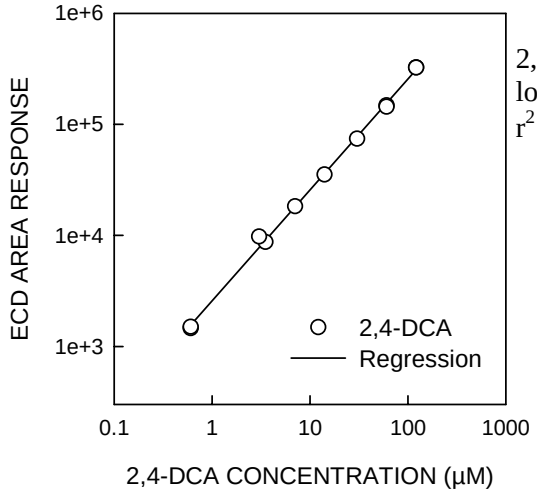


3,4,5-TrCA Calibration:
log (ECD area) = 1.0428 log (µM 3,4,5-TrCA) + 4.886
r^2 = 0.997

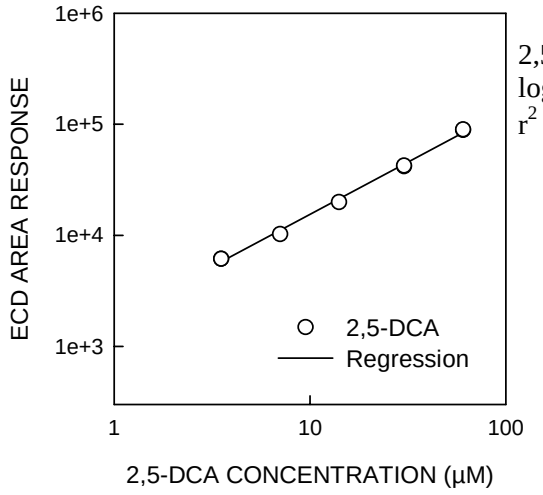
Figure A-3. Sample calibration curves for 2,4,5-trichloroaniline (2,4,5-TrCA), 2,4,6-trichloroaniline (2,4,6-TrCA), 3,4,5-trichloroaniline (3,4,5-TrCA).



2,3-DCA Calibration:
log (ECD area) = 0.935 log (µM 2,3-DCA) + 3.6829
r^2 = 0.999

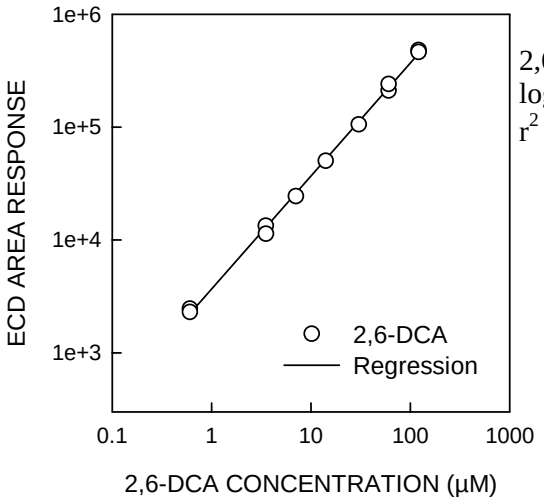


2,4-DCA Calibration:
log (ECD area) = 0.9992 log (µM 2,4-DCA) + 3.41
r^2 = 0.998

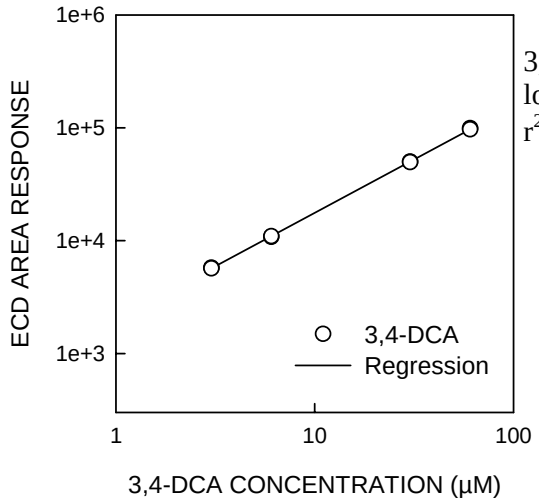


2,5-DA Calibration:
log (ECD area) = 0.9418 log (µM 2,5-DCA) + 3.2477
r^2 = 0.996

