Biodegradation (2010) 21:691–702
DOI 10.1007/s10532-010-9335-2

ORIGINAL PAPER

Microbial transformation of pentachloronitrobenzene under nitrate reducing conditions

Didem Okutman Tas · Spyros G. Pavlostathis

Received: 18 June 2009 / Accepted: 22 January 2010 / Published online: 4 February 2010

Abstract The effect of pentachloronitrobenzene (PCNB) on denitrification was assessed with two denitrifying cultures (PCNB-free control and PCNB-acclimated) developed from a contaminated estuarine sediment. PCNB was transformed to pentachloroaniline (PCA) in the PCNB-acclimated culture repeatedly amended with 0.1 μM PCNB, but further dechlorination or degradation of PCA was not observed for almost 1 year. The effect of PCNB on denitrification was also investigated with the PCNB-free control culture. PCNB at an initial concentration of 13 μM was transformed to PCA simultaneously with nitrate reduction but only after the nitrate concentration was at or below 20 mg N/l. PCNB addition at an initial concentration of 13 μM to the control denitrifying culture developed as PCNB-free culture resulted in a transient accumulation of nitric oxide (NO) and nitrous oxide (N_2O). 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 pentachlorobenzene (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 maintained under active nitrate reducing conditions. Based on the results of this study, the presence of nitrate at low concentrations in anoxic/anaerobic soil and sediments is not expected to negatively affect the biotransformation of PCNB to PCA, but dechlorination or degradation of PCA is not expected under active nitrate reducing conditions.

Keywords Biotransformation · Denitrification · Inhibition · Pentachloroaniline · PCNB

D. Okutman Tas · S. G. Pavlostathis (✉)
School of Civil and Environmental Engineering, Georgia Institute of Technology, Atlanta, GA 30332-0512, USA
e-mail: spyros.pavlostathis@ce.gatech.edu

D. Okutman Tas
e-mail: okutmand@itu.edu.tr

Present Address:
D. Okutman Tas
Department of Environmental Engineering, Istanbul Technical University, Istanbul 34469, Turkey

Introduction

Pentachloronitrobenzene (PCNB) is an organochlorine fungicide used as a seed dressing and applied in a wide range of crops. As a result of its worldwide extensive use, PCNB is widespread and released into all environmental compartments. The U.S. EPA has classified PCNB as a possible cancer causing substance, has included it in the Toxicity Class III chemicals list, as well as in the list of 'Waste Minimization Priority Chemicals' (U.S. EPA 2003). Pentachloroaniline (PCA) is the main abiotic and first

biotic transformation product of PCNB under anoxic/anaerobic conditions (Hakala et al. 2007; Klupinski et al. 2004; Okutman Tas and Pavlostathis 2005, 2007, 2008; Okutman Tas et al. 2006; Susarla et al. 1996; Tamura et al. 1995). PCA has a lower aqueous solubility, a higher tendency to partition into natural organic matter compared to PCNB (Okutman Tas and Pavlostathis 2005), and a higher bioconcentration factor (1000) compared to that of PCNB (750) (U.S. EPA 2003). Therefore, PCA is of concern because of its higher potential to bioaccumulate in the food chain, especially in environments where PCA dechlorination does not take place. Under low redox conditions (e.g., methanogenic and sulfidogenic), microbial transformation of PCA resulted in its sequential dechlorination down to di- and in some cases to mono-chloroanilines, but the resulting di- and mono-chloroanilines were not further transformed (Kuhn and Suflita 1989; Okutman Tas and Pavlostathis 2005; Susarla et al. 1996). Reported EC_{50} values for fifteen chloroaniline congeners show a general trend of decreasing toxicity with decreasing degree of chlorination (Argese et al. 2001).

The biotransformation potential and kinetics of PCNB under methanogenic conditions is well documented (Kuhn and Suflita 1989; Okutman Tas and Pavlostathis 2005; Okutman Tas et al. 2006; Susarla et al. 1997). Aside from carbonate (methanogenesis), a wide variety of alternative electron acceptors can also be used by microorganisms under anoxic/anaerobic conditions. Nitrate, sulfate, and metals are alternative electron acceptors commonly found in subsurface environments, are involved in subsurface processes (e.g., nitrate, sulfate, and metal reduction), and may be used for bioremediation applications. A few studies have been conducted on the biotransformation potential of PCNB under alternative electron accepting conditions, other than methanogenic conditions, such as sulfate reducing (Kuhn et al. 1990), and iron reducing conditions (Okutman Tas and Pavlostathis 2007). Nitrate, because of its high mobility in soil and sediment, is a ubiquitous electron acceptor, resulting from many agricultural and/or industrial activities. Because of the increased occurrence of nitrate pollution worldwide (Prasad 1996), the co-existence of nitrate and PCNB is expected, especially in agricultural soils. Several researchers have previously reported biotransformation of halogenated organic compounds under denitrifying conditions (Bae et al. 2002, 2004; Coschigano et al. 1994; Freedman et al. 2004; Gerritse et al. 1999; Häggblom and Young 1999; Petersen et al. 1994; Sherwood et al. 1996). Although there are denitrifying microorganisms which degrade halogenated organics, a decrease of denitrification activity in the presence of organic contaminants (e.g., nitroaromatics) has been previously observed (Fuller and Manning 1998; Siciliano et al. 2000). The effect of nitrate reduction on the reductive biotransformation of PCNB was recently assessed with a mixed fermentative/methanogenic culture (Okutman Tas and Pavlostathis 2008). PCNB at an initial concentration of 3 μM was transformed to PCA simultaneously with nitrate reduction in cultures amended with 10 to 200 mg N/l nitrate. PCA sequentially dechlorinated to dichlorinated anilines (mainly 2,5-DCA) in the nitrate-free control culture, and the culture which was amended with 10 mg N/l nitrate. However, nitrate concentrations at or above 50 mg N/l resulted in accumulation of denitrification intermediates (i.e., NO, N_2O), which in turn led to partial PCA dechlorination to tetrachloroanilines. Although the dynamics of simultaneous nitrate reduction, fermentation, methanogenesis, and PCA dechlorination were investigated in the above-mentioned study, the culture used was maintained at low redox conditions which are not representative of typical nitrate reducing conditions. Thus, to our knowledge, the biotransformation potential of PCNB under long-term, active nitrate reducing conditions has not been previously investigated.

