JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Temperature and pH Effect on the Microbial Reductive Transformation of Pentachloronitrobenzene

DIDEM OKUTMAN TAS AND SPYROS G. PAVLOSTATHIS*

School of Civil and Environmental Engineering, Georgia Institute of Technology, Atlanta, Georgia 30332-0512

The effect of pH and temperature on the microbial reductive transformation of pentachloronitrobenzene (PCNB), an organochlorine fungicide, was investigated with a mixed fermentative/methanogenic culture developed from a contaminated estuarine sediment. Culture series 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 pentachloroaniline (PCA) in all culture series. However, sequential dechlorination of PCA was observed only 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 effect of temperature on the PCA dechlorination rate was modeled using an Arrhenius relationship, which accounts for both enzyme activation and deactivation. The dechlorination of PCA and chlorinated aniline intermediates was simulated using a branched-chain Michaelis−Menten model, and kinetic constants were determined.

KEYWORDS: Biotransformation; chloroanilines; dechlorination; kinetics; PCNB

INTRODUCTION

Pentachloronitrobenzene (PCNB) is an organochlorine fungicide produced in the United States since 1962 (*1*). Most of its usage is found in the southeastern United States because of its effectiveness against phytopathogenic fungi associated with crops predominantly found in this region (*2*). PCNB is also used in the northern tier of the United States on turf (golf courses, sod farms, commercial and industrial turf, and lawns) and nonturf ornamentals. The estimated annual PCNB usage was reported as 770 000−1 000 000 lb, which is applied on cotton (400 000 lb/year), potatoes (60 000 lb/year), and turf (250 000−500 000 lb/year)(*3*). PCNB is also used extensively in many European and Asian countries.

Complete reduction of the nitro group is the first step in the reductive transformation of PCNB, resulting in the formation of pentachloroaniline (PCA) (*4*−*8*). Microbial reductive transformation of PCA resulted in its sequential dechlorination down to di- and in some cases monochloroanilines, but the resulting monochloroanilines were not transformed further (*5*−*11*). Because there is an about 4−5 order of magnitude increase in aqueous solubility and a >3 order of magnitude decrease in the octanol−water partition coefficient between PCA and di- or monochloroanilines (*12*), reductive dechlorination is expected to reduce the bioaccumulation of PCNB, PCA, and other highly chlorinated anilines. Furthermore, many of the less chlorinated anilines generated by reductive dechlorination are biodegradable under aerobic conditions by microorganisms that are isolated from soil, sediments, and wastewater treatment systems (*13*−*17*).

In a previous paper, we reported that the transformation of PCNB to PCA was relatively fast and occurred under both abiotic and biotic reductive conditions (*7*). However, PCA sequential dechlorination was observed only in the presence of biological activity. Subsequently, the reductive dechlorination kinetics of PCA and all available chloroaniline congeners were investigated and reported (*8*). The previous studies were performed at constant temperatures and pH values. However, 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 22−30 °C and at about neutral pH (*5*−*7*). However, depending on depth, location, and climate, PCNB-contaminated soil and sediment environments in a wide range of temperature and pH values are encountered (*18*−*20*).

The objective of the research reported here was to assess 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 a mixed, fermentative/methanogenic culture derived from a contaminated sediment. The kinetics of the

* Author to whom correspondence should be addressed [telephone (404) 894-9367; fax (404) 894-8266; e-mail spyros.pavlostathis@ce.gatech.edu].

10.1021/jf0637675 CCC: $37.00
Published on Web 06/07/2007

sequential dechlorination of PCA under different incubation temperature and pH values were simulated using a branched-chain Michaelis−Menten model.

MATERIALS AND METHODS

Enrichment Culture. An enrichment culture developed from a contaminated sediment was used in this study (*7*). The culture was fed weekly with glucose (330 mg/L), autoclaved culture media (*21*), and a PCNB/methanol solution (0.09 μM/53 mg/L), resulting in a retention time of 84 days. The culture was kept in the dark in a 22 °C constant-temperature room and stirred once a day, and its pH was kept around 7 with $NaHCO_3$ addition. The steady-state biomass concentration of the sediment-free culture was 315 ± 40 mg/L (expressed as particulate organic carbon; POC). Detailed information relative to the culture development was reported elsewhere (*7*). The predominant PCNB biotransformation pathway was as follows: PCNB → 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 enrichment culture has sustained its PCNB biotransformation as well as sequential PCA dechlorination capacity over 4.5 years.

