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Enhancement of anaerobic carbon tetrachloride biotransformation in methanogenic sludge with redox active vitamins

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Abstract

Carbon tetrachloride (CT) is an important groundwater pollutant which is only subject to biotransformation in the absence of oxygen. The anaerobic biotransformation of CT is influenced by electron shuttling compounds. The purpose of this study was to evaluate the impact of redox active vitamins on CT (100 μM) metabolism in a methanogenic sludge consortium (0.5 g VSS l^{-1}) supplied with volatile fatty acids as electron donor (0.2 g COD l^{-1}). The redox active vitamins, tested at concentrations ranging from 0.5 to 20 μM, were riboflavin (RF) and two forms of vitamin B12, cyanocobalamin (CNB12) and hydroxycobalamin (HOB12), and these were compared with a redox mediating quinone, anthraquinone-2,6-disulfonate (AQDS). Substoichiometric concentrations of RF, CNB12, HOB12 at molar ratios of vitamin:CT as low as 0.005 significantly increased rates of CT-bioconversion. These are the lowest molar ratios of vitamin B12 reported having an impact on dechlorination. Additionally, this study constitutes the first report of RF having a role in reductive dechlorination. At molar ratios of 0.1 vitamin:CT, RF, CNB12, HOB12 increased the first order rate constant of CT bioconversion by 4.0-, 13.3-and 13.6-fold, respectively. The redox active vitamins also enhanced the rates of abiotic CT conversion in heat killed sludge treatments, but the rates were approximately 4- to 5-fold lower than the corresponding vitamin enhanced rates of biological CT conversion. The addition of CNB12 or HOB12 to the live methanogenic sludge consortium increased the yield of inorganic chloride (Cl^-) from CT-converted. Chloroform was a transient intermediate in CNB12 or HOB12 supplemented cultures. In contrast, the addition of RF increased the yield of chloroform from CT-converted. Taken as a whole the results clearly demonstrate that very low concentrations of redox active vitamins could potentially play an important role in accelerating the anaerobic the bioremediation of CT as well as influencing the proportions of biotransformation products formed.

Abbreviations: AQDS – anthraquinone-2,6-disulfonate; COD – chemical oxygen demand; CNB12 – cyanocobalamin; HOB12 – hydroxycobalamin; RF – riboflavin; VFA – volatile fatty acids; VSS – volatile suspended solids

Introduction

Carbon tetrachloride (CT) is an important priority pollutant contaminating groundwater in the United States (U.S.) (Barbash & Roberts 1986). CT is ranked as the 40th most important pollutant in the 2003 Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) list maintained by the U.S. Environmental Protection Agency (EPA) and the U.S. Agency for Toxic Substance and Disease Registry (ATSDR). CT has been observed at approximately 25% of National

Priority List sites, which are the most serious hazardous waste sites in the U.S. (ATSDR 2003). CT is a solvent that has a long history of use as a cleaning fluid, degreasing agent, as well as a fumigant of grain. Industrially it was used in the synthesis of refrigeration fluids and propellants for aerosol cans (ATSDR 2003). CT was used in large quantities as a dry-cleaning agent until it was banned in the 1950s, when it was gradually replaced by trichloroethylene and perchloroethylene (Wentz 1995). While most uses of CT have been halted, improper disposal and spills in the past as well as leaking storage tanks, have led to its widespread contamination of groundwater and sediments.