The objective of the research reported here was to assess the biotransformation potential of PCNB in a PCNB-acclimated denitrifying culture derived from a contaminated sediment, as well as to investigate possible inhibitory effects of PCNB on a denitrifying culture enriched in the absence of PCNB.

Materials and methods

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 location and details on the sediment sampling and analysis have been reported elsewhere (Gess and Pavlostathis 1997; Prytula and Pavlostathis 1996). PCNB and PCA were not detected in the sediment sample used as inoculum

in this study. The cultures were initiated by diluting 80 g of sediment in 1.5 l of mineral media in helium-flushed, 2 l glass flask reactors, capped with Teflon-lined stoppers. The medium had the following composition (in mg/l): K_2HPO_4, 900; KH_2PO_4, 500; NH_4Cl, 500; $CaCl_2{\cdot}2H_2O$, 100; $MgCl_2{\cdot}6H_2O$, 200; $FeCl_2{\cdot}4H_2O$, 100. Also, 1 mL/l vitamin stock solution (Wolin et al. 1963) and 1 mL/l trace metal stock solution (Mah and Smith 1981) were added to the media. 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 0.1 μM PCNB dissolved in methanol (53 mg/l). The nitrate concentration was kept in excess (>100 mg N/l) with frequent additions 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 helium-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/l (expressed as particulate organic carbon; POC), 7.0 ± 0.1 and 7.8 ± 0.2, respectively. The batch assay reported here was performed with the two, sediment-free, third generation denitrifying cultures.

Nitrate reduction and PCNB biotransformation assay

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. Using aliquots from the PCNB-acclimated and PCNB-free control cultures, two cultures were prepared and amended with an initial PCNB concentration of 13 μM dissolved in methanol (referred to as PCNB-acclimated and PCNB-amended control cultures, respectively). Another control culture was also set up with the PCNB-free culture without any PCNB amendment (PCNB-free control). The initial concentration of carbon sources in all cultures was as follows: glucose (666 mg/l), yeast extract (34 mg/l), and methanol (1,535 mg/l). The chemical oxygen demand (COD) of the glucose and methanol added to each serum bottle was 710 and 2,300 mg/l, respectively, and the initial nitrate concentration was 60 mg N/l. The initial biomass concentration was 230 ± 35 mg POC/l (mean ± SD; $n = 3$). The cultures were incubated in a 22°C 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.

Because of the potential of some culture media components (i.e., Fe(II), vitamin B12), as well as culture products to abiotically mediate the reductive transformation of PCNB, three abiotic controls were set up: autoclaved culture media, autoclaved PCNB-acclimated culture, and autoclaved PCNB-free control culture. The autoclaved culture media control contained only media anaerobically transferred to helium pre-flushed 160 ml serum bottles, autoclaved at 121°C for 30 min. For the autoclaved culture controls, aliquots of the two active cultures were transferred to helium pre-flushed 160 ml serum bottles and were autoclaved twice in two consecutive days at 121°C for 30 min. Then, all abiotic controls were amended with PCNB resulting in an initial PCNB concentration of 13 μM and were incubated in a 22°C constant temperature room, manually shaken once a day.

Chemicals

PCNB, PCA, and pentachlorobenzene (PeCB) stock solutions were prepared by dissolving neat standards (98%) obtained from Sigma–Aldrich (St. Louis, MO, USA) in HPLC-grade (99.99%) methanol obtained from Fisher Scientific (Pittsburgh, PA, USA).

Analytical methods

Liquid/liquid extraction was performed in order to quantify PCNB and its (bio)transformation products and extracts were analyzed by gas chromatography (electron capture detection) as previously described (Okutman Tas and Pavlostathis 2005). Details on the extraction efficiency and detection limits have been

reported elsewhere (Okutman Tas and Pavlostathis 2005).

Nitrate and nitrite concentrations were determined using a Dionex DX-100 ion chromatography unit (Dionex Corporation, Sunnyvale, CA, USA) equipped with a conductivity detector, a Dionex IonPac AG14A (4 × 50 mm) precolumn, and a Dionex IonPac AS14A (4 × 250 mm) analytical column. The unit was operated in autosuppression mode with 1 mM $NaHCO_3$/8 mM Na_2CO_3 eluent at a flow rate of 1 ml/min. Calibration curves were generated using standards prepared by dissolving reagent-grade sodium salts of each compound in deionized (DI) water. All standards and samples were filtered through 0.2 μm membrane filters prior to injection. The minimum detection limits for nitrate and nitrite were 0.05 and 0.1 mg N/l, respectively.

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 burette and recording the volume of displaced solution, after correcting to atmospheric pressure. Gas composition was determined by a gas chromatography unit (Agilent Technologies, Model 6890N; Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with two columns and two thermal conductivity detectors. 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, USA). Both columns were operated with helium as the carrier gas at a constant flow rate of 6 mL/min. The 10:1 split injectors were maintained at 150°C, the oven was set at 40°C and the detectors temperature was set at 150°C. The minimum detection limits for NO and N_2O were 0.5 and 0.007 mL/l, respectively.

Volatile fatty acids (VFAs; C_2 to C_7) were measured using an HP 5890 Series II GC (Hewlett Packard, Palo Alto, CA, USA) equipped with a flame ionization detector and a 35-m Stabilwax-DA, 0.53-mm i.d. column (Restek, Bellefonte, PA, USA). 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, USA). Culture samples were filtered through 1.2 μm glass fiber filters (Whatman GF/C, Springfield Mill, England). After filtration the filter contents were rinsed with DI water, dried at 95°C for 15 min, and then combusted at 900°C.