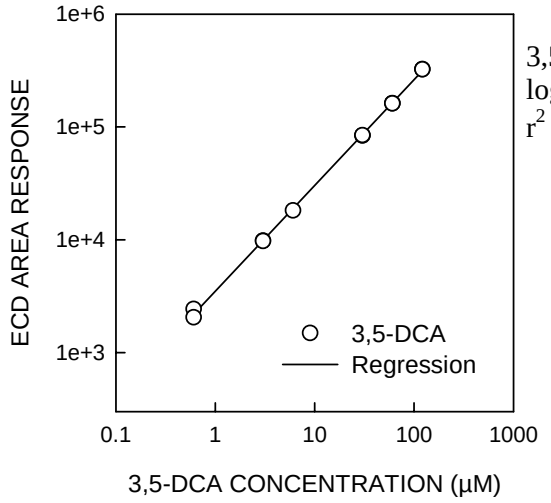
Figure A-4. Sample calibration curves for 2,3-dichloroaniline (2,3-DCA), 2,4-dichloroaniline (2,4-DCA), 2,5-dichloroaniline (2,5-DCA).



2,6-DCA Calibration:
log (ECD area) = 1.001 log (µM 2,6-DCA) + 3.5659
r^2 = 0.998



3,4-DCA Calibration:
log (ECD area) = 1.0428 log (µM 3,4-DCA) + 4.886
r^2 = 0.997



3,5-DA Calibration:
log (ECD area) = 0.938 log (µM 3,5-DCA) + 3.544
r^2 = 0.999

Figure A-5. Sample calibration curves for 2,6-dichloroaniline (2,6-DCA), 3,4-dichloroaniline (3,4-DCA), 3,5-dichloroaniline (3,5-DCA).

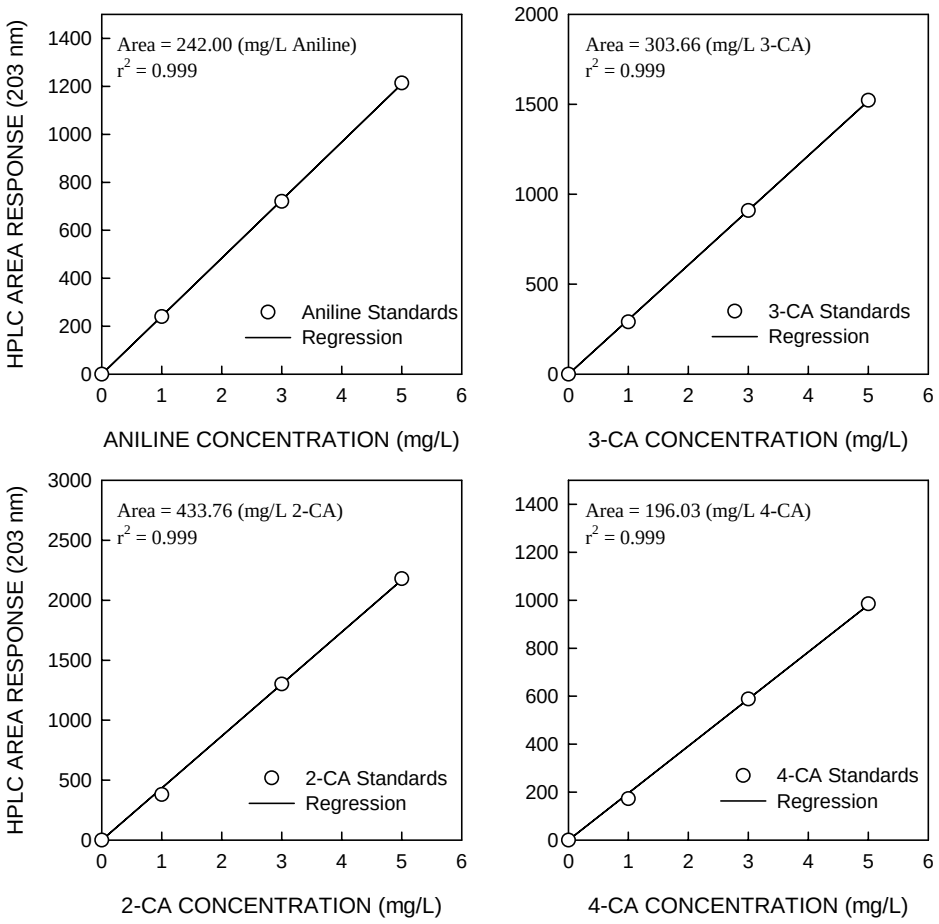


Figure A-6. Sample calibration curves with HPLC for aniline, 2-chloroaniline (2-CA), 3-chloroaniline (3-CA), 4-chloroaniline (4-CA).