Under aerobic conditions, CT is poorly degraded. However under anaerobic conditions, the bioconversion of CT is reported under a wide variety of redox conditions. CT biotransformation has been observed to take place by pure cultures of methanogens (Egli et al. 1987, 1990), acetogenic bacteria (Egli et al. 1988), fermentative bacteria (Galli & McCarty 1989), sulfate reducing bacteria (Egli et al. 1987) and iron reducing bacteria (Picardal et al. 1993) without any apparent benefit to the microbe responsible for the degradation suggesting cometabolism (Holliger & Schraa 1994). CT is sequentially reduced forming chloroform (CF), dichloromethane (DCM) and even traces of chloromethane (CM) as products. In some studies, radiolabeled CO_2 as well as CS_2 were also observed from the anaerobic conversion of radiolabeled CT indicating the occurrence of substitutive reactions involving nucleophilic addition of water or thiol groups (Egli et al. 1988; Hashsham et al. 1995). Heat-killed cells of methanogens and anaerobic mixed cultures also catalyze the dechlorination of CT predominantly *via* the substitutive reactions (Egli et al. 1990; Van Eekert et al. 1998) forming similar products as the living cells including CO_2 and CS_2. Reduced enzyme cofactors of the anaerobic microorganisms have been implicated as the catalyst of the observed transformations. The heat stable cobalt-containing cofactor, vitamin B_{12}, was shown to directly catalyze the dechlorination of CT when supplied with an appropriate reducing agent such as Ti(III)-citrate or hydrogen sulfide (Assaf-Anid & Lin 2002; Gantzer & Wackett 1991; Krone et al. 1991; Tanaka 1997). Vitamin B_{12} is a common cofactor of strict anaerobes, especially those involved in C_1 metabolism. The unique nickel containing coenzyme F430 of methanogens was additionally implicated as a catalyst in the reductive dechlorination of CT to CF, DCM, CM and methane (Gantzer & Wackett 1991; Krone et al. 1989). Recently extracellular zinc containing porphorinogen-type molecules found in a methanogen (*Methanosarcina thermophila*) were also shown to catalyze the reductive dechlorination of CT (Koons et al. 2001).

CT degradation under denitrifying conditions by *Pseudomonas stutzeri* KC has also been reported with CO_2 as the major product and with little or no formation of CF as an intermediate (Criddle et al. 1990). The responsible catalyst is a siderophore excreted by the organism, which has been identified as pyridine-2,6-bis(thiocarboxylic acid) (PDTC) complexed with copper (Lewis et al. 2001). Biodegradation proceeds through the formation of phosgene and thiophosgene as intermediates (Lewis & Crawford 1995). Under iron reducing conditions, CT reduction to CF is catalyzed by reactive metal oxide surfaces formed from the adsorption of biogenic Fe(II) to iron oxides (Kim & Picardal 1999; McCormick et al. 2002). Similar results were obtained in abiotic systems with Fe(II) adsorbed onto iron oxides (Pecher et al. 2002). Recently CT reductive dechlorination was also observed in anaerobic enrichment cultures respiring humus or anthraquinone-2,6-disulfonate (AQDS) (Cervantes et al. 2004). AQDS is commonly used as a model for redox active quinone moieties in humus (Nurmi & Tratnyek 2002). Rates of CT dechlorination were increased in methanogenic mixed cultures by AQDS and in the cultures of the iron-reducing bacterium, *Shewanella putrefaciens* by humus (Cervantes et al. 2004; Collins & Picardal 1999). The reduced form of AQDS, anthrahydroquinone-2,6-disulfonate (AH_2QDS), was shown to directly reduce CT to CF chemically (Cervantes et al. 2004; Curds & Reinhard 1994).

The results taken as a whole suggest an important role of electron shuttles in the anaerobic biotransformation of polyhalogenated methanes. Compounds mediating reduction include enzyme cofactors, siderophores, quinones and adsorbed Fe(II). The objective of this study was to compare several redox active vitamins for their ability to increase rates of CT dechlorination in an anaerobic mixed culture. Additionally their impact on the types of products formed was evaluated. Vitamin B12, riboflavin and AQDS were selected as

redox mediators for this study. Riboflavin was included since previous results indicated it was effective in mediating the reductive biotransformation of azo dyes (Field & Brady 2003; Semde et al. 1998).

Methods

Microorganisms

Methanogenic granular sludge was obtained from an industrial anaerobic treatment plant treating distillery wastewaters (Nedalco BV, Bergen op Zoom, The Netherlands). The content of volatile suspended solids (VSS) in the Nedalco sludge was 10.0%. The microbial cultures were 20 stored under nitrogen gas at 4 °C.