Results

Enrichment cultures

PCNB was biotransformed to PCA in the sediment microcosm (first generation culture) in 2 days under denitrifying conditions. However, dechlorination or degradation of PCA was not observed for almost 1 year of incubation in the sediment-free, third generation culture maintained with addition of glucose, yeast extract and PCNB in methanol every 7 days and excess nitrate at a concentration equal to or higher than 100 mg N/l (Fig. 1). Glucose and methanol were consumed within 3 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 ± SD; $n = 14$). The glucose and methanol consumption as well as the nitrate reduction rate and extent were similar in both the PCNB-acclimated and the PCNB-free control denitrifying cultures (data not shown). Figure 2 shows a typical gas production and composition 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. Accumulation of VFAs was not observed in both denitrifying cultures at the end of each 7-day feeding cycle.

Abiotic transformation of PCNB

Three autoclaved abiotic controls—culture media, PCNB-acclimated culture, and PCNB-free control culture—were monitored for over 30 days. The PCNB concentration in the autoclaved culture media remained constant over the incubation period (coefficient of variation less than 5%; $n = 6$) and PCA was not detected. A relatively low decline of the PCNB concentration over the incubation period was observed in the autoclaved PCNB-free control and

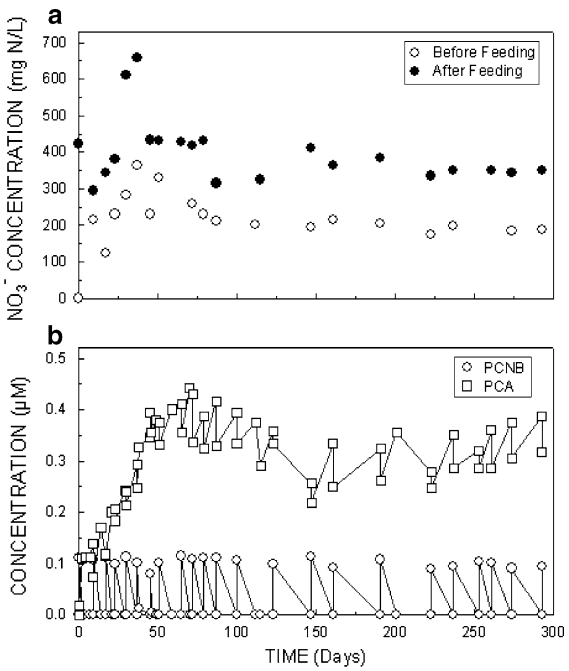

Fig. 1 Concentration profiles of nitrate (**a**) PCNB and its transformation product PCA (**b**) in the PCNB-acclimated culture (multiple feeding cycles; third generation culture)

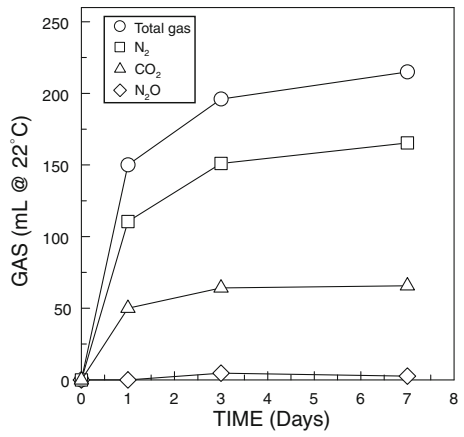

Fig. 2 Gas production profiles in the PCNB-free control culture during a typical feeding cycle (third generation culture)

PCNB-acclimated culture samples and traces of PCA were detected. Dechlorination of PCA was not observed in any of the abiotic controls over the incubation period. The pseudo-first-order rate constants for the transformation of PCNB to PCA at an initial PCNB concentration of 13 μM were (mean ± SE; $n \geq 6$) 0.0021 ± 0.0004 day^{-1} ($r^2 = 0.821$) and 0.0041 ± 0.0015 day^{-1} ($r^2 = 0.717$) for the autoclaved PCNB-free control and PCNB-acclimated culture samples, respectively. The lack of measurable PCNB to PCA transformation in the autoclaved media compared to that observed in the two autoclaved culture samples indicates that biotically derived reductants and/or other factors in these cultures facilitates the PCNB to PCA transformation. Although the observed PCNB to PCA rates in the two autoclaved culture samples were more than an order of magnitude lower than those observed in active denitrifying cultures as discussed below, the rate was statistically ($P < 0.05$) higher in the autoclaved PCNB-acclimated culture sample than in the autoclaved PCNB-free control culture sample.

Under low redox conditions, the PCNB to PCA transformation is a relatively fast process occurring abiotically, its rate depending on the inorganic reducing agent(s) (e.g., sulfide, Fe(II)) concentration as well as biotically derived reductants and/or other factors (Hakala et al. 2007; Klupinski et al. 2004; Okutman Tas and Pavlostathis 2005). Abiotic control experiments conducted with autoclaved reduced culture media containing 67 mg sulfide-S/l and autoclaved samples of a fermentative/methanogenic culture grown in the same, reduced media resulted in pseudo-first-order rate constants for the transformation of PCNB to PCA at an initial PCNB concentration of about 0.1 μM equal to 0.851 ± 0.004 and 40.8 ± 3.7 day^{-1} (mean ± SE), respectively, showing the beneficial effect of biotically derived reductants (Okutman Tas and Pavlostathis 2005). Therefore, the low redox conditions maintained in the previous study resulted in a more than 100-fold higher abiotic PCNB to PCA transformation rates, than in the present study.