Temperature Assay. The effect of temperature on PCNB (bio)-transformation and PCA dechlorination was assessed using five culture series amended with PCNB and incubated at 4, 15, 22, 35, and 45 °C. Three replicates were used in this part of the study. Assays were conducted in 160 mL serum bottles 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 1 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). To avoid any electron donor limitation and to achieve complete biotransformation, three 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 concentration of PCNB tested in this study was higher than its aqueous solubility. Therefore, to eliminate bioavailability limitations, PCNB was introduced to the culture dissolved in methanol. All nutrients were added to the culture in excess to eliminate any limitations. The initial pH of the cultures varied from 6.8 to 7, and the initial biomass concentration was 320 ± 10 mg POC/L [mean ± standard deviation (SD); $n = 3$]. 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.

pH Assay. The effect of pH on PCNB (bio)transformation was assessed using six culture series with the following pH values (mean ± SD; $n = 3$): 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. Three replicates were used in this part of the study. 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 amounts of glucose (330 mg/L), yeast extract (17 mg/L), and PCNB (3 μM) dissolved in methanol (234 μL). The initial biomass concentration in these cultures was 285 ± 15 mg POC/L (mean ± SD; $n = 3$). 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, VFAs, and gas composition. The average pK_a values of CAs are as follows: PCA, −2.1; TeCAs, −0.79; TrCAs, 0.6; DCAs, 1.85; monoCAs, 3.06 (*12*). It is noteworthy that the pK_a values of PCA and all TrCAs are lower than the pH values used in this assay.

Chemicals. PCNB, PCA, and other chlorinated aniline stock solutions were prepared by dissolving neat standards (98%) obtained from Sigma-Aldrich (St. Louis, MO) in HPLC-grade (99.99%) methanol obtained from Fisher Scientific (Pittsburgh, PA). The 2,3,5-TrCA and 2,3,5,6-TeCA standards were obtained from Neosyn Laboratories (New Milford, CT) and VWR (Buffalo Grove, IL), respectively.

Analytical Methods. PCNB, PCA, and other chloroanilines were liquid/liquid extracted from culture samples with isooctane, which contained 0.5 mg/L of 1,3,5-tribromobenzene (1,3,5-TrBB) used as an internal standard. The extracts were analyzed by gas chromatography (electron capture detection) as previously described (*7*). Because of detection limitations by GC-ECD, aniline and di- and monochlorinated anilines were also measured with a high-performance liquid chromatography (HPLC) unit as previously described (*7*). Details on extraction efficiency and detection limits have been reported elsewhere (*7*).

Gas production was measured by connecting the culture headspace via a needle to an acid-brine solution (10% NaCl w/v, 2% H_2SO_4 v/v) filled graduated buret and recording the volume of displaced solution, after correcting to atmospheric pressure. Gas composition (CH_4, CO_2) was determined by gas chromatography (thermal conductivity detection) as previously described (*7*).

POC was determined using a Shimadzu Total Organic Carbon (TOC) Analyzer equipped with a Solids Sample Module (SSM) and an infrared detector for CO_2 measurement (Shimadzu Instrument, Columbia, MD). Culture samples were filtered through 1.2 μm glass fiber filters (Whatman GF/C, Springfield Mill, U.K.). After filtration, the filters were rinsed with deionized (DI) water and dried at 95 °C for 15 min, and the filter contents were combusted at 900 °C.

Kinetic Modeling. The PCNB to PCA (bio)transformation rates under various incubation temperature and pH conditions were measured by assuming pseudo-first-order kinetics (*7*). The kinetic parameters for the reductive dechlorination of PCA and other less chlorinated anilines (CAs) under electron donor saturation conditions were estimated using a simplified Michaelis−Menten-type equation (*8*)

$$-\frac{dC}{dt} = \frac{k'C}{K_C + C} \quad (1)$$

where C is the concentration of CA (μM), k' is the maximum CA dechlorination rate for a constant biomass concentration, and K_C is the half-velocity coefficient for CA dechlorination (μM). To avoid bioavailability limitations and inhibition effects, PCNB was introduced to the cultures dissolved in methanol and at relatively low concentrations. The concentration of electron donor was relatively high to eliminate any limitation related to reducing equivalents. A Michaelis−Menten half-lifetime was derived for the condition of $C = 0.5C_0$ and was used to calculate half-lifetime of CAs (*8*):

$$t_h = \frac{0.5C_0 - K_C \ln(0.5)}{k'} \quad (2)$$

A sequential chloroaniline dechlorination model was developed based on eq 1 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 (*22*). Detailed information on the kinetic modeling has been reported elsewhere (*8*).

RESULTS AND DISCUSSION

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 4−45 °C. Pseudo-first-order rate constant values for the PCNB to PCA (bio)transformation reported in **Table 1** show that the transformation rate increased with increasing temperature. Complete transformation was achieved in all cultures in <6 h. It is noteworthy that the PCNB to PCA transformation is a relatively fast process and occurs under both abiotic and biotic reductive conditions (*7*, *23*). The rate of abiotic transformation depends on the reducing agent (sulfide) concentration and the presence of certain media components

Table 1. Rate Constants (k_{obs}) for the Biotransformation of PCNB to PCA as a Function of Incubation Temperature[a]

incubation temperature (°C)	k_{obs}[b] (day^{-1})	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 ± SD; $n = 3$); initial PCNB concentration 3 μM and pH 6.9 ± 0.1. [b] Nonlinear regression best estimate (mean ± SE; $n \geq 3$).

[e.g., Fe(II)] as well as biotically generated reductants (*7*). PCNB was abiotically transformed to PCA in autoclaved culture media, which contained 67 mg/L sulfide, 28.1 mg/L Fe(II), and 2 μg/L vitamin B_{12}, but at much lower rates as compared to the biotic assays (*7*). 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 day^{-1} [mean ± standard error (SE)], respectively. However, dechlorination of PCA was not observed in any of the abiotic controls (*7*).