Basal media

The basal medium contained ($g\,l^{-1}$): 0.3 $NH_4CH_3CO_2$, 0.08 K_2HPO_4, 0.05 $MgSO_4 \cdot 7H_2O$, 0.015 $NaHCO_3$ and 0.035 $Ca(OH)_2$. The basal medium additionally contained and $1\,ml\,l^{-1}$ of trace elements solution and $5\,ml\,l^{-1}$ of vitamins solution. The trace element solution contained ($mg\,l^{-1}$): 50 H_3BO_3, 2800 $FeSO_4 \cdot 7H_2O$, 106 $ZnSO_4 \cdot 7H_2O$, 680 $MnSO_4 \cdot 7H_2O$, 50 $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$, 175 AlK $(SO_4)_2 \cdot 12H_2O$, 113 $NiSO_4 \cdot 6H_2O$, 2360 $CoSO_4 \cdot 7H_2O$, 100 $NaSeO_3 \cdot 5H_2O$, 157 $CuSO_4 \cdot 5H_2O$, 1000 EDTA, 200 resazurin. The vitamins solution contained ($mg\,l^{-1}$): 20 biotin, 50 *p*-aminobenzoate, 50 pantothenate, 20 folic acid dihydrate, 50 lipoic acid, 100 pyridoxine, 50 nicotinamide, 50 thiamine. The buffer solution was 0.01 M phosphate. The media was supplemented with volatile fatty acids (VFA) solution (acetate, propionate and butyrate) in 1:1:1 proportion of chemical oxygen demand (COD) from a stock solution to a final concentration of $200\,mg\,COD\,l^{-1}$, taking into account acetate added with $NH_4CH_3CO_2$. The final concentration of each VFA was as follows: acetate, 1.04 mM; propionate, 0.6 mM; and butyrate, 0.42 mM.

Batch assays

The experiments were performed in triplicate using 120 ml serum bottles with a liquid volume of 50 ml (70 ml headspace). The basal medium, buffer and VFA solutions were combined and the anaerobic methanogenic sludge was added to provide a final concentration of 0.5 g volatile suspended solids (VSS) l^{-1}. Afterwards the redox mediators were added from concentrated stock solutions to provide the desired final concentrations. The final concentration of redox mediators was: 10 μM for the initial set of CT experiments, and 0.5, 2, 10, 20 μM for the cyanocobalamin and riboflavin concentration gradient CT experiments. The bottles were flushed with nitrogen gas for 2 min in the liquid phase, two minutes in the headspace, and then sealed with Viton stoppers (Maag Technic AG, Dubendorf, Switzerland) and aluminum crimps. After sealing the bottles, they were flushed for another two minutes. The CT was added from a concentrated stock solution to the bottles at the final concentration of 100 μM referred to the liquid volume. After addition of CT, the bottles were shaken on a reciprocal shaker (120 strokes per minute) at 32 °C in a temperature controlled room (±2 °C) during the entire experiment. Two kinds of chemical controls were prepared in order to prove that there were no leaks in the treatments and to calculate the background level of chloride of the medium. The first kind containing nutrient basal medium, buffer, VFA and CT but without sludge, and the second kind, containing nutrient basal medium, buffer and VFA without CT and without sludge. In all experiments sludge blanks were prepared with basal medium, buffer, VFA and with sludge but without CT and these were incubated in parallel to obtain the background chloride levels for correction of the chloride levels in treatments. The experiments with heat killed sludge followed the same procedure as that for the living sludge, but the bottles containing everything except for the redox mediator were autoclaved for 20 min at 120 °C and afterwards the headspace of the bottles were flushed with filter sterilized N_2. Filter sterilized redox mediators were added later and CT addition was done as indicated for living sludge. For abiotic experiments, the concentrations tested for the redox mediators were 1, 10 and 20 μM and AQDS was not tested.

To investigate the possible degradation of redox active vitamins in the treatments, an experiment was conducted with basal medium, buffer, VFA and sludge but without CT as described above. The treatments were supplemented with

either riboflavin or cyanocobalamin at a final concentration of 100 μM and incubated at 32 °C. The controls cultures lacking redox active vitamins were also included to correct for background absorbance. The redox active vitamins concentration was quantified spectrophotometrically after allowing them to be reoxidized in air, for which a standard curve for each was prepared. The extinction coefficient was 11,900 $M^{-1}\,cm^{-1}$ at 450 nm and 9700 $M^{-1}\,cm^{-1}$ at 550 nm for the oxidized forms of riboflavin and cyanocobalamin respectively in the corresponding 0.01 M phosphate buffer solution at pH 7.