Biotransformation of PCNB

The concentration profiles of PCNB, its biotransformation product PCA and nitrate during the batch biotransformation assay conducted with the PCNB-acclimated and the PCNB-amended control denitrifying enrichment cultures at an initial PCNB concentration of 13 μM are shown in Fig. 3. 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, enrichment culture. PCNB transformation to PCA started after 4 days of a lag period in the PCNB-acclimated culture, whereas in the PCNB-amended control culture, PCNB transformation started after 8 days of a lag period and more than 90%

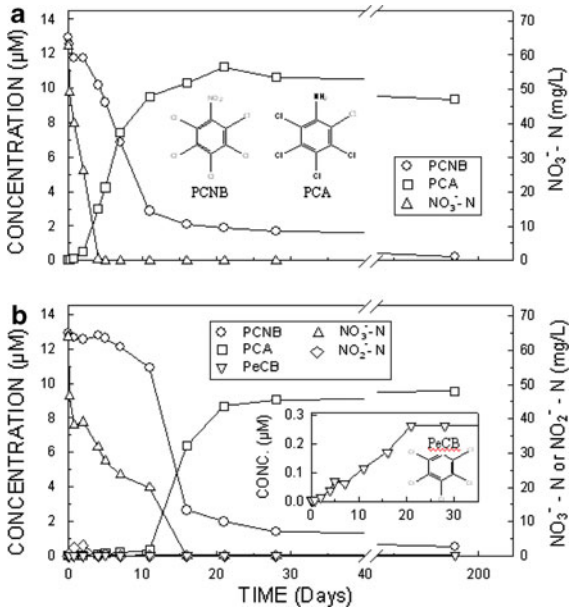

Fig. 3 Time course of PCNB, its biotransformation product PCA, nitrate and nitrite during the batch biotransformation assay conducted with the PCNB-acclimated (**a**) and the PCNB-amended control (**b**) enrichment cultures (Both cultures were amended with 13 μM PCNB and 60 mg NO_3^--N/l; *inset* in **b** *panel* shows the production of low levels of PeCB in the PCNB-amended control culture)

of PCNB was transformed to PCA in both cultures within 30 days. PCNB biotransformation was not observed in both cultures until the nitrate concentration decreased to about 20 mg N/l. A low degree of nitro group removal resulting in the formation of PeCB, which persisted throughout the incubation period, was only observed in the PCNB-amended control culture (Fig. 3b, inset). In both cultures, PCNB was transformed to PCA only when the nitrate level was at or below 20 mg N/l. Inhibition by denitrification intermediates such as NO and N_2O and energetic limitations aside (see below), the observed lack of PCNB to PCA biotransformation at nitrate levels above approximately 20 mg N/l may be the result of competition for electron donor(s) among different physiological bacterial groups in the mixed culture. Dechlorination of PCA was not observed in either culture over a 190-day incubation period in spite the fact that all nitrate and denitrification intermediates had been depleted and a significant concentration of VFAs remained in these cultures (see below), thus ruling out any electron donor limitations.

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 (mean ± SE) 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 1.7-fold faster than that attained in the PCNB-amended control culture. 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 ± SE) (Okutman Tas and Pavlostathis 2005). Similar PCNB transformation rates were also observed under iron reducing conditions (Okutman Tas and Pavlostathis 2007). Thus, the PCNB to PCA biotransformation rate under nitrate reducing conditions was relatively low as compared to that under fermentative/methanogenic and iron reducing conditions. Compared to the above-mentioned PCNB to PCA transformation rates under abiotic conditions, the biotransformation rates were more than an order of magnitude higher. Thus, the observed PCNB to PCA transformation in the two enrichment cultures was biotically mediated and enhanced.

PCNB effect on denitrification

Complete nitrate reduction in the PCNB-acclimated culture which was amended with 13 μM PCNB and the PCNB-free control culture at an initial nitrate concentration of 60 mg N/l occurred in about 4 days, whereas the same transformation required 16 days in the PCNB-amended control culture at an initial PCNB concentration of 13 μM (Fig. 4). The pseudo-first-order rate constants of the nitrate reduction in the PCNB-acclimated culture and PCNB-amended control culture were 0.56 ± 0.07 ($r^2 = 0.978$) and 0.13 ± 0.02 ($r^2 = 0.915$) day^{-1}, respectively, whereas the nitrate reduction rate was 0.73 ± 0.04 ($r^2 = 0.996$) day^{-1} in the PCNB-free control culture. Thus, the presence of PCNB at an initial concentration of 13 μM resulted in a decrease of the nitrate reduction rate, especially in the PCNB-amended control culture. In a recent study conducted with a PCNB-acclimated, mixed fermentative/methanogenic culture the presence of 3 μM PCNB did not affect the nitrate reduction rate at initial nitrate concentrations between 10 and 200 mg N/l and the mean pseudo-first-order rate for the nitrate removal was 1.07 ± 0.01 day^{-1} ($r^2 = 0.999$) (Okutman Tas and Pavlostathis 2008).

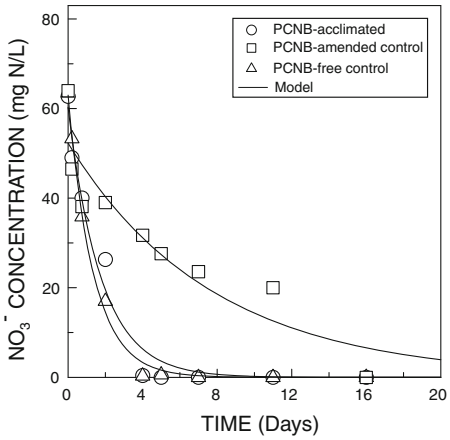

Fig. 4 Time course of nitrate during the batch biotransformation assay conducted with the PCNB-acclimated, the PCNB-amended control, and the PCNB-free control culture (*lines* are first-order fits to the nitrate data) (the cultures were fed with 60 mg NO_3^--N/l and 13 μM PCNB, except the PCNB-free control culture, which received only nitrate)

The results of the present study agree well with those of the previous study with respect to the effect of different PCNB levels on the nitrate reduction rate.