PCA sequential dechlorination was observed in cultures incubated in a temperature range of 4−35 °C (**Figure 1**). It took about 10 days for the complete sequential 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 occurred very slowly at 4 °C and was not observed in the cultures incubated at 45 °C (data not shown).

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 (data not shown). 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 up to 35 °C, the extent of methane production was the same at the end of the incubation period. Although the methane production rate was highest at 35 °C, the maximum PCA dechlorination rate was observed at 22 °C and significantly decreased at 35 °C. We have previously reported that although methanogens in the mixed enrichment culture were completely inhibited by 2-bromoethanesulfonate (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 (*7*). On the basis of 16S rRNA gene analysis, among the dechlorinating bacterial groups tested (*Dehalococcoides*, *Dehalobacter*, *Desulfuromonas*, *Geobacter*, and *Anaeromyxobacter*) only *Dehalococcoides* was detected in the mixed fermentative/methanogenic 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, and more detailed studies to elucidate the microbiology of the process are warranted (*8*).

The dechlorination product distribution at four different incubation temperature values was calculated on the basis of the mole fractions among all dechlorination products measured during the dechlorination of PCA (**Table 2**). The extent of ortho dechlorination of PCA resulting in the formation of 2,3,4,5-

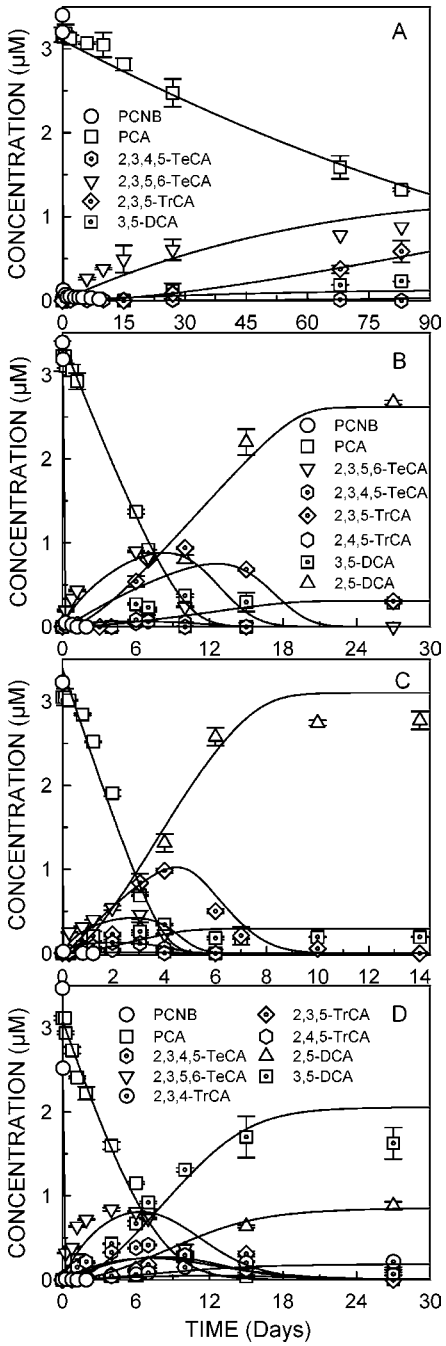

Figure 1. Time course of PCNB and its biotransformation products during a batch biotransformation assay conducted at incubation temperatures of 4 °C (**A**), 15 °C (**B**), 22 °C (**C**), and 35 °C (**D**) using the PCNB-enriched culture at pH 6.9 ± 0.1. Error bars represent mean values ± one standard deviation; lines are model fits.

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

Table 2. Dechlorination Reactions Carried out by the PCA-Dechlorinating, Enrichment Culture and Product Distribution at Different Incubation Temperatures

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

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 stereospecificities. 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 2**). 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 temperatures from 4 to 22 °C (**Table 2**). 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 2**). These results also indicate that reductive dechlorination of PCA at very low temperatures (e.g., 4 °C) is possible, albeit at lower rates than those attained at higher temperature values (e.g., 15−35 °C).

The effect of temperature on the PCA dechlorination rate was simulated by using an Arrhenius relationship, which accounts for both enzyme activation and deactivation (*24*)

$$R_d = \frac{\beta T\left[\exp\frac{-E_a}{RT}\right]}{1+\left[\exp\frac{\Delta S}{R}\right]\left[\exp\frac{-\Delta H}{RT}\right]} \quad (3)$$

where R_d is the PCA dechlorination rate (μM/day), β is the dechlorination proportionality factor (μM/days·K), T is the absolute temperature (K), E_a is the activation energy (kcal/mol), R is the gas constant (= 1.9872×10^{-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). Nonlinear regression of PCA dechlorination rate data obtained with the enriched culture amended with 3 μM PCNB and incubated in a temperature range of 4−45 °C based on eq 3 resulted in the following parameter estimates: $\beta = 1.008 \times 10^8$ μM/days·K; $E_a = 14.183$ kcal/mol; $\Delta S = 0.358$ kcal/mol·K; $\Delta H = 109.5$ kcal/mol. On the basis of these estimates and eq 3, the rate of PCA dechlorination increased with increasing temperature up to 29 °C and then declined as the temperature rose to 35 °C (**Figure 2**).