Analytical methods

Analysis for CT, chloroform (CF), dichloromethane (DCM) and perchloroethylene (PCE) was performed in the headspace by a gas chromatograph (GC Hewlett Packard 5890 series)equipped with an electron capture detector (ECD) and a GS-GASPRO column (30 m × 0.317 mm, J&W Scientific). Standards for CT, CF and DCM were prepared in serum bottles maintaining the same conditions established for the treatments with respect to temperature, headspace and liquid volume. The operation conditions were the following: injector temperature, 200 °C, detector temperature, 275 °C, oven temperature, 200 °C, gas carrier was He. Headspace samples (100 μl) were injected to the GC-ECD each time during the experiment. The retention times for CT, CF DCM and PCE were 5.2, 5.0, 4.7 and 7.1 min, respectively. Analysis for the liquid phase was performed by liquid chromatography, (Ion Chromatograph equipped with Dionex IP25 isocratic pump, Dionex EG40

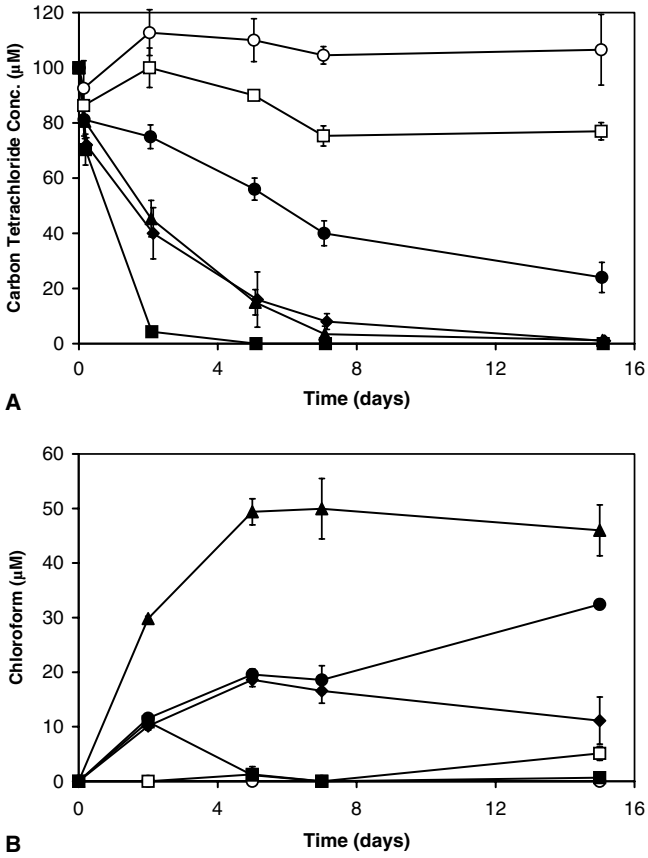

Figure 1. The time course of the anaerobic biotransformation of CT (100 μM) by anaerobic 5 granular sludge. *Panel A* shows the disappearance of CT. *Panel B* shows the accumulation of CF. *Legend:* no redox mediator added, (closed circles); 10 μM of anthraquinone disulfonate, (closed diamonds); 10 μM of riboflavin, (closed triangles); 10.0 μM of cyanocobalamin, (closed squares); abiotic medium control (open circles); and killed sludge control (open squares). Error bars indicate standard deviation.

eluent generator, Dionex CD 20 conductivity detector and a LC20 chromatography enclosure in which a Dionex IonPac AS11-HC column analytical 4×250 mm was installed. The eluent solution was KOH at a 7 mM concentration for 30 min to separate the chloride peak. The retention time for the chloride peak was 4 min.

Chemicals

9, 10-Anthraquinone-2,6–disulfonic acid disodium salt (98% purity), cyanocobalamin (99% purity), hydroxocobalamin acetate salt (99% purity), riboflavin (98% purity) were obtained from Sigma Aldrich (St. Louis, MO, USA). Additionally HPLC grade CT, CF, DCM and PCE were obtained from Sigma Aldrich. Glacial acetic acid (98% purity) was purchased from Spectrum Chemicals (New Brunswick, NJ, USA) and propionic acid (99% purity) and butyric acid (99% purity) were purchased from Sigma Aldrich.

Results

Comparison redox active vitamins on CT-bioconversion

CT (100 μM) was incubated with methanogenic sludge in the presence and absence of redox mediating compounds supplied at 10 μM. The effect of cyanocobalamin (CNB12), riboflavin (RF) and anthraquinone-2,6-disulfonate (AQDS) on the time course of CT eliminationis shown in Figure lA. The graph illustrates no loss of CT incubated in sterile medium, and only small losses of CT incubated with heat-killed sludge. CT removal occurred in all cases with living sludge. However the rate of CT degradation was significantly increased in treatments amended with redox mediating compounds. CT was completely eliminated within 2 days with CNB12 amended cultures or 7 days with RF and AQDS-amended cultures. In contrast, the untreated culture had only removed about 75% of the CT after 14 days. Hydroxycyanocobalamin (HOB12) was also tested and provided results identical to CNB12. The first order rate constant (k) of the two vitamin B12 compound amended cultures were more than 13-fold greater than that of the control culture without addition of redox mediator (Table 1). RF- and AQDS-amended cultures had k values that were 4- and 3.75-fold greater than the control culture, respectively.

