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H_2 consumption during the microbial reductive dehalogenation of chlorinated phenols and tetrachloroethene *

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Abstract

Competition for molecular hydrogen exists among hydrogen-utilizing microorganisms in anoxic environments, and evidence suggests that lower hydrogen concentrations are observed with more energetically favorable electron-accepting processes. The transfer of electrons to organochlorines via reductive dehalogenation reactions plays an important role in hydrogen dynamics in impacted systems. We studied the flux of aqueous hydrogen concentrations in methanogenic sediment microcosms prior to and during reductive dehalogenation of a variety of substituted chlorophenols (CP) and tetrachloroethene (perchloroethylene, PCE). Mean hydrogen concentrations during reductive dehalogenation of 2,4-CP, 2,3,4-CP, and PCP were 3.6 nM, 4.1 nM, and 0.34 nM, respectively. Sediment microcosms that were not dosed with chlorophenols yet were actively methanogenic maintained a significantly higher mean hydrogen concentration of 9.8 nM. During active PCE dehalogenation, sediment microcosms maintained a mean hydrogen concentration of 0.82 nM. These data indicate that during limiting hydrogen production, the threshold ecosystem hydrogen concentration is controlled by microbial populations that couple hydrogen oxidation to thermodynamically favorable electron accepting reactions, including reductive dehalogenation of chloroaromatic and chloroaliphatic compounds. We also present revised estimates for the Gibbs free energy available from the reductive dehalogenation of a variety of substituted chlorophenols based on recently published values of vapor pressure, solubility, and pK_a for these compounds.

Introduction

Chlorinated phenols are among the many halogenated aromatic compounds used for a variety of biocide applications (Liu et al. 1996). The widespread use of such chemicals as wood preservatives, insecticides, and herbicides is of tremendous concern due to their toxicity within the environment. The industrial application of chlorophenols over several decades has resulted in large-scale contamination of terrestrial environments and groundwater supplies (Haggblom & Valo 1995). In addition to its deliberate dispersal, halogenated phenols may also enter the environment as intermediates formed during the anaerobic microbial metabolism of other anthropogenic herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Boyle et al. 1999). Currently, chlorinated phenols are listed as priority pollutants by the U.S. Environmental Protection Agency (EPA) (Federal Register 1984).

Numerous reports have illustrated that, under anoxic conditions, microbial transformation of chlorophenols proceeds via reductive dehalogenation (O'Conner & Young 1996; McAllister et al. 1996; Magar et al. 2000; Takeuchi et al. 2000; Tartakovsky et al. 2001). Reductive dehalogenation is defined as the addition of electrons to a molecule with the concomitant removal of a halogen substituent (Mohn & Tiedje 1992). Several studies have documented rates and pathways of dehalogenation of chlorinated phenols under sulfate-reducing and methanogenic redox

conditions (Haggblom & Young 1995; Masunaga et al. 1996a; Haggblom et al. 1993; Haggblom 1998). In particular, one investigation demonstrated the complete dechlorination of pentachlorophenol (PCP) using a mixture of sludges acclimated to transform monochlorinated phenols (Mikesell & Boyd 1986). Many physicochemical characteristics, including solubility, octanol-water partition coefficient, pH, and position of chlorine substituents, as well as toxic effects on cellular functions, are known to influence the onset, rate and pathway of anaerobic microbial metabolism of halogenated phenols (Escher et al. 1996; Mohn & Kennedy 1992).

Hydrogen gas is produced in anoxic sediments through the oxidation of organic matter, and the available H_2 is rapidly consumed (oxidized) via microbial-mediated terminal electron accepting processes such as iron reduction, sulfate-reduction, and methanogenesis (Hoehler et al. 1998). Thus, the metabolism of molecular hydrogen plays as an important role in electron transfer between fermenting and respiring microorganisms in anaerobic sediments. Lovely & Goodwin (1988) have presented evidence that the thermodynamics of a predominant terminal hydrogen-consuming reaction impart an important control on observed hydrogen concentrations when hydrogen production is rate-limiting. Data suggest that microorganisms that couple oxidative metabolism to an energetically more favorable electron acceptor are more competitive for available hydrogen than organisms utilizing electron acceptors of lower energy yield, resulting in the maintenance of a lower hydrogen concentration (Conrad 1999).

Evidence presented to date suggests that the transfer of electrons to halogenated organic compounds may indeed have a direct effect on hydrogen dynamics in anoxic sediments (Christensen et al. 2000). Electron transfer coupled to the reductive dehalogenation of chlorinated aromatic and aliphatic compounds may provide microbes a thermodynamic advantage over natural electron accepting processes such as microbial sulfate reduction and methanogenesis (Table 1). Several investigations have reported that during the anaerobic microbial metabolism of chloroethylene compounds, hydrogen serves as an important electron transfer agent. In those studies, microorganisms acclimated for sequential reductive dehalogenation of chlorinated aliphatics maintained a lower hydrogen concentration compared to conditions without dehalogenation but that favored methanogenesis (Smatlak et al. 1996; Yang & McCarty 1998). Recently, we reported that a lower hydrogen concentration was maintained in sediment slurries with PCE serving as a terminal electron acceptor compared to sediments in which sulfate-reduction was the predominant electron accepting process (Mazur & Jones 2001).