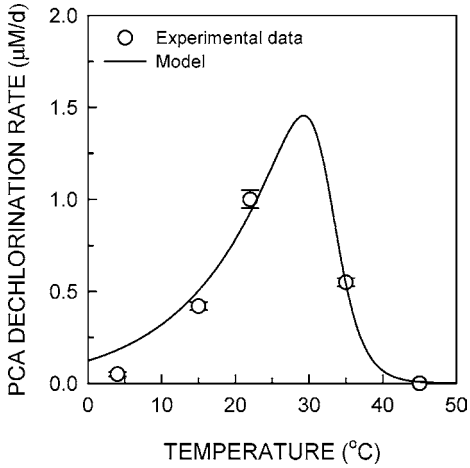

Figure 2. PCA dechlorination rate as a function of incubation temperature. Cultures were amended with 3 μM PCA and maintained at pH 6.9 ± 0.1; error bars represent mean values ± one standard deviation.

Zhuang and Pavlostathis (*25*) 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; $\Delta S = 0.113$ kcal/mol·K; and $\Delta H = 33.95$ kcal/mol. Similarly, Armenante et al. (*26*) 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, respectively. The estimated E_a value for the dechlorination of PCA in the present study is comparable to previously reported E_a values for the microbial reductive dechlorination of chlorinated solvents (*25*) and chlorophenols (*26*).

On the basis of 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

$$Q_{10} = \frac{k_T + 10}{k_T} = \exp\left[\frac{10E_a}{RT_1T_2}\right] \quad (4)$$

where k is the PCA dechlorination rate (μM/day), E_a is the activation energy (kcal/mol), R is the gas constant (= 1.987×10^{-3} kcal/mol·K), and 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 4−29 °C. On the basis of 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 vinyl chloride (VC) has been reported at 4 °C in anoxic microcosms prepared with cold temperature-adapted aquifer and river sediments from Alaska (*27*). Backman et al. (*28*) showed that *Arthrobacter chlorophenolicus* A6 was able to degrade large amounts of 4-chlorophenol in soil at 5 °C. The ability of PCNB-biotransforming bacteria to maintain metabolic activity at lower

Table 3. Rate Constants (k_{obs}) for the Biotransformation of PCNB to PCA as a Function of Incubation pH[a]

pH[b]	k_{obs}[c] (day^{-1})	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 ± SD; $n = 3$); initial PCNB concentration 3 μM and incubation temperature 22 °C. [b] pH values throughout the incubation period (mean ± SD; $n = 3$). [c] Nonlinear regression best estimate (mean ± SE; $n \geq 3$).

temperatures may aid in their survival in sediments and soils, especially in cold climates or seasons.

He et al. (*29*) reported similar VC dechlorination rates to ethane by *Dehalococcoides* at temperatures between 22 and 30 °C. However, they did not observe VC dechlorination at 4 and 35 °C. Wu et al. (*18*, *19*) 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 patterns at temperatures ranging from 4 to 66 °C in sediment slurries taken from Woods Pond Sediment (Lenox, MA) (*19*). Wu et al. (*18*) reported that ortho, meta, and para dechlorinations of 2,3,4,6-tetrachlorobiphenyl took place at different temperatures in freshwater sediments taken from two different climates and suggested the presence of different dechlorinating enzymes and microorganisms with different temperature optima for PCB dechlorination in these sediments.

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 3** 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 pH values of 2.7 ± 0.1 and 4.1 ± 0.1, where complete PCNB transformation was achieved in about 40 and 9 days, respectively (**Figure 3**).

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 3A**,**B**). PCA sequentially dechlorinated down to dichlorinated anilines at the following culture pH values: 6.2 ± 0.1, 6.6 ± 0.1, 7.2 ± 0.1, and 7.6 ± 0.1 (**Figure 3**). PCA removal rates were progressively slower as the incubation pH decreased. In all cases, complete PCA removal was accomplished within 10 days of incubation