The N_2 production rate and extent were similar in the PCNB-free control and the PCNB-acclimated culture amended with 13 μM PCNB (Fig. 5a, b). In both cultures, nitrate was completely reduced to N_2 in 4 days and intermediate products of denitrification (e.g., NO, N_2O) were not observed in either culture. However, a transient inhibition and accumulation of NO and N_2O were observed in the PCNB-amended control denitrifying culture (Fig. 5c). Likewise, the CO_2 production rate and extent were very similar in the PCNB-free control culture and the PCNB-acclimated culture amended with PCNB (Fig. 5a, b). However, a lower CO_2 production rate was observed in the PCNB-amended control denitrifying culture as a result of transient inhibition brought about by denitrification intermediates (Fig. 5c). After 16 days of incubation, 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

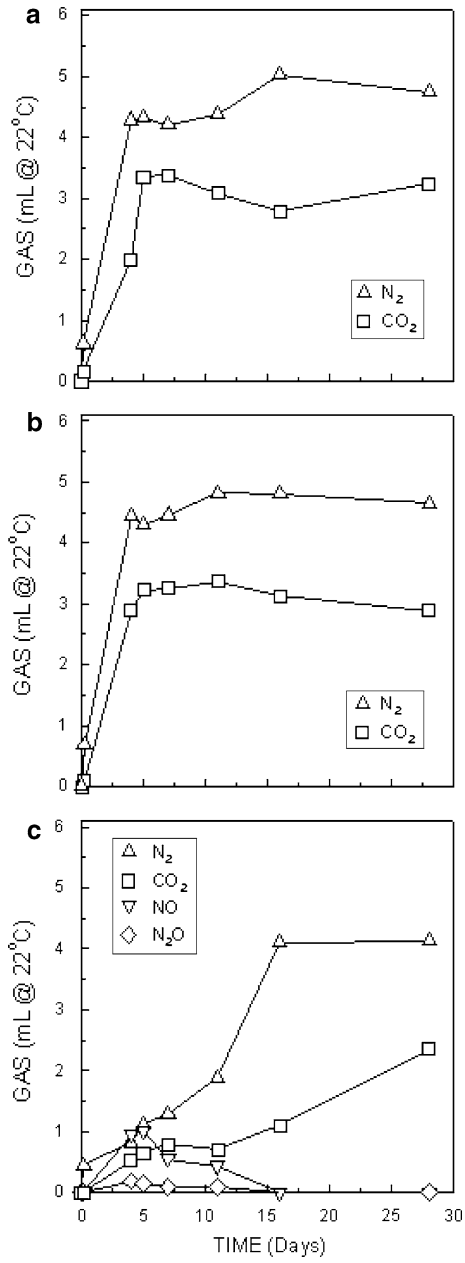

Fig. 5 Comparison of gas composition during the batch biotransformation assay conducted with the PCNB-free control culture (**a**), the PCNB-acclimated culture (**b**), and the PCNB-amended control culture (**c**) (The cultures were fed with 60 mg NO_3^--N/l and 13 μM PCNB, except the PCNB-free control culture, which received only nitrate)

microorganisms (Mancinelli and McKay 1983; Zumft 1993).

The profiles of VFAs production in the three cultures are shown in Fig. 6. Accumulation of acetic,

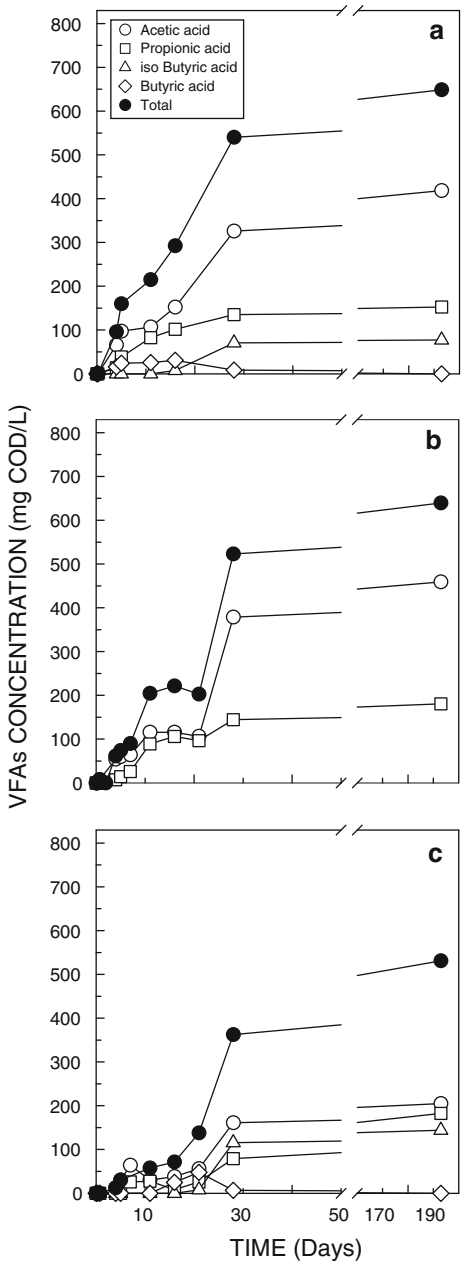

Fig. 6 Comparison of VFAs production and consumption during the batch biotransformation assay conducted with the PCNB-free control culture (**a**), the PCNB-acclimated culture (**b**), and the PCNB-amended control culture (**c**) (The cultures were fed with 60 mg NO_3^--N/l and 13 μM PCNB, except the PCNB-free control culture, which received only nitrate)

propionic, iso butyric and butyric acid was observed in both the PCNB-free control and the PCNB-amended control cultures, whereas only acetic and propionic acid accumulation was observed in the PCNB-acclimated culture. A relatively low production of VFAs was observed in the PCNB-amended control culture within 16 days of incubation (Fig. 6c), suggesting that some degree of acclimation to PCNB and PCA had taken place in the PCNB-acclimated culture over the long-term enrichment. The total COD required for the complete reduction of 60 mg/l NO_3^--N to N_2, neglecting microbial growth, 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 used 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 around the 7th day of incubation, due to NO and N_2O detection limitations (Table 1). At the end of the 28-day incubation period, close to 100% nitrogen balance closure was observed for all three cultures. Nitrogen balance calculations showed that nitrate was completely reduced to N_2, i.e., denitrification was the main nitrate reduction pathway in all three denitrifying cultures. In contrast, a *Clostridium* sp. which dechlorinated chlorinated phenols, was reported to reduce nitrate and nitrite to ammonium (Madsen and Licht 1992). In a recent study, addition of nitrate at or above 50 mg N/l to a PCA-dechlorinating, mixed fermentative/methanogenic culture amended with 3 μM PCNB resulted in accumulation of denitrification intermediates (i.e., NO, N_2O), which in turn led to partial PCA dechlorination to tetrachloroanilines

Table 1 Total nitrogen balance (%) during the incubation period of the three denitrifying cultures

Time (days)	PCNB-free control culture	PCNB-acclimated culture[a]	PCNB-amended control culture[a]
0	100.0	100.0	100.0
0.2	94.4	88.5	80.4
5	72.8	81.2	78.9
7	75.2	92.5	68.6
11	83.1	98.4	75.0
16	101.8	107.2	85.0
28	108.0	100.0	92.4

Initial biomass concentration 230 ± 35 mg POC/l (mean ± SD; $n = 3$) and initial nitrate concentration 60 mg N/l in all three cultures

[a] Cultures were amended with 13 μM PCNB

and partial nitrate reduction to ammonia was observed (Okutman Tas and Pavlostathis 2008).