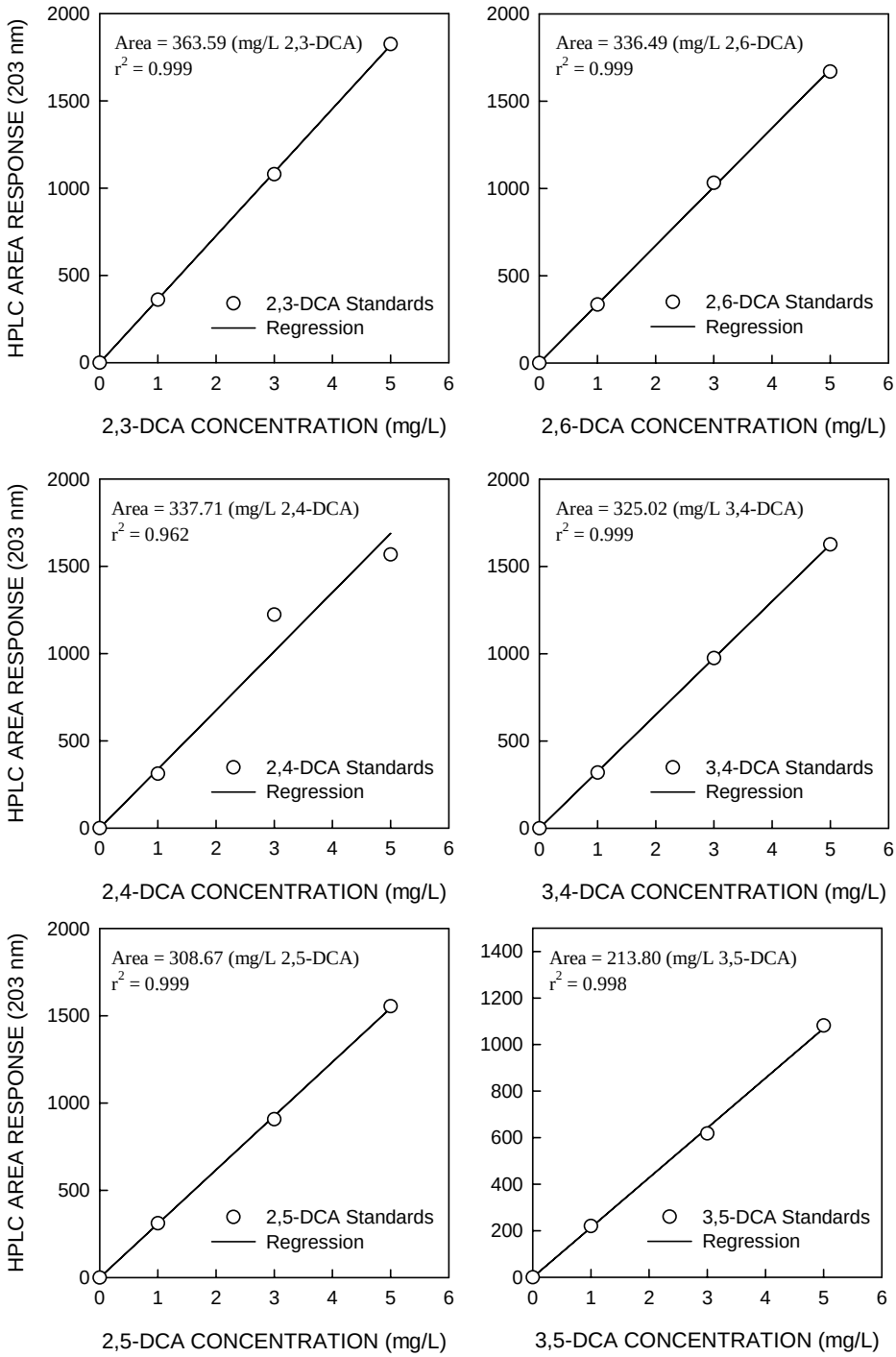


Figure A-7. Sample calibration curves with HPLC for 2,3-dichloroaniline (2,3-DCA), 2,4-dichloroaniline (2,4-DCA), 2,5-dichloroaniline (2,5-DCA), 2,6-dichloroaniline (2,6-DCA), 3,4-dichloroaniline (3,4-DCA), 3,5-dichloroaniline (3,5-DCA).