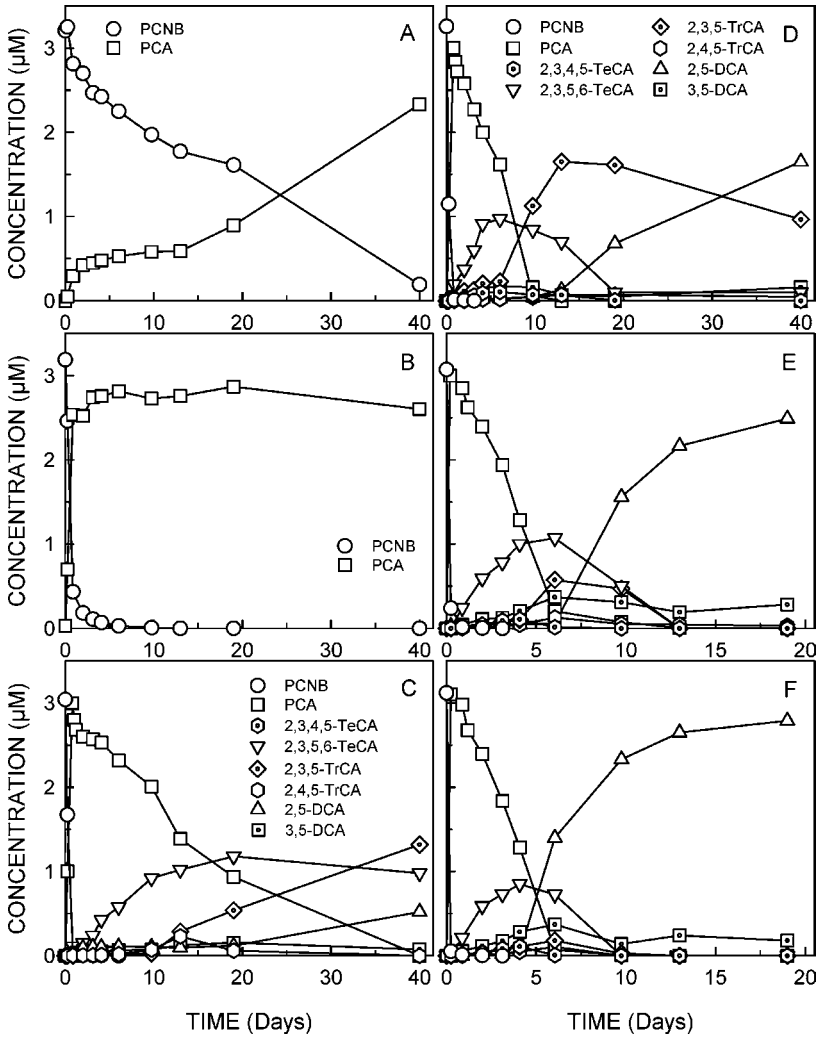

Figure 3. Time course of PCNB and its biotransformation products during a batch biotransformation assay conducted at pH 2.7 ± 0.1 (**A**), 4.1 ± 0.1 (**B**), 6.2 ± 0.1 (**C**), 6.6 ± 0.1 (**D**), 7.2 ± 0.1 (**E**), and 7.6 ± 0.1 (F) using the PCNB-enriched culture at an incubation temperature of 22 °C.

Table 4. Maximum Dechlorination Rates (k'), Half-Velocity Coefficients (K_C), and Half-Lifetimes (t_h) for the Dechlorination of CAs at Different Incubation Temperature Values[a]

	k' (μM/day)				K_C (μM)				t_h (days)			
compound	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 ± SD; $n = 3$); 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.

Table 5. Maximum Dechlorination Rates (k'), Half-Velocity Coefficients (K_C), and Half-Lifetimes (t_h) for the Dechlorination of CAs at Two Incubation pH Values[a]

	k' (μM/day)		K_C (μM)		t_h (days)	
compound	pH 6.2 ± 0.1[b]	pH 7.2 ± 0.1	pH 6.2 ± 0.1	pH 7.2 ± 0.1	pH 6.2 ± 0.1	pH 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 ± SD; $n = 3$); initial PCNB concentration 3 μM and incubation temperature 22 °C. [b] pH value throughout the incubation period (mean ± SD; $n = 3$).

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 (data not shown). The lowest methane production rate was observed in the pH 6.2 ± 0.1 culture. In all cultures the extent of methane production was the same. Although there are methanogens that live in extreme pH environments, the optimum pH reported for methanogenesis is near neutral (*30*, *31*).

The PCA dechlorination product distribution for all five cultures incubated at different pH values was calculated on the basis of 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, whereas 2,5-DCA was the predominant end product (**Figure 3**). Therefore, the culture incubation pH did not have any significant effect on the PCA dechlorination pathway (for a pH range of 6.2−7.6).

Zhuang and Pavlostathis (*25*) 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 (*32*). Rutgers et al. (*33*) 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 data relative to the observed pH range for the microbial reductive dechlorination of various chlorinated compounds agree with the conclusion reached by Fennell and Gossett (*34*), 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 and the presence of certain media components [e.g., Fe(II)] as well as biotically generated reductants. A decrease in pH should lead to decreased reducing capacity as the redox potential will be shifted toward 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. (*23*) reported an increase in the PCNB degradation rate constant to PCA as the pH increased at constant goethite and total Fe(II) concentrations. They also observed an increase in the sorbed Fe(II) concentration with increasing pH, but the increase was not proportional to the observed PCNB transformation rate.