Experiments conducted with enriched cultures and bacterial isolates, utilizing H_2 as an electron donor, have shown that chlorinated phenols can also serve as favorable electron acceptors (Van de Pas et al. 2001; Holliger et al. 1999). Loffler et al. (1999), using pure and enriched microbial cultures, demonstrated a lower hydrogen concentration during the dechlorination of 2-chlorophenol compared to either sulfidogenic or methanogenic cultures. Results from an acclimated microbial culture derived from sewage sludge indicated that hydrogen competition between sulfate reducing bacteria and a dechlorinating organism(s) may inhibit the rate of PCP dehalogenation (Madsen & Aamand 1991). In addition, studies with Chesapeake Bay estuarine sediments reported stimulation of reductive transformation of 2,4-dichlorophenol by the addition of exogenous hydrogen in sulfate-depleted sediments, whereas dechlorination was not enhanced in the same system when supplied with ample sulfate (Warner et al. 2002).

Although several reports using defined cultures indicate that H_2 is an important electron donor for reductive transformation reactions, current information regarding the competition for H_2 during chlorophenol dehalogenation in anaerobic sediments remains limited. The primary objective of this study was to determine molecular hydrogen concentrations in methanogenic sediment slurries in the presence and absence of organochlorines and during the reductive transformation of a variety of substituted chlorophenols and PCE.

Materials and methods

Chemicals

The following chlorinated phenols were used in this study: pentachlorophenol (PCP), 2,3,4,5-tetrachlorophenol (2,3,4,5-CP), 3,4,5-trichlorophenol (3,4,5-CP), 2,3,4-trichlorophenol (2,3,4-CP), 2,4-dichlorophenol (2,4-CP), 3,4-dichlorophenol (3,4-CP), and 4-chlorophenol (4-CP). Chlorophenols were purchased from AccuStandard Inc., New Haven, CT. Neat solutions of PCE, trichloroethylene (TCE) (Aldrich Chemical Co., Milwaukee, WI), and *cis*-1,2,-dichloroethylene (*cis*-1,2-DCE) (Supelco Park,

Table 1. Energetics of various dechlorination reactions and environmentally relevant terminal electron transfer reactions

Reaction	$\Delta G^{\circ\prime}$ (kJ/mol of H_2)	
	A Original from Dolfing and Harrison (1992)	B Recalculated (new VP, S, and pK_a; pH 7.0)[a]
PCP + H_2 → 2,3,4,5-CP + H^+ + Cl^-	−156.9	−152.4
2,3,4,5-CP + H_2 → 3,4,5-CP + H^+ + Cl^-	−141.0	−141.3
3,4,5-CP + H_2 → 3,4-CP + H^+ + Cl^-	−142.3	−159.8
2,3,4-CP + H_2 → 3,4-CP + H^+ + Cl^-	−152.9	−158.1
3,4-CP + H_2 → 4-CP + H^+ + Cl^-	−144.7	−151.3
2,4-CP + H_2 → 4-CP + H^+ + Cl^-	−131.3	−148.6
4-CP + H_2 → Phenol + H^+ + Cl^-	−164.5	−150.0
PCE + H_2 → TCE + H^+ + Cl^-	−173[b]	
SO_4^{2-} + $4H_2$ → S^{2-} + $4H_2O$	−38[c]	
CO_2 + $4H_2$ → CH_4 + $2H_2O$	−33[c]	

[a] Best available property values.

Compound	Vapor pressure (kP_a)[d]	Solubility (mM)[e]	pK_a[f]
4-CP	0.018	206.3	9.29
2,4-CP	0.015[g]	38.8	7.85
3,4-CP	0.0019	58.1	8.62
2,3,4-CP	0.00095	9.5	6.97
3,4,5-CP	0.00040	5.4[i]	7.80
2,3,4,5-CP	0.00020	14.5	5.64
PCP	0.00012[h]	6.0	4.72

[b] From Dolfing and Janssen (1994).
[c] From Zinder (1993).
[d] Vapor pressure values calculated using SPARC calculator (Hilal et al. 1994).
[e] From Ma et al. (1993).
[f] From Wightman and Fein (1999).
[g] From Bidleman and Renberg (1985).
[h] From Lei et al. (1999).
[i] Solubility of 3,4,5-CP at pH 5 was assumed equal to that of 2,3,4-CP; both species are expected to be in the protonated form at pH 5, and solubility values calculated by SPARC for the protonated form are similar.

Bellefonte, PA) were used to prepare chlorinated aliphatic analytical standards. Scott Speciality Gases (99%, Aldrich Chemical Co.) were used to prepare methane standards. Hydrogen standards were prepared using Grade 5.5 (99.999%) hydrogen (BOC Group Inc., Murray Hill, N.J.) and were calibrated using a 500 ppb (v/v) hydrogen standard purchased from Scott-Marrin, Inc.(Riverside, CA).