The evolution of CF as a biotransformation product in the conversion of CT is shown in Figure 1B. In the CNB12 amended culture, CF was initially formed in small amounts and then subsequently degraded. In the control and AQDS-amended cultures, CF accumulated in the range of 11–32 μM. The RF amendment significantly increased the yield of CF, which reached approximately 50 μM after only 5 days. The conversion of CT also paralleled the release of inorganic chloride (Cl^-) indicating organochlorine mineralization as shown in Figure 2. The highest release was observed for the CNB12 treated culture; followed by the AQDS treated culture. A lower level of chlorine mineralization was observed in the

Table 1. First order rate constant (k) and chlorine balance on day 5 in the experiment evaluating the impact of different redox mediating compounds at 10 μM on the biological conversion of CT

Treatments	k (day^{-1})	Chlorine recovery day 5			$\sum$ products[a] (%CT-C1 removal)
		CT-C1	CF-C1	Cl^-	
		% initial CT-C1			
HKS[b]	0.038 (±0.001)	85.1 (±1.5)	0.9 (±1.0)	10.7 (±2.1)	77.7
LS[b]	0.077 (±0.010)	52.8 (±3.8)	13.8 (±0.7)	26.6 (±1.3)	85.6
LS + AQDS	0.289 (±0.016)	15.1 (±10.3)	13.2 (±0.9)	41.9 (±3.3)	64.9
LS + RF	0.309 (±0.019)	13.8 (±4.4)	34.9 (±1.7)	33.0 (±1.1)	78.8
LS + HOB12	1.040 (±0.038)	1.8 (±0.2)	2.0 (±0.4)	62.6 (±3.7)	65.8
LS + CNB12	1.021 (±0.051)	0.8 (±0.1)	0.8 (±0.6)	61.5 (±0.6)	62.8

[a] $100 \times (\text{CF-Cl} + Cl^-)/\text{CT-Cl}_{\text{removed}}$.
[b] HKS – heat killed sludge; LS – living sludge.

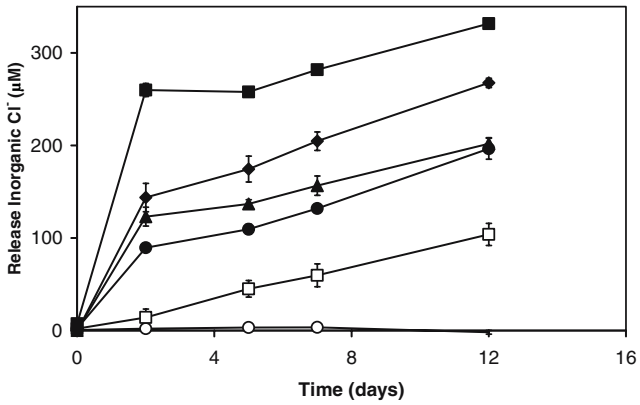

Figure 2. The time course of inorganic chloride accumulation from the anaerobic biotransformation of CT (100 μM) by anaerobic granular sludge. *Legend:* no redox mediator added, (closed circles); 10 μM of anthraquinone disulfonate, (closed diamonds); 10 μM of 15 riboflavin, (closed triangles); 10.0 μM of cyanocobalamin, (closed squares); abiotic medium control (open circles); and killed sludge control (open squares). Error bars indicate standard deviation.