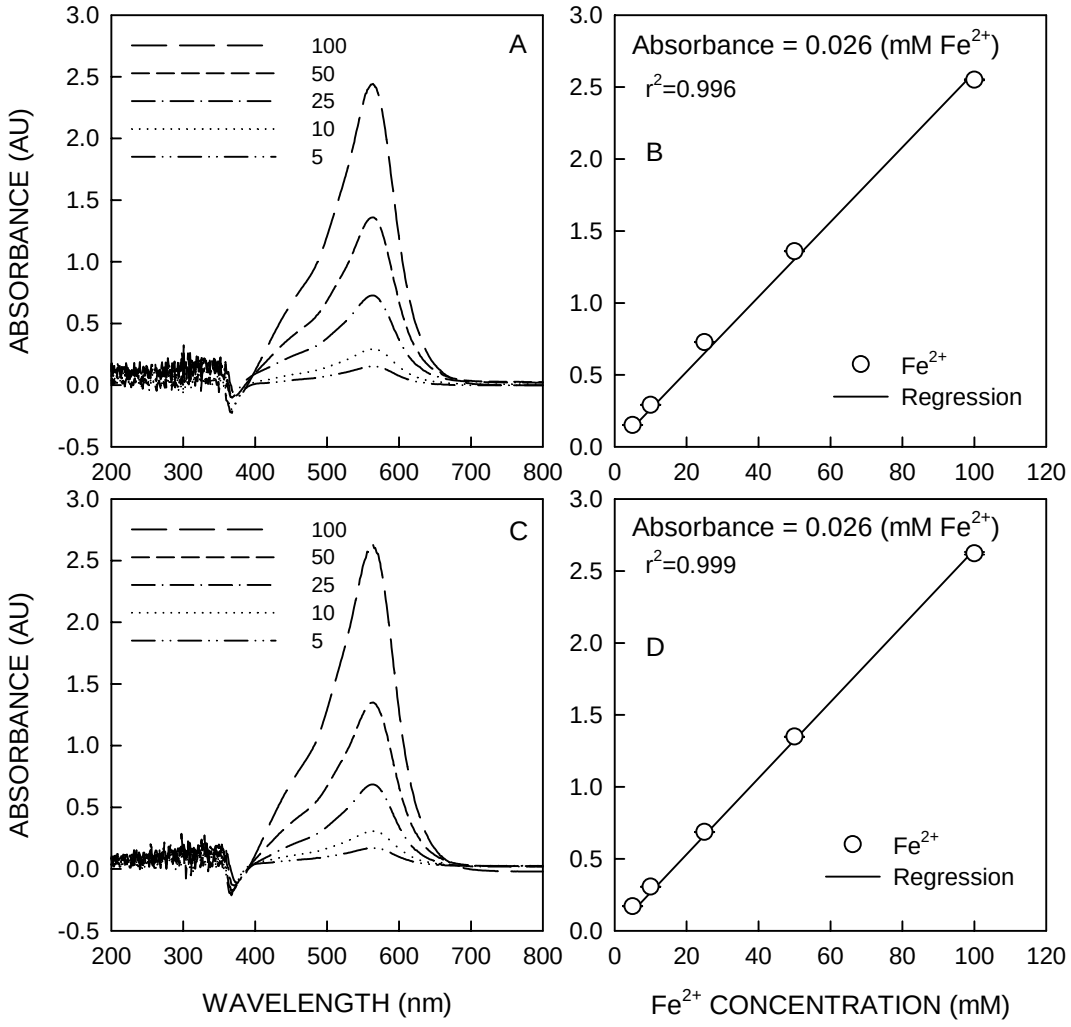
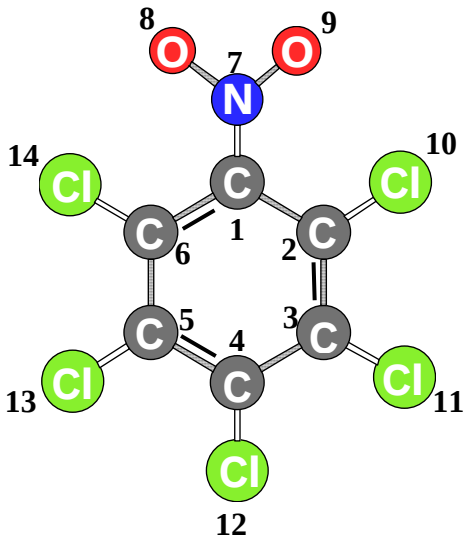