Sediment collection and microcosm preparation

Sediment samples were collected at a depth of approximately 20–25 cm below the surface of a freshwater tidal wetland area along the West Branch Canal Creek located at Aberdeen Proving Ground, Maryland. Sediments were placed in glass jars, sealed, and stored at 4 °C prior to use. The sediment, collected from a site designated as WB-26, was characterized as methanogenic and has a documented history of exposure to several volatile organic compounds (VOCs), including TCE, 1,1,2,2-tetrachloroethane (PCA), carbon tetrachloride (CT), and chloroform (CF) (Lorah et al. 1997).

Sediment slurries were prepared inside an anaerobic chamber containing an atmosphere of 99% N_2 and 1% H_2. The collected sediment was passed through a 1-mm sieve, and thoroughly mixed with anoxic (N_2-sparged) water collected from a local freshwater river located in Athens, GA, to achieve a sediment solids concentration of approximately 100 g/L slurry. Forty-five milliliters of the slurry was dispensed into 60-mL amber serum bottles that were sub-

sequently sealed with Teflon-lined, butyl-rubber septa and aluminum crimp caps. Individual chlorophenols (2,3,4,5-CP; 3,4,5-CP; 2,3,4-CP; and 2,4-CP) were added to the bottles at a final concentration of approximately 25 μM from saturated aqueous stock solutions. Because of concerns about aqueous solubility constraints, PCP (dissolved in hexane) was added to sterile, empty serum bottles and the hexane was evaporated to dryness prior to addition of the sediment slurry. The final concentration of PCP in sediment microcosms was 25 μM. In re-dosing experiments, PCP was added to empty, sterile bottles as described above followed by anoxic transfer of the microcosm slurry to the solvent evaporated bottle. Published solubility values for PCP at room temperature vary over two orders of magnitude. A thorough review of the literature on PCP solubility revealed that the variability in the reported values for PCP solubility is primarily due to differences in measurement pH. Near a pH of 5, reported solubility values range from 0.04 to 0.08 mM (Arcand et al. 1995; Blackman et al. 1955; Ma et al. 1993). At neutral pH, PCP exists primarily in the ionized form; therefore, the observed solubility is much higher. Measured solubility values near neutral pH range from 4.3 to 7.5 mM (Arcand et al. 1995; Huang et al. 2000; Wightman and Fein 1999). In separate experiments, PCE was added to sediment slurries from a PCE-saturated aqueous stock solution. At specified times, an organic carbon solution (N_2-sparged) was added to the sediment slurries to ensure an ample supply of electron donor. The solution consisted of the following constituents at the indicated final concentration: 0.5 g/L dextrin; 0.5 g/L soluble starch; 0.25 g/L yeast extract; 0.1 g/L sodium pyruvate; and 0.5 g/L cellobiose. Sediment slurries containing no organochlorine compounds were also prepared to evaluate microbial processes and hydrogen concentrations in the absence of supplemented alternative electron acceptors. Sterile control microcosms were prepared by autoclaving sediment slurries at 120 °C for 30 minutes over three consecutive days. All experimental microcosms were prepared in triplicate. The composited sediment sample used in this study had a total organic content of 6.1% (w/w) and the pH of the sediment slurries was approximately 6.5.

Analytical techniques

Chlorinated phenols were analyzed using high-performance liquid chromatography (HPLC) equipped with a variable wavelength ultraviolet detection system. Subsamples (1 mL) of the slurry material were extracted into an equal volume of acetonitrile, vortexed for 60 seconds, and centrifuged before being transferred to automatic sampling vials. All metabolites were analyzed using a 1050 series Hewlett-Packard HPLC and were quantified using authentic standards. The solvent system consisted of 65% acetonitrile, 33% water, and 2% acetic acid, and separation was achieved using a *u*Bondapack C-18 analytical column (3.9 × 100 mm column, Waters Inc., Milford, Massachusetts.).

Gas chromatography techniques were used to quantify PCE, TCE, *cis*-1,2 DCE, methane and hydrogen. Subsamples (50 μL) of the sediment slurry were extracted into hexane (1 mL) and analyzed for chlorinated aliphatics by gas chromatography. PCE and TCE were analyzed by direct injection (1 μL) using a 5890 Hewlett-Packard gas chromatograph equipped with an electron capture detector (ECD). Separation was achieved with a 30 m × 0.32 mm i.d., 0.25 mm film thickness DB-5 capillary column (J&W Scientific, Folsom, CA). The detector and injection-port temperatures were 300 and 150 °C, respectively. The temperature program was as follows: 60 °C, hold 5 minutes, 10 °C min^{-1} to 90 °C, no hold, 40 °C/min to 220 °C. Volatile organic dehalogenation products were concentrated from aqueous samples by purge and trap followed by desorption of the VOCs from the adsorbent and GC analysis using a Hewlett Packard (HP) 6890 gas chromatograph equipped with a HP 5971A mass spectrometer. VOCs were separated and quantified using a 30 m × 0.32 mm id, 1.8 μm film thickness HP-624 capillary column. The injection and detector temperatures were 250 and 280 °C, respectively. The temperature program was as follows: 32 °C, hold 6 min, 6 °C min^{-1} to 142 °C, no hold, 20 °C min^{-1} to 160 °C, hold for 1 min.