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 on the basis of data reported in **Table 2**. The resulting system of the simultaneous ordinary differential equations was solved numerically using MATLAB version 7, ode23 suite, which uses a Runge−Kutta solver (*22*). The values of k' and K_C for PCA and all CAs observed at each incubation temperature were estimated using Berkeley Madonna 8.0.1 software (*35*) and are reported in **Table 4**. 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 1**. The simulated progress curves for PCA and all CAs agree reasonably 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 4**). These results confirm that the sequential dechlorination rates of CAs increase with

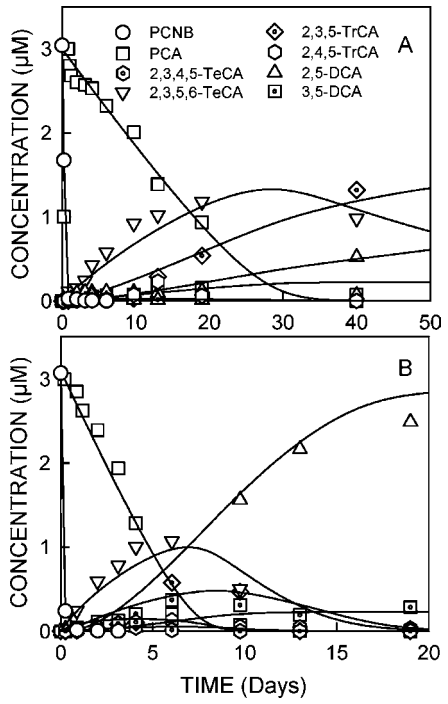

Figure 4. Time course of PCNB and its biotransformation products during a batch biotransformation assay conducted at pH 6.2 ± 0.1 (**A**) and 7.2 ± 0.1 (**B**) using the PCNB-enriched culture at an incubation temperature of 22 °C. Lines are model fits.

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 2**).

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 ± 0.1 to 7.6 ± 0.1 were simulated using a Michaelis−Menten model and the observed branching and product distribution at each dechlorination step reported in **Table 2**. The values of k' and K_C for PCA and all CAs observed at each incubation pH value were estimated using Berkeley Madonna 8.0.1 software (*35*). The results for two representative pH values (6.2 ± 0.1 and 7.2 ± 0.1) are shown in **Table 5**. The maximum CA dechlorination rates increased with increasing pH. A 3-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 4-fold increase was observed when the incubation pH increased from 6.2 ± 0.1 to 7.6 ± 0.1. The TeCAs and TrCAs maximum dechlorination rates increased 4-fold when the pH increased from 6.2 ± 0.1 to 7.2 ± 0.1, and a 2-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 4**. The simulated progress curves for PCA and all CAs agree reasonably well with the experimental data. As previously mentioned under Materials and Methods, the pK_a values of PCA and all TeCAs, TrCAs, and DCAs are lower than the pH values used in this assay. Therefore, the observed differences in the dechlorination rates as a function of culture pH are not related to the protonation/deprotonation of the parent compounds. 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 5**). Similar to our results, Young and Gossett (*36*) reported 4- and 2-fold decreases 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.

Impact and Significance. The results of this study indicate that pH and temperature are two important parameters which affect the reductive (bio)transformation rate and extent of PCNB. Reductive transformation of PCNB was observed at all tested temperature (4−45 °C) and pH (2.7−7.6) values, and its half-lifetime ranged from 0.08 to 30 days. A half-lifetime value of 1.68 days was reported for the aerobic biodegradation of PCNB by a pure culture of *Alcaligenes xylosoxidans* PCNB-2 isolated from an agricultural soil (*37*). However, it should be emphasized that the observed, short half-lifetime was under laboratory conditions in the presence of high microbial population density of a PCNB-degrading bacterium, conditions which do not reflect real soil environment. Indeed, it was reported that the half-lifetime for the complete aerobic degradation of PCNB and its transformation products was 1124 days in soil with representative mixed microbial populations (*38*). Thus, the biotransformation of PCNB in soils is expected to be much faster under anaerobic, reductive conditions compared to aerobic conditions.

Incubation at different pH values resulted in differences in the biotransformation rate and extent. Similarly, differences were observed in terms of biotransformation rate and extent as well as dechlorination products as a function of temperature. Sequential dechlorination of PCA was observed only 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 incubation temperature had a pronounced effect of the biotransformation product distribution. As the temperature increased from 4 to 22 °C, 2,5-DCA was the predominant product and the dechlorination rate increased with increasing temperature. However, at 35 °C the predominant product was 3,5-DCA and its production rate was relatively similar to the production rate of 2,5-DCA at 22 °C.

It is noteworthy that reported EC_{50} values for 15 chloroaniline congeners show a general trend of decreasing toxicity with decreasing degree of chlorination (*39*). The toxicity of DCAs (the predominant products of PCNB biotransformation) depends on the type of the isomers (*40*). Therefore, the microbial reductive transformation of PCNB will lead to the formation of less toxic products (mainly DCAs). In addition, changes in the temperature as well as pH values in PCNB-contaminated sites could result in the accumulation of biotransformation products with various toxicities. The microbial transformation rates and pathway are a function of the microbial community composition, which in turn is the result of acclimation and enrichment. Culture enrichment conditions resulting in the development of specific dechlorinating species that have different enzymes and/or enzymatic activities may result in different dechlorination pathways which may not be observed in all contaminated sediment and soil environments. In addition, caution is advised in the interpretation and comparison of dechlorination rates and pathways reported by different studies conducted with mixed cultures under widely varying experimental conditions.