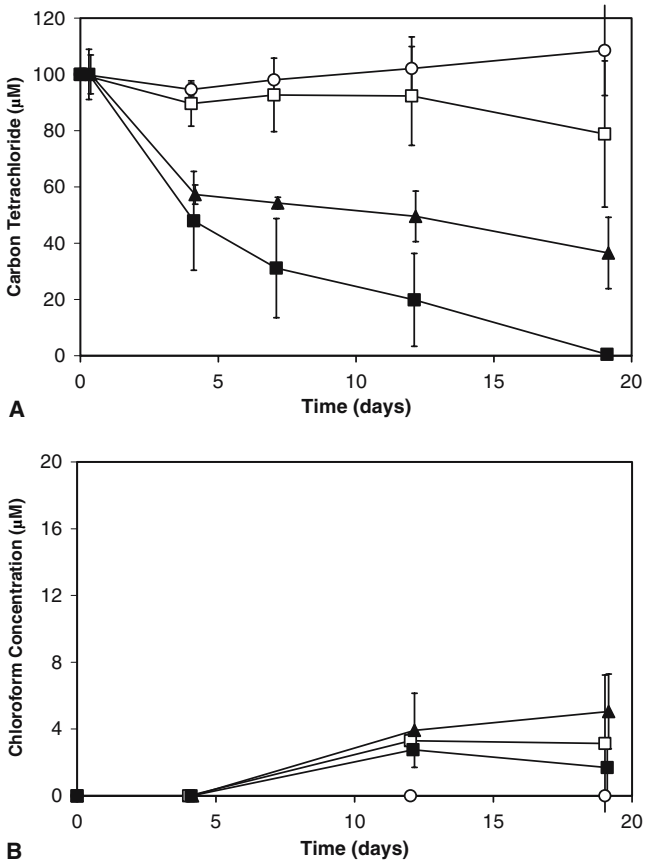

Figure 3. The time course of abiotic transformation of CT (100 μM) by killed anaerobic granular sludge in the absence or presence of 10.0 μM redox mediators. *Panel A* shows the disappearance of CT. *Panel B* shows the accumulation of CF. *Legend*: no redox mediator added, (open squares); 10 μM of riboflavin, (closed triangles); 10.0 μM of cyanocobalamin, (closed squares); and medium only control (open circles). Error bars indicate standard deviation.

RF-amended and untreated cultures. Chlorine was also partially mineralized the treatment with heat-killed sludge.

A chlorine balance was established on day 5 of the experiment. The organochlorine of CT in cultures amended with either of the two vitamin B12 compounds was mineralized Cl^- by 62%. The organochlorine mineralization of 33–42% achieved with the other redox mediators was also significantly higher than the 27% mineralization observed in the unamended culture. The recovery of chlorine as CF was greatest in the cultures with RF and lowest in the cultures with vitamin B12 compounds. The combined recovery of chlorine as Cl^- and CF was high and accounted for 63% to 86% of the CT chlorine removed from the various treatments (Table 1).

Heat killed controls

The role of the redox mediating vitamins on the abiotic transformation of CT (100 μM) by heat killed sludge was examined at three concentrations, 1, 10 and 20 μM. The time-course of the abiotic CT conversion is shown in Figure 3A for treatments in the presence and absence of 10 μM of RF or CNB12. The figure illustrates significant enhancement of abiotic CT conversion by RF and CNB12. However, the rates of the redox active vitamin enhanced abiotic CT conversion (Table 2)

Table 2. First order rate constant *(k)* and chlorine balance on day 19 in the experiment evaluating the impact of different redox mediating compounds at variable concentrations on the abiotic conversion of CT in the presence of heat killed sludge

Treatments	k (day^{-1})	Chlorine recovery day 19			$\sum$ products[a] (%CT-Cl removal)
		CT-Cl	CF-Cl	Cl^-	
		% initial CT-Cl			
No mediator	0.003 (±0.014)	63.8 (±0.2)	2.2 (±2.8)	12.5 (±8.9)	40.5
HOB12 1 μM	0.010 (±0.006)	34.2 (±1.4)	2.1 (±2.1)	24.2 (±7.8)	40.0
HOB12 10 μM	0.077 (±0.076)	5.4 (±8.2)	2.0 (±0.3)	48.6 (±4.1)	53.4
HOB12 20 μM	0.081 (±0.025)	0.2 (±0.1)	1.1 (±0.3)	52.1 (±3.7)	53.3
CNB12 1 μM	0.021 (±0.011)	18.6 (±18.7)	2.9 (±1.4)	32.9 (±7.7)	44.0
CNB12 10 μM	0.208 (±0.150)	0.4 (±0.7)	1.2 (±1.1)	43.2 (±1.5)	44.5
CNB12 20 μM	0.249 (±0.048)	0.1 (±0)	1.5 (±0.4)	43.3 (±2.3)	44.8
RF 1 μM	0.090 (±0.009)	40.9 (±3.5)	2.9 (±0.2)	17.7 (±1.5)	34.9
RF 10 μM	0.075 (±0.021)	33.6 (±11.7)	3.5 (±1.5)	20.5 (±7.6)	36.1
RF 20 μM	0.063[b]	21.0[b]	6.8 (±5.0)	22.8 (±1.1)	37.5

[a]Definition in Table 1 footnote.
[b]Only one replicate.