Figure A-8. Sample calibration curves of Fe^{2+}. UV-visible spectra in DI water (A) and calibration curve (B). UV-visible spectra in autoclaved culture media (C) and calibration curve (D)(A 0.5 N HCl solution was used as the blank).

APPENDIX B

SUMMARY OF MOPAC OUTPUT DATA



Pentachloronitrobenzene (PCNB)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			-0.3826	
2	C	1.4019	1	-0.0610	4.0610
3	C	1.3983	2	-0.1742	4.1742
4	C	1.3956	3	-0.0916	4.0916
5	C	1.3954	4	-0.1740	4.1740
6	C	1.3979	5	-0.0609	4.0609
7	N	1.5074	1	1.3107	3.6893
8	O	1.2119	7	-0.5802	6.5802
9	O	1.2118	7	-0.5802	6.5802
10	Cl	1.6722	2	0.1705	6.8295
11	Cl	1.6738	3	0.1479	6.8521
12	Cl	1.667	4	0.1575	6.8425
13	Cl	1.6744	5	0.1472	6.8529
14	Cl	1.6723	6	0.1709	6.829

Pentachloroaniline (PCA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			-0.0455	4.0455
2	C	1.4062	1	-0.1796	4.1796
3	C	1.3941	2	-0.1029	4.1029
4	C	1.3954	3	-0.1697	4.1697
5	C	1.3958	4	-0.1031	4.1031
6	C	1.4062	5	-0.1794	4.1794
7	N	1.4194	1	0.0905	4.9095
8	H	0.9958	7	0.0459	0.9541
9	H	0.9958	7	0.0460	0.9540
10	Cl	1.6825	2	0.1099	6.8901
11	Cl	1.6738	3	0.1268	6.8732
12	Cl	1.6748	4	0.1243	6.8757
13	Cl	1.6738	5	0.1264	6.8736
14	Cl	1.6825	6	0.1102	6.8898

2,3,4,5-tetrachloroaniline (2,3,4,5-TeCA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.1120	3.8880
2	C	1.3400	1	-0.1371	4.1370
3	C	1.4037	2	-0.0066	4.0066
4	C	1.4040	3	-0.1097	4.1097
5	C	1.3903	4	-0.0070	4.0070
6	C	1.4153	5	-0.1936	4.1936
7	N	1.3857	1	-0.3399	5.3399
8	H	0.9942	7	0.2158	0.7842
9	H	1.1021	7	0.2081	0.7919
10	Cl	1.7002	2	0.0109	6.9891
11	Cl	1.6967	3	0.0197	6.9804
12	Cl	1.6930	4	0.0330	6.9670
13	Cl	1.6950	5	0.0374	6.9626
14	H	1.1021	6	0.1570	0.8430

2,3,4,6-tetrachloroaniline (2,3,4,6-TeCA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.0991	3.9009
2	C	1.4186	1	-0.0667	4.0667
3	C	1.4035	2	-0.0614	4.0614
4	C	1.3952	3	-0.0540	4.0540
5	C	1.3944	4	-0.1149	4.1149
6	C	1.4161	1	-0.0676	4.0675
7	N	1.3903	1	-0.4687	5.4687
8	H	0.9834	7	0.2349	0.7651
9	H	0.9830	7	0.2346	0.7654
10	Cl	1.6989	2	0.0274	6.9726
11	Cl	1.6939	3	0.0360	6.9640
12	Cl	1.6947	4	0.0236	6.9764
13	H	1.1031	5	0.1679	0.8321
14	Cl	1.6968	6	0.0096	6.9904