Headspace samples (200 μL) were analyzed for methane by gas chromatography using a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector. Methane was separated on a Poropak N column (6′ × 1/8″ o.d., 80/100 mesh; Alltech, Deerfield, IL) at 50 °C with a N_2 carrier flow of 15 ml min^{-1}.

Hydrogen concentration from sediment slurries was measured by direct injection of headspace samples into a Trace Analytical model RGA3 gas chromatograph (Menlo Park, CA) equipped with 1 ml loop and a reduction gas detector (RGD2). Hydrogen was analyzed every 3–4 days to ensure equilibrium during the continuous production and consumption of

aqueous hydrogen in the sediment slurries (Mazur & Jones 2001). The concentration of dissolved hydrogen in the aqueous solution was calculated by assuming equilibrium as follows: $H_{2(\text{dissolved})} = (\text{LP})/(\text{RT})$, where $H_{2(\text{dissolved})}$ is the concentration of dissolved H_2 in moles per liter; L is the Ostwald coefficient for H_2 (Wilhelm et al. 1977); R is the universal gas constant; P is pressure (atm); and T is the temperature (K). All hydrogen concentration data are reported as the aqueous-phase concentration.

Calculation of Gibbs free energy values

The Gibbs free energy change ($\Delta G^{\circ\prime}$) was calculated for the reductive dechlorination of each chlorinated phenol with hydrogen as the electron donor. These values were calculated for each possible reaction pathway (i.e., each potential dechlorination site) and adjusted for the species present at pH 7. For example, the reaction for the reductive dechlorination of PCP at the *ortho* position is:

$$\begin{aligned}(\alpha_0)_{\text{PCP}}\,C_6Cl_5OH + [1\text{-}(\alpha_0)_{\text{PCP}}]C_6Cl_5O^- + H_2 \\ \Leftrightarrow (\alpha_0)_{2,3,4,5\text{-CP}}C_6HCl_4OH \\ + [1\text{-}(\alpha_0)_{2,3,4,5\text{-CP}}]C_6HCl_4O^- \\ + Cl^- + [1 + (\alpha_0)_{\text{PCP}}\text{-}(\alpha_0)_{2,3,4,5\text{-CP}}]H^+, \quad (1)\end{aligned}$$

where $(\alpha_0)_{\text{PCP}}$ and $(\alpha_0)_{2,3,4,5\text{-CP}}$ are the fraction of PCP and 2,3,4,5-CP, respectively, in the protonated form. At neutral pH, the highly chlorinated phenols tend to be in the ionized form (e.g., $(\alpha_0)_{\text{PCP}} = 0.005$ at pH 7), while the less chlorinated phenols tend to be in the protonated form (e.g., $(\alpha_0)_{4\text{-CP}} = 0.99$ at pH 7). The $\Delta G^{\circ\prime}$ for reaction (1) is calculated from the Gibbs free energy of formation (ΔG°_f) of each species involved in the reaction, as follows:

$$\begin{aligned}\Delta G^{\circ\prime} \quad = \quad & \left(\sum_i \nu_i \Delta G^{\circ}_{f,i}\right)_{\text{products}} \\ & - \left(\sum_i \nu_i \Delta G^{\circ}_{f,i}\right)_{\text{reactants}}, \quad (2)\end{aligned}$$

where $\sum$ refers to the summation of the terms and ν_i is the stoichiometric coefficient of species i. This calculation differs from the Gibbs free energy change values calculated at biological standard state ($\Delta G^{\circ\prime}$) presented by Dolfing & Harrison (1992), since Dolfing & Harrison (1992) calculated $\Delta G^{\circ\prime}$ as if the chlorinated aromatics were solely present in the ionized form.

The Gibbs free energy of formation of each chlorinated phenol was calculated using the approach described by Dolfing & Harrison (1992). In this approach, the ΔG°_f value for the compound in solution is calculated by adjusting the ΔG°_f value of the gaseous form of the compound, using the following equation:

$$\Delta G^{\circ}_{f\,\text{aq}} = \Delta G^{\circ}_{f\,\text{gas}} + RT \ln\left(\frac{P}{P^0}\right) - RT \ln S_0, \quad (3)$$

where P is the vapor pressure, P^0 is atmospheric pressure and S_0 is the solubility of the neutral form of the compound. The Gibbs free energy of formation of the ionized form of a compound is related to the Gibbs free energy of formation of the neutral form ($\Delta G_{f\,\text{aq}}$) as follows:

$$\Delta G^{\circ}_{f\,\text{ion}} = \Delta G^{\circ}_{f\,\text{aq}} - RT \ln(K_a). \quad (4)$$

In the present study, the ΔG°_f calculations by Dolfing & Harrison (1992) were repeated using improved estimates of the solubility and vapor pressure of each compound. The original ΔG°_f values presented by Dolfing & Harrison (1992) for the gaseous forms of each compound were used as the starting point for the calculations. These $\Delta G_{f\,\text{gas}}$ values were calculated by applying Benson's method.