Discussion

Biotransformation under nitrate reducing conditions

Under active nitrate reducing conditions applied in the present study, PCNB was transformed to PCA, which was not further dechlorinated in the enrichment culture maintained over 1 year (Fig. 1b). Several researchers have previously reported biotransformation of halogenated organics by various denitrifying microorganisms. Häggblom et al. (1993, 1996) reported degradation of 3- and 4-chlorobenzoate 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 monochlorophenols (2-CP, 3-CP, 4-CP) and dichlorophenols (2,4-DCP, 2,6-DCP) under denitrifying conditions using an activated sludge inoculum. However, they only observed stoichiometric nitrate consumption concomitantly during 2-CP degradation and a dechlorination intermediate was not detected, suggesting that the 2-CP degradation was coupled to nitrate reduction. Kostyál et al. (1997) reported that dechlorination of di-, tri- and tetrachlorophenols in a pulp and paper mill wastewater treated in a fluidized-bed bioreactor was negligible under denitrifying conditions.

Denitrification inhibition

As discussed above, amendment of the glucose/methanol fed, PCNB-free denitrifying, mixed culture with PCNB resulted in a relatively slower nitrate reduction and a transient inhibition of fermentation (Figs. 4, 5c, 6c). 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 denitrification activity in response to increasing 2,4,6-trinitrotoluene (TNT) concentrations. They reported that nitrous oxide reductase was much more sensitive to TNT than nitrate, nitrite, and nitric oxide reductases. An investigation with soils chronically exposed to halogenated and nitroaromatic compounds showed that Gram-negative bacteria tended to predominate (Fuller and Manning 1998). As discussed above, in the present study NO and N_2O were observed only in the PCNB-amended control culture and not in the PCNB-acclimated culture, both amended with an initial PCNB concentration of 13 μM.

Effect of nitrate reduction on dechlorination

Many studies have shown that nitrate inhibits the transformation of halogenated organic compounds by anaerobic microbial communities, specifically by inhibiting reductive dehalogenation (Genthner et al. 1989; Häggblom et al. 1993; Milligan and Häggblom 1999; Okutman Tas and Pavlostathis 2008; Picardal et al. 1995). 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 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). Milligan and Häggblom (1999) observed inhibition of the 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.

Production of N_2O during denitrification may inhibit reductive dechlorination. Nelson et al. (2002) showed the necessity of complete removal of nitrate and sulfate for the reductive dechlorination of perchloroethene (PCE) even though excess H_2 was provided to the culture to eliminate any threshold limitation for dehalorespirers. These researchers also observed inhibition of PCE dechlorination in microcosm experiments conducted at high N_2O (100 μM) concentrations. In the present study, the observed delay in PCNB transformation to PCA may have been the result of transient production of both NO and N_2O. In a recent study conducted with a PCNB-acclimated, mixed fermentative/methanogenic culture, amendment with 3 μM PCNB and a range of nitrate concentrations from 10 to 200 mg N/l did not affect significantly the rate of PCNB transformation to PCA, but accumulation of both NO and N_2O at or above 50 mg N/l nitrate led to partial PCA dechlorination to

tetrachloroanilines (Okutman Tas and Pavlostathis 2008).

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 ($\leq$20 mg N/l = 1.4 mM), PCA dechlorination or biodegradation was not observed. Although denitrifying cultures have been reported which under active denitrifying conditions degrade chlorinated organics with a low degree of chlorination, other studies have reported lack of degradation of mono- and dichlorophenols (Bae et al. 2002; Kostyál et al. 1997). In mixed cultures, dechlorination and denitrification processes can take place simultaneously by different populations. However, there is no evidence of dechlorination of highly chlorinated organics under denitrifying conditions. The results of the present study show that although PCNB was transformed to PCA, dechlorination of PCA was not mediated by the enriched denitrifying culture. It is noteworthy that a mixed, methanogenic and PCA-dechlorinating culture was successfully developed within 35 days of incubation using as inoculum the same sediment which was also used in the present study for the development of the denitrifying cultures (Okutman Tas and Pavlostathis 2005). The presence of halorespiring bacteria in the mixed, methanogenic and PCA-dechlorinating culture, such as *Dehalococcoides*, was confirmed based on 16S rRNA gene analysis using a nested PCR approach (Okutman Tas et al. 2006). Therefore, long-term, active nitrate reducing conditions maintained in the PCNB-acclimated culture in the present study prevented the development of halorespiring, PCA-dechlorinating bacteria.

Redox processes and thermodynamic considerations

In natural anoxic environments, several biogeochemical redox processes take place sequentially or in parallel and are affected by several factors. The distribution of products formed as a result of the sequential reductive dechlorination of pentachloroaniline (PCA) and pentachlorobenzene (PeCB) under methanogenic conditions was examined based on calculated thermodynamic, chromatographic, and electronic properties and then compared to the product distribution achieved by enrichment cultures (Okutman Tas et al. 2010). Theoretical dechlorination pathways were consistent with the formation of some of the products, especially for the less chlorinated compounds. Thus, a combination of several factors (e.g., thermodynamic, electronic, chromatographic) as well as enzyme specificity may possibly control isomer distribution during the sequential reductive dechlorination (Okutman Tas et al. 2010).