2,3,5,6-tetrachloroaniline (2,3,5,6-TeCA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.1621	3.8380
2	C	1.4250	1	-0.1576	4.1576
3	C	1.3970	2	-0.0045	3.9955
4	C	1.3956	3	-0.1786	4.1786
5	C	1.3956	4	-0.0049	3.9951
6	C	1.4247	1	-0.1577	4.1577
7	N	1.3703	1	-0.3838	5.3838
8	H	0.9887	7	0.2421	0.7579
9	H	0.9888	7	0.2422	0.7578
10	Cl	1.7012	2	0.0070	6.9930
11	Cl	1.6965	3	0.0201	6.9799
12	H	1.1012	4	0.1682	0.8318
13	Cl	1.6971	5	0.0200	6.9800
14	Cl	1.7012	6	0.0067	6.9933

2,3,4-trichloroaniline (2,3,4-TrCA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.0932	3.9068
2	C	1.4181	1	-0.0694	4.0694
3	C	1.4027	2	-0.0595	4.0595
4	C	1.3974	3	-0.0612	4.0612
5	C	1.3884	4	-0.1151	4.1151
6	C	1.4111	1	-0.1355	4.1355
7	N	1.3938	1	-0.4764	5.4764
8	H	0.9831	7	0.2295	0.7705
9	H	0.9825	7	0.2293	0.7707
10	Cl	1.6978	2	0.0202	6.9798
11	Cl	1.6950	3	0.0293	6.9707
12	Cl	1.6961	4	0.0142	6.9859
13	H	1.1011	5	0.1536	0.8464
14	H	1.1022	6	0.1479	0.8521

2,3,5-trichloroaniline (2,3,5-TrCA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.1019	3.8981
2	C	1.4193	1	-0.0785	4.0785
3	C	1.3969	2	-0.0522	4.0522
4	C	1.3958	3	-0.1208	4.1208
5	C	1.3944	4	-0.0515	4.0515
6	C	1.4104	1	-0.1391	4.1391
7	N	1.3938	1	-0.4761	5.4761
8	H	0.9828	7	0.2311	0.7689
9	H	0.9823	7	0.2303	0.7697
10	Cl	1.6960	2	0.0204	6.9797
11	Cl	1.6975	3	0.0142	6.9858
12	H	1.1015	4	0.1655	0.8345
13	Cl	1.6996	5	-0.0032	7.0032
14	H	1.1022	6	0.1580	0.8420

2,3,6-trichloroaniline (2,3,6-TrCA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.0983	3.9017
2	C	1.4195	1	-0.0721	4.0721
3	C	1.3981	2	-0.0606	4.0606
4	C	1.3894	3	-0.1157	4.1157
5	C	1.3966	4	-0.1166	4.1166
6	C	1.4157	1	-0.0735	4.0735
7	N	1.3913	1	-0.4697	5.4697
8	H	0.9828	7	0.2333	0.7667
9	H	0.9831	7	0.2333	0.7667
10	Cl	1.6970	2	0.0199	6.9801
11	Cl	1.6974	3	0.0116	6.9884
12	H	1.1013	4	0.1561	0.8439
13	H	1.1021	5	0.1548	0.8452
14	Cl	1.6995	6	0.0010	6.9990

2,4,5-trichloroaniline (2,4,5-TrCA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.1459	3.8541
2	C	1.4224	1	-0.1510	4.1510
3	C	1.3957	2	-0.0575	4.0575
4	C	1.4031	3	-0.1201	4.1201
5	C	1.3911	4	-0.0027	4.0027
6	C	1.4192	1	-0.2107	4.2107
7	N	1.3721	1	-0.3923	5.3923
8	H	0.9883	7	0.2387	0.7613
9	H	0.9867	7	0.2317	0.7683
10	Cl	1.7030	2	-0.0155	7.0155
11	H	1.1032	3	0.1632	0.8368
12	Cl	1.6970	4	0.0070	6.9930
13	Cl	1.6983	5	0.0103	6.9897
14	H	1.1015	6	0.1528	0.8472

2,4,6-trichloroaniline (2,4,6-TrCA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.1481	3.8519
2	C	1.4239	1	-0.1464	4.1464
3	C	1.3964	2	-0.0610	4.0610
4	C	1.3963	3	-0.1170	4.1170
5	C	1.3912	4	-0.0611	4.0611
6	C	1.4245	1	-0.1465	4.1465
7	N	1.3697	1	-0.3854	5.3854
8	H	0.9874	7	0.2396	0.7604
9	H	0.9889	7	0.2400	0.7600
10	Cl	1.7044	2	-0.0142	7.0141
11	H	1.1027	3	0.1623	0.8377
12	Cl	1.6989	4	-0.0069	7.0069
13	H	1.1028	5	0.1622	0.8378
14	Cl	1.7039	6	-0.0137	7.0137