Table 1 lists the solubility and vapor pressure values used in this analysis. Measured vapor pressure values were available for 2,4-CP (Bidleman & Renberg 1985) and for PCP (Lei et al. 1999). Vapor pressure values for the other chlorinated phenols were calculated with the SPARC (Sparc Performs Automated Reasoning in Chemistry) model (Hilal et al. 1994). The S_0 values were calculated from solubility data measured near pH 5 (Ma et al. 1993), as described by Wightman & Fein (1999). For 3,4,5-trichlorophenol, no measured solubility data were available; therefore, its solubility was estimated based on that of a similar compound. Calculated S_0 values from the SPARC model (Hilal et al. 1996) demonstrated that the S_0 value for 3,4,5-trichlorophenol should be similar to the value for 2,3,4-trichlorophenol.

Results and discussion

Free-energy calculations presented in Table 1 indicate that the reductive dehalogenations of chlorinated phenols may serve as energetically favorable electron accepting processes. Values reported in column "A" of

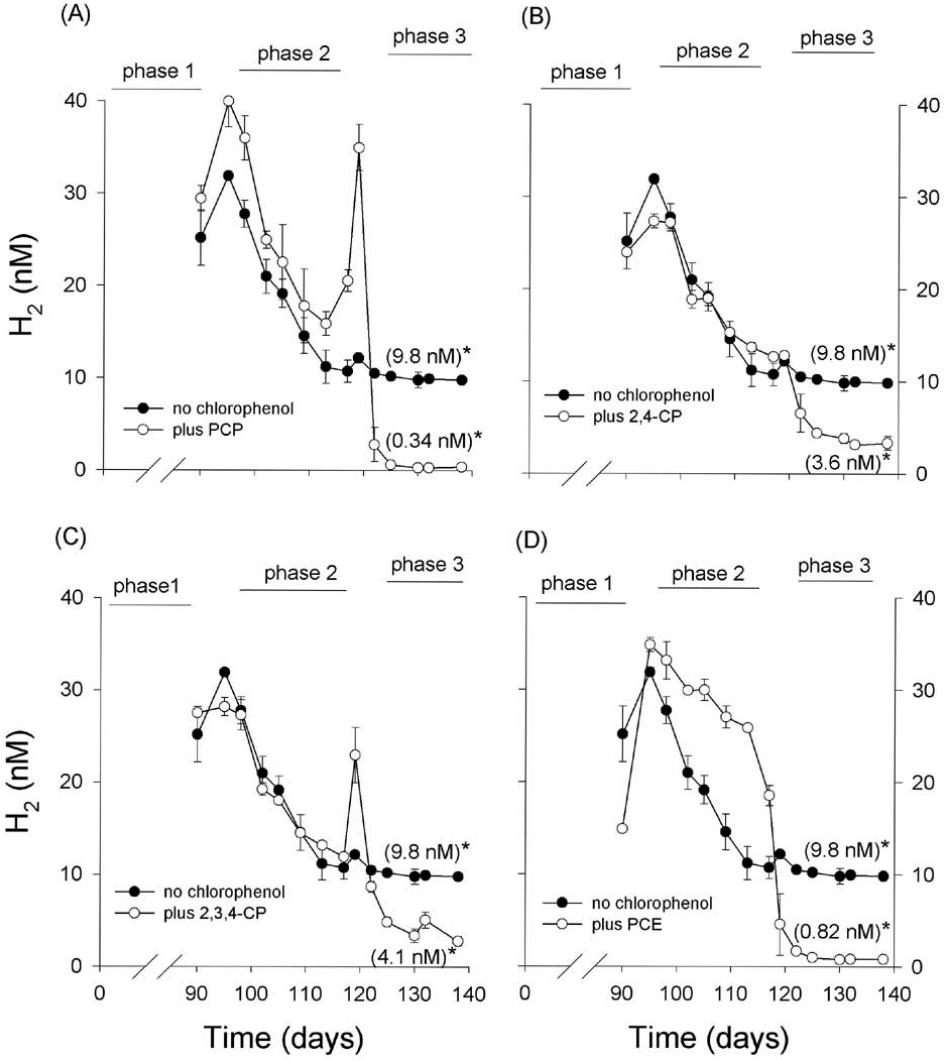

Figure 1. Profiles of H_2 concentration in natural sediment slurries (no chlorophenol addition) and in sediment slurries dosed with (a) PCP, (b) 2,4-CP, (c) 2,3,4-CP, and (d) PCE. Phase 1 represents an initial 90 day period of acclimation to the specific organochlorine; phase 2 represents the time period following addition of a mixture of readily degradable organic compounds; phase 3 represents the time period of observed dehalogenation of the dosed organochlorine. The numbers in parenthesis represent the mean hydrogen concentration during the phase 3 (dehalogenation) time period (days 125–138) as represented by the 'solid bar'.