The results of the present study have significant implications relative to the fate and biotransformation of PCNB under variable pH and temperature environmental conditions typically encountered in soil and sediments and should be useful in optimizing pH and temperature conditions for the enhancement

of bioremediation applications related to soil and sediments contaminated with PCNB and CAs.

LITERATURE CITED

(1) International Agency for Research on Cancer (IARC). *Some Organochlorine Pesticides*; Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man 5; IARC: Lyons, France, 1974; pp 211−218.

(2) U.S. Geological Survey (USGS). Maps of annual pesticide use (PCNB); Sacramento, CA, 1998; available at http://ca.water-.usgs.gov/pnsp/use92/pcnb.html.

(3) U.S. Environmental Protection Agency. *Reregistration Eligibility Decision for Pentachloronitrobenzene*; List A, Case No. 0128; U.S. EPA, Office of Prevention, Pesticides and Toxic Substances: Washington, DC, June 2006.

(4) Murthy, N. B. K; Kaufman, D. D. Degradation of pentachloronitrobenzene (PCNB) in anaerobic soils. *J. Agric. Food Chem.* **1978**, *26*, 1151−1156.

(5) Tamura, K.; Hasegawa, Y.; Kudo, T.; Yamaguchi, I. Isolation and characterization of PCNB degrading bacterium, *Pseudomonas aeruginosa* strain I-41. *J. Pestic. Sci.* **1995**, *20*, 145−151.

(6) Susarla, S.; Masunaga, S.; Yonezawa, Y. Transformations of chloronitrobenzenes in anaerobic sediment. *Chemosphere* **1996**, *32*, 967−977.

(7) Okutman Tas, D.; Pavlostathis, S. G. Microbial reductive transformation of pentachloronitrobenzene under methanogenic conditions. *Environ. Sci. Technol.* **2005**, *39*, 8264−8272.

(8) Okutman Tas, D.; Thomson, I. N.; Löffler, F. E.; Pavlostathis, S. G. Kinetics of the microbial reductive dechlorination of pentachloroaniline. *Environ. Sci. Technol.* **2006**, *40*, 4467−4472.

(9) Kuhn, E. P.; Suflita, J. M. Sequential reductive dehalogenation of chloroanilines by microorganisms from a methanogenic aquifer. *Environ. Sci. Technol*. **1989**, *23*, 848−852.

(10) Struijs, J.; Rogers, J. E. Reductive dehalogenation of dichloroanilines by anaerobic microorganisms in fresh and dichlorophenol-acclimated pond sediment. *Appl. Environ. Microbiol.* **1989**, *55*, 2527−2531.

(11) Susarla, S.; Yonezawa, Y.; Masunaga, S. Reductive dehalogenation of chloroanilines in anaerobic estuarine sediment. *Environ. Technol.* **1997**, *18*, 75−83.

(12) Reuther, C.; Crommentuijn, T.; van de Plassche, E. J. *Maximum Permissible Concentrations and Negligible Concentrations for Aniline Derivatives*; Report 601501003; National Institute of Public Health and the Environment: Bilthoven, The Netherlands, 1998.

(13) Reber, H.; Helm, V.; Karanth, N. G. K. Comparative studies on the metabolism of aniline and chloroanilines by *Pseudomonas multivorans* strain An 1. *Appl. Microbiol. Biotechnol.* **1979**, *7*, 181−189.

(14) Surovtseva, E. G.; Ivoilov, V. S.; Karasevich, Y. N.; Vasileva, G. K. Chlorinated anilines, a source of carbon, nitrogen and energy for *Pseudomonas diminuta*. *Microbiology* **1985**, *54*, 756−760.

(15) Surovtseva, E. G.; Sukhikh, A. P.; Ivoilov, V. S. Isozymes of the pathway for aniline and 4-chloroaniline preparatory metabolism in *Alcaligenes* sp. *Microbiology* **1992**, *61*, 99−106.

(16) Surovtseva, E. G.; Ivoilov, V. S.; Vasileva, G. K.; Belyaev, S. S. Degradation of chlorinated anilines by certain representatives of the genera *Aquaspirillum* and *Paracoccus*. *Microbiology* **1996**, *65*, 553−559.

(17) Boon, N.; Goris, J.; De Vos, P.; Verstraete, W.; Top, E. M. Bioaugmentation of activated sludge by an indigenous 3-chloroaniline-degrading *Comamonas testosteroni* strain, 12gfp. *Appl. Environ. Microbiol.* **2000**, *66*, 2906−2913.

(18) Wu, Q. Z.; Bedard, D. L.; Wiegel, J. Influence of incubation temperature on the microbial reductive dechlorination of 2,3,4,6-tetrachlorobiphenyl in two freshwater sediments. *Appl. Environ. Microbiol.* **1996**, *62*, 4174−4179.

(19) Wu, Q. Z.; Bedard, D. L.; Wiegel, J. Effect of incubation temperature on the route of microbial reductive dechlorination of 2,3,4,6-tetrachlorobiphenyl in polychlorinated biphenyl (PCB)-contaminated and PCB-free freshwater sediments. *Appl. Environ. Microbiol.* **1997**, *63*, 2836−2843.