3,4,5-trichloroaniline (3,4,5-TrCA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.1425	3.8575
2	C	1.4170	1	-0.2143	4.2143
3	C	1.4032	2	-0.0015	3.9985
4	C	1.4047	3	-0.1252	4.1252
5	C	1.3915	4	-0.0013	3.9987
6	C	1.4174	1	-0.2142	4.2142
7	N	1.3753	1	-0.3990	5.3990
8	H	0.9861	7	0.2306	0.7694
9	H	0.9860	7	0.2306	0.7694
10	H	1.1005	2	0.1515	0.8485
11	Cl	1.6997	3	0.0098	6.9902
12	Cl	1.6943	4	0.0226	6.9774
13	Cl	1.6986	5	0.0112	6.9889
14	H	1.1013	6	0.1511	0.8489

2,3-dichloroaniline (2,3-DCA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.1428	3.8573
2	C	1.4225	1	-0.1563	4.1563
3	C	1.3957	2	-0.0040	4.0040
4	C	1.3941	3	-0.1838	4.1838
5	C	1.3858	4	-0.0684	4.0683
6	C	1.4198	1	-0.2158	4.2158
7	N	1.3744	1	-0.3979	5.3979
8	H	0.9878	7	0.2368	0.7632
9	H	0.9860	7	0.2289	0.7711
10	Cl	1.7031	2	-0.0080	7.0079
11	Cl	1.6996	3	0.0021	6.9979
12	H	1.0996	4	0.1497	0.8503
13	H	1.1010	5	0.1372	0.8628
14	H	1.0997	6	0.1366	0.8634

2,4-dichloroaniline (2,4-DCA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.1369	3.8632
2	C	1.4218	1	-0.1489	4.1489
3	C	1.3956	2	-0.0640	4.0640
4	C	1.3991	3	-0.1209	4.1209
5	C	1.3860	4	-0.0680	4.0680
6	C	1.4210	1	-0.2087	4.2087
7	N	1.3732	1	-0.3959	5.3959
8	H	0.9871	7	0.2368	0.7632
9	H	0.9863	7	0.2288	0.7712
10	Cl	1.7058	2	-0.0236	7.0236
11	H	1.1022	3	0.1587	0.8414
12	Cl	1.7007	4	-0.0172	7.0172
13	H	1.1017	5	0.1471	0.8529
14	H	1.1002	6	0.1389	0.8611

2,5-dichloroaniline (2,5-DCA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.1436	3.8564
2	C	1.4215	1	-0.1555	4.1555
3	C	1.3911	2	-0.0626	4.0626
4	C	1.3986	3	-0.1830	4.1830
5	C	1.3906	4	-0.0059	4.0059
6	C	1.4203	1	-0.2145	4.2145
7	N	1.3736	1	-0.3966	5.3966
8	H	0.9873	7	0.2373	0.7627
9	H	0.9863	7	0.2297	0.7703
10	Cl	1.7061	2	-0.0261	7.0261
11	H	1.1018	3	0.1494	0.8506
12	H	1.0998	4	0.1510	0.8490
13	Cl	1.7016	5	-0.0152	7.0152
14	H	1.1008	6	0.1484	0.8516

2,6-dichloroaniline (2,6-DCA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.1459	3.8541
2	C	1.4237	1	-0.1505	4.1505
3	C	1.3924	2	-0.0669	4.0669
4	C	1.3928	3	-0.1825	4.1825
5	C	1.3924	4	-0.0674	4.0674
6	C	1.4232	1	-0.1508	4.1508
7	N	1.3718	1	-0.3912	5.3912
8	H	0.9883	7	0.2379	0.7621
9	H	0.9879	7	0.2380	0.7620
10	Cl	1.7060	2	-0.0244	7.0244
11	H	1.1012	3	0.1474	0.8526
12	H	1.0989	4	0.1416	0.8584
13	H	1.1017	5	0.1471	0.8529
14	Cl	1.7061	6	-0.0240	7.0240