Table 1 are those from Dolfing & Harrison (1992), and have been widely cited in the literature. $\Delta G^{\circ\prime}$ values in column "B" are newly calculated values based on calculated or recently published data for chlorophenol vapor pressure (Bidleman & Renberg 1985; Lei et al. 1999), solubility (Ma et al. 1993), and pK_a (Wightman & Fein 1999). The "best available" property values for the recalculated $\Delta G^{\circ\prime}$ values reported in column "B" are presented in Table 1. It should be noted that the $\Delta G^{\circ\prime}$ values in column "A" were calculated for the ionic forms of the chlorophenols, which in some cases are not the environmentally relevant species. Thus, in column "B", $\Delta G^{\circ\prime}$ values were calculated for the chlorophenol species distribution that would be present at pH 7.

During the initial 90 day period of sediment slurry incubation and acclimation to the added organochlorines, reductive dehalogenation was observed in freshwater sediment slurries individually dosed with PCP, 3,4,5-CP, 2,3,4-CP, 2,4-CP, and PCE (25 μM). No dehalogenation was observed in microcosms containing 2,3,4,5-CP (25 μM) or in any of the organochlorine-

dosed autoclaved controls. Following the 90-day acclimation and dehalogenation period, an organic carbon mixture was added to all sediment microcosms to ensure an ample supply of oxidizable carbon for continual microbial activity. During the next 20–25 days, background levels of hydrogen (Figure 1; days 90–115) and methane were quantified and the microcosms were then re-dosed with the original organochlorine compound (day 115). Concentrations of hydrogen and methane were again measured over time in the dehalogenating microcosms and values were compared to microcosms receiving no organochlorine compound (characterized as methanogenic).

During the time period (days 90–97) immediately following addition of the organic carbon mixture (but prior to re-addition of the chlorophenols), hydrogen levels were high (greater than 20 nM) in all sediment microcosms (Figure 1a–c). These results were attributed to an initial burst of hydrogen produced from the oxidation of the added organic carbon mixture. During this time, dissolved hydrogen concentrations measured in the "natural" microcosms (containing no chlorophenol) and those originally dosed with 2,4-CP, 2,3,4-CP, and 3,4,5-CP were similar. At 22 to 25 days following the addition of the organic carbon mixture (days 112–115), aqueous hydrogen values measured in the microcosms originally dosed with 2,4-CP, 2,3,4-CP, and 3,4,5-CP ranged from approximately10 to13 nM (Figures 1b-c, 2b). These values were comparable to H_2 concentrations reported in previous studies that were representative of methanogenic conditions (Lovely & Goodwin 1988; Yang & McCarty 1998). Higher hydrogen values (approximately 15–20 nM) (Figures 1a and 3) and lower methane concentrations (data not shown) were observed in microcosms originally dosed with PCP and 2,3,4,5-CP, suggesting partial inhibition of hydrogen-mediated methanogenesis due to the inhibitory effects of residual concentrations of PCP and 2,3,4,5-CP and/or their dehalogenation products. Although PCP dehalogenation was observed in the original PCP-dosed sediment microcosms, residual levels (20 μM) of its dehalogenation product (2,3,4,5-CP) accumulated.

Over a 10 day period immediately following addition of the organic carbon mixture, methane concentrations rapidly increased in all sediment microcosms (data not shown). Further, rates of methane production and final concentrations of methane in microcosms originally dosed with 2,4-CP, 2,3,4-CP, or 3,4,5-CP were similar to methane values in sediment slurries without chlorophenol addition. The consumption of

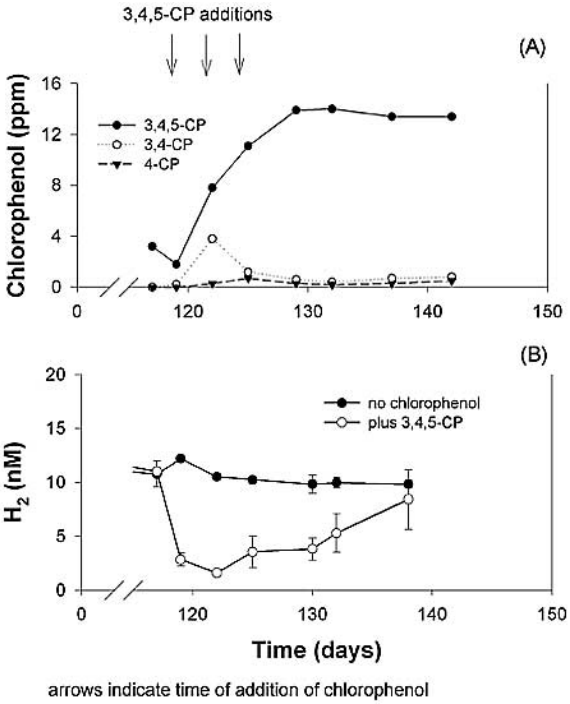

Figure 2. (a) Profile of 3,4,5-CP reductive dehalogenation in live sediment slurries; (b) profile of H_2 concentrations in 3,4,5-CP dosed and non-dosed sediment slurries. Arrows indicate times of dosing with 3,4,5-CP.