Despite the fact that dechlorination reactions are often more thermodynamically favorable than anaerobic metabolic processes such as sulfate reduction or methanogenesis, in many cases dechlorination takes place simultaneously with other metabolic processes (Maithreepala and Doong 2004; Vanderloop et al. 1999). It should be pointed out that the free energy that can be obtained from the various redox reactions of the electron acceptors may affect microbial dechlorination reactions. The free energy released during the transformation of PCNB to PCA is higher than that associated with metabolic processes such as sulfate reduction, methanogenesis and nitrate reduction (Okutman Tas and Pavlostathis 2007). The free energy released during the sequential dechlorination of chlorinated anilines is higher than that released during sulfate reduction, methanogenesis, and iron reduction (from goethite, hematite, and magnetite), but lower than the free energy released during nitrate reduction (Okutman Tas and Pavlostathis 2007). Therefore, the observed transformation of PCNB to PCA only at low nitrate concentrations ($\leq$20 mg N/l) and the lack of PCA dechlorination under active denitrifying conditions can be attributed to the free energy released during these transformation processes, a condition that led to the predominance of nitrate reducers in the mixed, PCNB-acclimated culture enriched and maintained with a stoichiometric excess of nitrate as compared to the available electron donor.

Conclusions

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 ($\leq$20 mg N/l) regardless of prior acclimation to PCNB. However, reductive dechlorination or biodegradation of PCA was not achieved under active denitrifying conditions maintained over a long period. PCNB addition to denitrifying cultures resulted in temporary inhibition leading to a transient NO and N_2O accumulation. As discussed above, PCA has the same acute toxicity as PCNB, but it is more hydrophobic than PCNB. Therefore, accumulation of this potentially toxic metabolite may create significant problems in anoxic, subsurface systems that do not support reductive dechlorination. The results of the present study therefore have significant implications relative to the fate and biotransformation of PCNB and PCA under nitrate reducing conditions commonly encountered in agricultural fields.

Acknowledgments This work was supported in part by a U.S. Geological Survey grant administered through the Georgia Water Resources Institute.

References

Argese E, Bettiol C, Agnoli F, Zambon A, Mazzola M, Ghirardini AV (2001) Assessment of chloroaniline toxicity by the submitochondrial particle assay. Environ Toxicol Chem 20:826–832. doi:10.1897/1551-5028(2001)020

Bae HS, Yamagishi T, Suwa Y (2002) Evidence for degradation of 2-chlorophenol by enrichment cultures under denitrifying conditions. Microbiology 148:221–227

Bae HS, Yamagishi T, Suwa Y (2004) An anaerobic continuous-flow fixed-bed reactor sustaining a 3-chlorobenzoate-degrading denitrifying population utilizing versatile electron donors and acceptors. Chemosphere 55:93–100. doi:10.1016/j.chemosphere.2003.10.022

Chang BV, Zheng JX, Yuan SY (1996) Effects of alternative electron donors, acceptors and inhibitors on pentachlorophenol dechlorination in soil. Chemosphere 33:313–320. doi:10.1016/0045-6535(96)00174-9

Chen IM, Chang BV, Yuan SY, Wang YS (2002) Reductive dechlorination of hexachlorobenzene under various additions. Water Air Soil Pollut 139:61–74. doi:10.1023/A:1015861217112

Chidthaisong A, Conrad R (2000) Turnover of glucose and acetate coupled to reduction of nitrate, ferric iron and sulfate and to methanogenesis in anoxic rice field soil. FEMS Microbiol Ecol 31:73–86

Coschigano PW, Häggblom MM, Young LY (1994) Metabolism of both 4-chlorobenzoate and toluene under denitrifying conditions by a constructed bacterial strain. Appl Environ Microbiol 60:989–995

Freedman DL, Swamy M, Bell NC, Verce MF (2004) Biodegradation of chloromethane by *Pseudomonas aeruginosa* strain NB1 under nitrate-reducing and aerobic conditions. Appl Environ Microbiol 70:4629–4634. doi:10.1128/AEM.70.8.4629-4634.2004

Fuller ME, Manning JF (1998) Evidence for differential effects of 2,4,6-trinitrotoluene and other munitions compounds on specific subpopulations of soil microbial communities. Environ Toxicol Chem 17:2185–2195. doi:10.1897/1551-5028(1998)017

Genthner BRS, Price WAI, Pritchard PH (1989) Anaerobic degradation of chloroaromatic compounds in aquatic sediments under a variety of enrichment conditions. Appl Environ Microbiol 55:1466–1471

Gerritse J, Drzyzga O, Kloetstra G, Keijmel M, Wiersum LP, Hutson R, Matthew D, Collins MD, Gottschal JC (1999) Influence of different electron donors and acceptors on dehalorespiration of tetrachloroethene by *Desulfitobacterium frappieri* TCE1. Appl Environ Microbiol 65:5212–5221

Gess P, Pavlostathis SG (1997) Desorption of chlorinated organic compounds from a contaminated estuarine sediment. Environ Toxicol Chem 16:1598–1605. doi:10.1897/1551-5028(1997)016

Häggblom MM, Young LY (1999) Anaerobic degradation of 3-halobenzoates by a denitrifying bacterium. Arch Microbiol 171:230–236. doi:10.1007/s002030050704

Häggblom MM, Rivera MD, Young LY (1993) Influence of alternative electron acceptors on the anaerobic biodegradability of chlorinated phenols and benzoic acids. Appl Environ Microbiol 59:1162–1167

Häggblom MM, Rivera MD, Young LY (1996) Anaerobic degradation of halogenated benzoic acids coupled to denitrification observed in a variety of sediment and soil samples. FEMS Microbiol Lett 144:213–219

Hakala JA, Chin YP, Weber EJ (2007) Influence of dissolved organic matter and Fe(II) on the abiotic reduction of pentachloronitrobenzene. Environ Sci Technol 41:7337–7342. doi:10.1021/es070648c

Klupinski TP, Chin YP, Traina SJ (2004) Abiotic degradation of pentachloronitrobenzene by Fe(II): reactions on goethite and iron oxide nanoparticles. Environ Sci Technol 38:4353–4360. doi:10.1021/es035434j