3,4-dichloroaniline (3,4-DCA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.1340	3.8660
2	C	1.4167	1	-0.2147	4.2147
3	C	1.4020	2	-0.0043	4.0043
4	C	1.3989	3	-0.1245	4.1245
5	C	1.3856	4	-0.0646	4.0646
6	C	1.4197	1	-0.2125	4.2125
7	N	1.3755	1	-0.4025	5.4025
8	H	0.9863	7	0.2282	0.7719
9	H	0.9852	7	0.2277	0.7723
10	H	1.1005	2	0.1480	0.8520
11	Cl	1.6988	3	0.0021	6.9979
12	Cl	1.6979	4	-0.0019	7.0019
13	H	1.1020	5	0.1478	0.8522
14	H	1.0999	6	0.1373	0.8627

3,5-dichloroaniline (3,5-DCA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.1410	3.8591
2	C	1.4175	1	-0.2193	4.2193
3	C	1.3961	2	-0.0003	4.0003
4	C	1.3973	3	-0.1847	4.1847
5	C	1.3916	4	-0.0009	4.0009
6	C	1.4184	1	-0.2188	4.2188
7	N	1.3765	1	-0.4033	5.4033
8	H	0.9863	7	0.2289	0.7711
9	H	0.9862	7	0.2289	0.7711
10	H	1.0996	2	0.1476	0.8524
11	CL	1.7031	3	-0.0138	7.0138
12	H	1.1000	4	0.1604	0.8396
13	CL	1.7024	5	-0.0130	7.0130
14	H	1.1000	6	0.1473	0.8527

2 -chloroaniline (2-CA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.1340	3.8660
2	C	1.4209	1	-0.1534	4.1534
3	C	1.3905	2	-0.0706	4.0706
4	C	1.3965	3	-0.1877	4.1876
5	C	1.3870	4	-0.0763	4.0763
6	C	1.4207	1	-0.2142	4.2142
7	N	1.3749	1	-0.4016	5.4016
8	H	0.9873	7	0.2346	0.7654
9	H	0.9858	7	0.2266	0.7734
10	Cl	1.7067	2	-0.0341	7.0341
11	H	1.1015	3	0.1431	0.8569
12	H	1.0987	4	0.1353	0.8647
13	H	1.1005	5	0.1313	0.8687
14	H	1.1000	6	0.1329	0.8671

3 -chloroaniline (3-CA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.1312	3.8688
2	C	1.4167	1	-0.2185	4.2185
3	C	1.3958	2	-0.0073	4.0073
4	C	1.3943	3	-0.1879	4.1879
5	C	1.3878	4	-0.0719	4.0719
6	C	1.4194	1	-0.2185	4.2185
7	N	1.3775	1	-0.4079	5.4079
8	H	0.9860	7	0.2261	0.7739
9	H	0.9856	7	0.2258	0.7742
10	H	1.0992	2	0.1436	0.8564
11	Cl	1.7037	3	-0.0251	7.0250
12	H	1.0991	4	0.1452	0.8549
13	H	1.1009	5	0.1334	0.8666
14	H	1.0997	6	0.1319	0.8681

4 -chloroaniline (4-CA)

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.1243	3.8757
2	C	1.4187	1	-0.2129	4.2129
3	C	1.3879	2	-0.0712	4.0712
4	C	1.3971	3	-0.1249	4.1249
5	C	1.3976	4	-0.0714	4.0714
6	C	1.4183	1	-0.2128	4.2128
7	N	1.3769	1	-0.4070	5.4070
8	H	0.9855	7	0.2254	0.7746
9	H	0.9855	7	0.2255	0.7745
10	H	1.0992	2	0.1335	0.8665
11	H	1.1009	3	0.1430	0.8570
12	Cl	1.7018	4	-0.0281	7.0281
13	H	1.1013	5	0.1431	0.8570
14	H	1.0997	6	0.1335	0.8665

Aniline

Atom #	Chemical Symbol	Bond Length (Angstroms)	Bond To Atom	Charge	Electron Density
1	C			0.1207	3.8793
2	C	1.41818	1	-0.2184	4.2184
3	C	1.38900	2	-0.0800	4.0800
4	C	1.39466	3	-0.1929	4.1928
5	C	1.39404	4	-0.0801	4.0801
6	C	1.41850	1	-0.2186	4.2186
7	N	1.37878	1	-0.4127	5.4126
8	H	0.98508	7	0.2227	0.7773
9	H	0.98521	7	0.2228	0.7772
10	H	1.09984	2	0.1272	0.8728
11	H	1.10108	3	0.1266	0.8734
12	H	1.09803	4	0.1289	0.8712
13	H	1.10063	5	0.1264	0.8736
14	H	1.09927	6	0.1273	0.8727

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