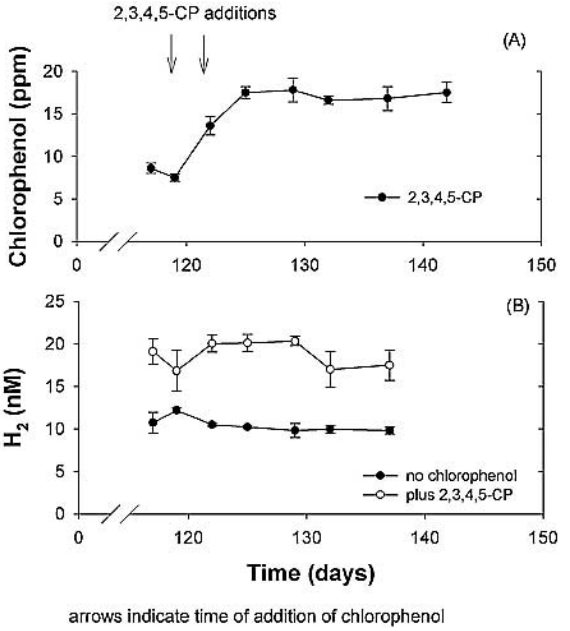

Figure 3. (a) Profile of 2,3,4,5-CP concentration in live sediment slurries; (b) profile of H_2 concentrations in 2,3,4,5-CP dosed and non-dosed sediment slurries. Arrows indicate times of dosing with 2,3,4,5-CP.

hydrogen coupled to methanogenesis indicated an important control on sediment hydrogen levels during this 10 day period.

According to the hydrogen threshold concept, dehalogenating organism(s) utilizing an energetically favorable chlorophenol as a terminal electron acceptor should display a lower hydrogen concentration during dehalogenation than during methane production (Lovely & Goodwin 1988). To determine if a lower hydrogen concentration was achieved during the reductive transformation of chlorinated phenols in our study, each sediment slurry was re-dosed with approximately 15–20 μM of the original chlorophenol 25 days after the addition of the organic mixture (day 116). The chlorophenol was dosed at frequent intervals (every 3–5 days) to ensure a constant supply of the chlorophenol. Due to water solubility constraints, microcosms containing PCP were re-dosed once to a concentration of 25 μM (see Methods section). Chlorophenol concentrations were frequently monitored in all microcosms, and H_2 values were compared to levels observed in natural slurries receiving no chlorophenol.

Results from PCP-dosed experiments demonstrated *ortho*-chlorine dehalogenation of PCP to produce 2,3,4,5-CP, followed by another *ortho*-chlorine dehalogenation to produce 3,4,5-CP. Little or no lag time was observed for dehalogenation upon re-dosing of PCP because dehalogenation products were identified rapidly following re-amendment (day 2). During the time of PCP dehalogenation, a significantly lower hydrogen level (mean of 0.34 nM; Figure 1a) was maintained compared to microcosms that were representative of methanogenic conditions. The methanogenic microcosms maintained a mean hydrogen concentration of approximately 9.8 nM during the additional 25 day incubation period (days 115 to 140). Lower dissolved hydrogen levels were also maintained in sediment microcosms during the period of active dehalogenation of 2,4-CP and 2,3,4-CP compared to sediments without added chlorophenol (Figure 1b, c, respectively). Analysis of the 2,3,4-CP microcosms indicated removal of the *ortho* Cl^- resulting in the formation of 3,4-CP which underwent subsequent *para*-dehalogenation to form 3-CP. During active dehalogenation of 2,3,4-CP, the mean aqueous hydrogen concentration was 4.1 nM. Dehalogenation of 2,4-CP dosed slurries proceeded with removal of the *ortho* Cl^- forming 4-CP. The mean aqueous hydrogen concentration was 3.6 nM during active dehalogenation of 2,4-CP. In both the 2,4-CP and 2,3,4-CP dosed microcosms, a continuous increase in 4-CP and 3-CP, respectively, occurred (data not shown) and further dehalogenation of these products was not observed during the final 25 day experimental period. Methane concentrations in all actively dehalogenating microcosms were significantly lower in comparison to the methanogenic microcosms. However, it is difficult to ascertain whether the lower methane concentrations were a direct result of reducing equivalents being channeled away from methane production in favor of dehalogenation or whether it was an apparent inhibitory effect of the added chlorophenol on methanogenesis.