(20) Boyd, C. E. *Bottom Soils, Sediment, and Pond Aquaculture*; Springer-Verlag: New York, 1995; 50 pp.

(21) Pavlostathis, S. G.; Prytula, M. T. Kinetics of the sequential microbial reductive dechlorination of hexachlorobenzene. *Environ. Sci. Technol.* **2000**, *34*, 4001−4009.

(22) MATLAB, version 7; The MathWorks, Inc.: Natick, MA, 2004.

(23) Klupinski, T. P.; Chin, Y. P.; Traina, S. J. Abiotic degradation of pentachloronitrobenzene by Fe(II): reactions on goethite and iron oxide nanoparticles. *Environ. Sci. Technol.* **2004**, *38*, 4353−4360.

(24) Bailey, J. E.; Ollis, D. F. *Biochemical Engineering Fundamentals*, 2nd ed.; McGraw-Hill: New York, 1986.

(25) Zhuang, P.; Pavlostathis, S. G. Effect of temperature, pH and electron donor on the microbial reductive dechlorination of chloroalkenes. *Chemosphere* **1995**, *31*, 3537−3548.

(26) Armenante, P. M.; Kafkewitz, D.; Lewandowski, G. A.; Jou, C. J. Anaerobic-aerobic treatment of halogenated phenolic compounds. *Water Res.* **1999**, *33*, 681−692.

(27) Bradley, P. M.; Richmond, S.; Chapelle, F. H. Chloroethene biodegradation in sediments at 4 °C. *Appl. Environ. Microbiol.* **2005**, *71*, 6414−6417.

(28) Backman, A.; Maraha, N.; Jansson, J. K. Impact of temperature on the physiological status of a potential bioremediation inoculant, *Arthrobacter chlorophenolicus* A6. *Appl. Environ. Microbiol.* **2004**, *70*, 2952−2958.

(29) He, J.; Ritalahti, K. M.; Aiello M. R.; Löffler, F. E. Complete detoxification of vinyl chloride by an anaerobic enrichment culture and identification of the reductively dechlorinating population as a *Dehalococcoides* species. *Appl. Environ. Microbiol.* **2003**, *69*, 996−1003.

(30) Ferry, J. *Methanogenesis: Ecology, Physiology, Biochemistry, & Genetics*; Chapman and Hall: New York, 1993.

(31) Rittmann, B. E.; McCarty P. L. *Environmental Biotechnology: Principles and Applications*; McGraw-Hill: New York, 2001.

(32) Holliger, C.; Schraa, G.; Stams, A. J. M.; Zehnder, A. J. B. A Highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. *Appl. Environ. Microbiol.* **1993**, *59*, 2991−2997.

(33) Rutgers, M.; van Bommel, S.; Breure, A. M.; van Andel, J. G.; Duetz, W. A. Effect of pH on the toxicity and biodegradation of pentachlorophenol by *Sphingomonas* sp. strain P5 in nutristat culture. *Environ. Toxicol. Chem.* **1998**, *17*, 792−797.

(34) Fennell, D. E.; Gossett, J. M. Microcosms for site-specific evaluation of enhanced biological reductive dehalogenation. In *Dehalogenation: Microbial Processes and Environmental Applications*; Häggblom, M. M., Bossert, I. D., Eds.; Kluwer Academic Publishers: Boston, MA, 2003.

(35) Berkeley Madonna, version 8.0.1; Berkeley, CA, 2000; available at http://www.berkeleymadonna.com.

(36) Young, R. G.; Gossett, J. M. Effect of environmental parameters and concentrations on dechlorination of chloroethenes. Presented at the Fourth International In Situ and On-Site Bioremediation Symposium, New Orleans, LA, 1997.

(37) Sutton, C.; Ravenscroft, J. E.; Steeger, T. M.; Jones, R. D.; Costello, K. *Environmental Fate and Ecological Risk Assessment for the Re-registration of Pentachloronitrobenzene*; Report 056502; U.S. EPA, Office of Prevention, Pesticides and Toxic Substances: Washington, DC, 2004.

(38) Shin, S. K.; Kim, J. E.; Kwon, G. S.; Kwon, J. W.; Oh, E. T.; So, J. S.; Koh, S. C. Isolation and identification of a pentachloronitrobenzene (PCNB) degrading bacterium *Alcaligenes xylosoxidans* PCNB-2 from agricultural soil. *J. Microbiol.* **2003**, *41*, 165−168.

(39) Argese, E.; Bettiol, C.; Agnoli, F.; Zambon, A.; Mazzola, M.; Ghirardini, A. V. Assessment of chloroaniline toxicity by the submitochondrial particle assay. *Environ. Toxicol. Chem*. **2001**, *20*, 826−832.

(40) Lo, H. H.; Brown, P. I.; Rankin, G. O. Acute nephrotoxicity induced by isomeric dichloroanilines in Fischer 344 rats. *Toxicology* **1990**, *63*, 215−231.

Received for review December 28, 2006. Revised manuscript received April 15, 2007. Accepted April 25, 2007. This work was supported in part by a U.S. Geological Survey grant administered through the Georgia Water Resources Institute.

JF0637675