Kostyál E, Nurmiaho-Lassila EL, Puhakka JA, Salkinoja-Salonen M (1997) Nitrification, denitrification, and dechlorination in bleached kraft pulp mill wastewater. Appl Microbiol Biotechnol 47:734–741. doi:10.1007/s002530051003

Kuhn EP, Suflita JM (1989) Sequential reductive dehalogenation of chloroanilines by microorganisms from a methanogenic aquifer. Environ Sci Technol 23:848–852. doi:10.1021/es00065a014

Kuhn EP, Townsend GT, Suflita JM (1990) Effect of sulfate and organic carbon supplements on reductive dehalogenation of chloroanilines in anaerobic aquifer sediments. Appl Environ Microbiol 56:2630–2637

Madsen T, Licht D (1992) Isolation and characterization of an anaerobic chlorophenol-transforming bacterium. Appl Environ Microbiol 58:2874–2878

Mah AR, Smith MR (1981) The methanogenic bacteria. In: Starr MP (ed) Prokaryotes: a handbook of habitats, isolation and identification of bacteria. Springer-Verlag, New York

Maithreepala RA, Doong RA (2004) Enhanced remediation of carbon tetrachloride by Fe(II)-Fe(III) systems in the presence of copper ions. Water Sci Technol 50:161–168

Mancinelli RL, McKay CP (1983) Effects of nitric oxide and nitrogen dioxide on bacterial growth. Appl Environ Microbiol 46:198–202

Middeldorp PJM, van Doesburg W, Schraa G, Stams AJM (2005) Reductive dechlorination of hexachlorocyclohexane (HCH) isomers in soil under anaerobic conditions. Biodegradation 16:283–290. doi:10.1007/s10532-004-1573-8

Milligan PW, Häggblom MM (1999) Biodegradation and biotransformation of dicamba under different reducing conditions. Environ Sci Technol 33:1224–1229. doi:10.1021/es981117e

Nelson DK, Hozalski RM, Clapp LW, Semmens MJ, Novak PJ (2002) Effect of nitrate and sulfate on dechlorination by a mixed hydrogen-fed culture. Bioremed J 6:225–236. doi:10.1080/10889860290777585

Okutman Tas D, Pavlostathis SG (2005) Microbial reductive transformation of pentachloronitrobenzene under methanogenic conditions. Environ Sci Technol 39:8264–8272. doi:10.1021/es050407+

Okutman Tas D, Pavlostathis SG (2007) The influence of iron reduction on the reductive biotransformation of pentachloronitrobenzene. Europ J Soil Biol 43:264–275. doi:10.1016/j.ejsobi.2007.03.003

Okutman Tas D, Pavlostathis SG (2008) Effect of nitrate reduction on the microbial reductive transformation of pentachloronitrobenzene. Environ Sci Technol 42:3234–3240. doi:10.1021/es702261w

Okutman Tas D, Thomson IN, Löffler FE, Pavlostathis SG (2006) Kinetics of the microbial reductive dechlorination of pentachloroaniline. Environ Sci Technol 40:4467–4472. doi:10.1021/es052103t

Okutman Tas D, Prytula MT, Mulholland JA, Pavlostathis SG (2010) Theoretical investigation of the sequential reductive dechlorination pathways of chlorobenzenes and chloroanilines. Biotechnol Bioeng 105:574–587. doi:10.1002/bit.22559

Petersen JN, Skeen RS, Amos KM, Hooker BS (1994) Biological destruction of CCl_4.1. Experimental-design and data. Biotechnol Bioeng 43:521–528

Picardal F, Arnold RG, Huey BB (1995) Effects of electron donor and acceptor conditions on reductive dehalogenation of tetrachloromethane by *Shewanella putrefaciens* 200. Appl Environ Microbiol 61:8–12

Prasad R (1996) Sustainable agriculture and fertilizer use. Current Sci 77:38–43

Prytula MT, Pavlostathis SG (1996) Effect of contaminant and organic matter bioavailability on the microbial dehalogenation of sediment-bound chlorobenzenes. Water Res 30:2669–2680

Sanford RA, Tiedje JM (1997) Chlorophenol dechlorination and subsequent degradation in denitrifying microcosms fed low concentrations of nitrate. Biodegradation 7:425–434. doi:10.1007/BF00056426

Sherwood JL, Petersen JN, Skeen RS, Valentine NB (1996) Effects of nitrate and acetate availability on chloroform production during carbon tetrachloride destruction. Biotechnol Bioeng 51:551–557

Siciliano SD, Roy R, Greer CW (2000) Reduction in denitrification activity in field soils exposed to long term contamination by 2,4,6-trinitrotoluene (TNT). FEMS Microbiol Ecol 32:61–68

Susarla S, Masunaga S, Yonezawa Y (1996) Transformations of chloronitrobenzenes in anaerobic sediment. Chemosphere 32:967–977. doi:10.1016/0045-6535(96)00006-9

Susarla S, Yonezawa Y, Masunaga S (1997) Reductive dehalogenation of chloroanilines in anaerobic estuarine sediment. Environ Technol 18:75–83

Tamura K, Hasegawa Y, Kudo T, Yamaguchi I (1995) Isolation and characterization of PCNB degrading bacterium, *Pseudomonas aeruginosa* Strain I-41. J Pesticide Sci 20:145–151

U.S. Environmental Protection Agency (2003) Waste minimization priority chemicals and chemical fact sheets website (http://www.epa.gov/epaoswer/hazwaste/minimize/chemlist.htm.) Office of Solid Waste and Emergency Response, Washington, DC

Vanderloop SL, Suidan MT, Moteleb MA, Maloney SW (1999) Biotransformation of 2,4-dinitrotoluene under different electron acceptor conditions. Water Res 33:1287–1295. doi:10.1016/S0043-1354(98)00320-0

Wolin EA, Wolin MJ, Wolfe RS (1963) Formation of methane by bacterial extracts. J Biol Chem 238:2882–2886

Zumft WG (1993) The biological role of nitric oxide in bacteria. Arch Microbiol 160:253–264. doi:10.1007/BF00292074