For chlorinated benzenes, the preferential dechlorination pathway has been found to correlate well with the free energy release of the dechlorination reactions (Dolfing & Harrison 1993; Beurskens et al. 1994; Masunaga et al. 1996b). In general, our results show that this correlation does not exist for chlorinated phenols. For example, both the original $\Delta G^{\circ\prime}$ values (Dolfing & Harrison 1992) and our recalculated $\Delta G^{\circ\prime}$ values at pH 7 predict that PCP would be preferentially dechlorinated at the *meta* position, while the observed product distribution indicates that PCP was preferentially dechlorinated at the *ortho* position. Our findings are in agreement with another study (Masunaga et al. 1996a) that demonstrated that the redox potentials for the dechlorination reactions are not good predictors of the preferential pathway for reductive dechlorination of chlorinated phenols. Among the possible dechlorination pathways for a given chlorophenol, the ranking of the magnitude of the $\Delta G^{\circ\prime}$ values is generally the same for both the recalculated and the original $\Delta G^{\circ\prime}$ values; however, the recalculated ΔG° values give the opposite order for *ortho*-substituted dichlorophenols. For 2,4-DCP, the recalculated $\Delta G^{\circ\prime}$ values predict that the compound would be dechlorinated at the *ortho* position, in agreement with the observed results.

An additional study with PCE-dosed sediment slurries was performed to determine if a lower hydrogen concentration was attained during dechlorination of a chloroaliphatic compound. As observed for the chlorophenol dosed experiments, a lower hydrogen concentration (mean of 0.82 nM) was observed during the time of PCE dehalogenation compared to the methanogenic microcosms (mean hydrogen conc. of 9.8 nM) (Figure 1d). The sequential dechlorination of PCE proceeded by the following reaction pathway: PCE $\rightarrow$ trichloroethylene (TCE) $\rightarrow$ *cis*-dichloroethylene (*cis*-DCE) $\rightarrow$ vinyl chloride. The lower hydrogen concentration maintained during re-

ductive transformation of PCE in the present study (<0.82 nM) was similar to the range of hydrogen concentrations (0.5–2 nM) reported by Mazur & Jones (2001), Lu et al. (2001), and Fennell et al. (1997) for PCE dehalogenation in anoxic estuarine sediment, anoxic aquifer material, and mixed microbial cultures, respectively.

Hydrogen concentration data in sediment slurries re-dosed with 3,4,5-CP provided interesting results (Figure 2b). Following 3,4,5-CP re-feed (days 115–125), reductive dehalogenation occurred via an initial *meta* Cl^- removal forming 3,4-CP (Figure 2a). The hydrogen concentration initially representative of methanogenic conditions at day 115 (approximately 11 nM) decreased to lower levels (approximately 3–4 nM; Figure 2b) during detectable 3,4,5-CP dehalogenation (days 118–122). End-product analysis indicated the subsequent dechlorination of 3,4-CP, resulting in the transient formation of low levels of 4-CP. Neither 3,4-CP nor 4-CP were found to accumulate in these sediment microcosms. However, following the initial transformation of 3,4,5-CP (apparent at day 118–122), dechlorination activity apparently decreased as the concentration of 3,4,5-CP increased upon subsequent re-feeds (days 122–126). From days 130–138, the concentration of hydrogen increased to levels once again representative of methanogenic conditions (Figure 2b). It is possible that toxic (inhibitory) levels of 3,4,5-CP accumulated following subsequent re-feeds of 3,4,5-CP that were inhibitory to the dechlorinating population. A report by Escher et al. (1996) detailed the toxic effects of chlorophenols by evaluating the potential for inhibition of electrochemical proton gradients across bacterial membranes. In that study, 2,3,4,5-CP was found to have the most toxic mode of action, while 3,4,5-CP was determined to have a greater uncoupling potency (i.e. more toxic) than either PCP or other di- or tri-chlorophenols tested.

Sediment microcosms dosed with 2,3,4,5-CP also suggest that toxicity of specific chlorophenols may directly influence hydrogen concentrations (Figure 3). In our study, the parent compound 2,3,4,5-CP was not transformed either during the initial 90-day acclimation period or upon subsequent refeeds (Figure 3a). Immediately following re-amendment of 2,3,4,5-CP at day 115 , the rate of methane production decreased in comparison to sediment slurries without chlorophenol addition (data not shown), and hydrogen levels (Figure 3b) remained at significantly higher (mean of 17.5 nM) values compared to sediment slurries without chlorophenol addition (mean of 9.8 nM; representative of methanogenic conditions). The lower methane concentration and higher hydrogen values in the 2,3,4,5-CP dosed experiments suggest inhibition of hydrogen consumption via both methane production and dehalogenation of 2,3,4,5-CP. Because of the resulting high hydrogen levels, it is likely that the microbial processes responsible for hydrogen production were not as affected by the addition of 2,3,4,5-CP.

In order to fully evaluate the reliability of hydrogen concentration as a redox indicator in anaerobic sediments, further studies are needed to address the toxic/inhibitory effects of organochlorines or other electron accepting organochemicals on dehalogenating, methanogenic, and other naturally-occurring hydrogen-metabolizing populations. Additionally, studies that examine the role of natural electron transfer mediators and alternative electron acceptors on hydrogen transfer in anaerobic sediments may provide useful information to fully characterize biodegradation rate data. Evaluation of H_2 concentration measurements in the present study indicated that a lower hydrogen concentration was maintained in sediment slurries during the reductive dehalogenation of PCE and most chlorophenols tested in comparison to the natural condition of methanogenesis.

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Disclaimer

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