Table 3. First order rate constant (k) and chlorine balance on day 6 in the experiment evaluating the impact of different concentrations of CNB12 on the biological conversion of CT

Treatments	k (day^{-1})	Chlorine recovery day 6			$\sum$ products[a] (%CT-C1 removal)
		CT-C1	CF-C1	Cl^-	
		% initial CT-Cl			
No CNB_{12}	0.185 (±0.038)	31.2 (±5.0)	10.5 (±0.9)	23.5 (±1.7)	49.4
CNB12 0.5 μM	0.510 (±0.078)	6.2 (±4.0)	9.6 (±1.2)	37.7 (±0.6)	50.4
CNB12 2 μM	0.805 (±0.064)	0.1 (±0.1)	7.3 (±1.1)	41.9 (±0.8)	49.3
CNB12 10 μM	1.049 (±0.060)	0.1 (±0.0)	3.8 (±0.6)	44.9 (±0.8)	48.8
CNB12 20 μM	2.130 (±0.220)	0.0	1.2 (±0.5)	45.1 (±0.8)	46.2

[a]Definition in Table 1 footnote.

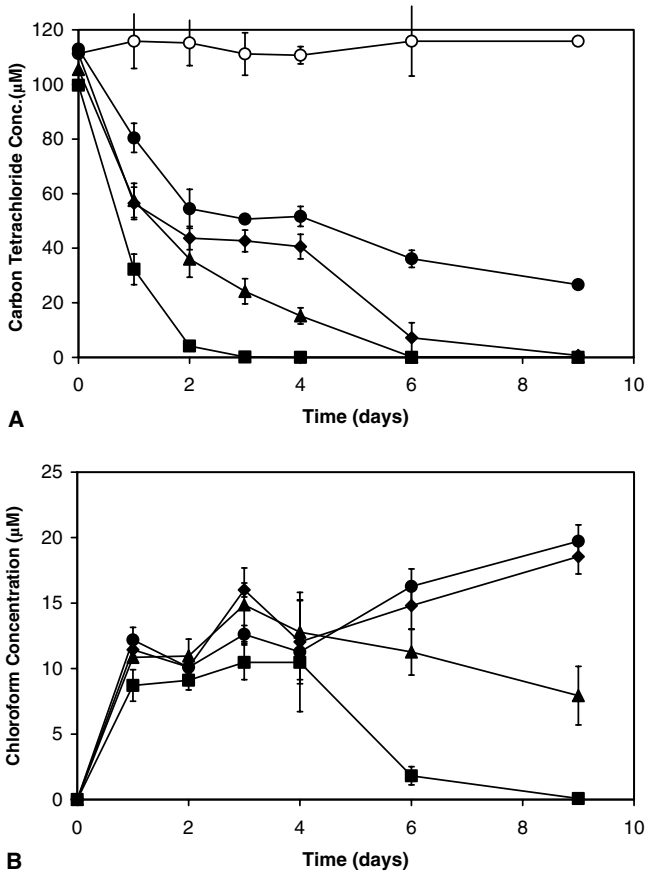

Figure 4. The time course of the anaerobic biotransformation of CT (100 μM) by anaerobic granular sludge in the presence of variable concentrations of cyanocobalamin. *Panel A* shows the disappearance of CT. *Panel B* shows the accumulation of CF. *Legend:* no cyanocobalamin added, (closed circles); 0.5 μM, (closed diamonds); 2.0 μM, (closed triangles); and 20.0 μM, (closed squares) of added cyanocobalamin, respectively; and abiotic medium control (open circles). Error bars indicate standard deviation.

were 4- to 5-fold lower than the corresponding redox active vitamin enhanced rates of biological CT conversion. The evolution of CF from the redox active vitamin enhanced abiotic conversion of CT (Figure 3B) was considerably lower than that observed in living sludge.

A chlorine balance was established on day 19 of the abiotic experiment (Table 2). CNB12 at 1–20 μM and HOB12 at 10–20 μM significantly enhanced the mineralization of the organochlorine in CT compared to the untreated heat killed sludge. RF at 20 μM significantly enhanced the accumulation of CF. The combined recovery of chlorine as Cl^- and CF accounted for 35–53% of the CT chlorine removed from the various treatments (Table 3). The highest values of recovery were observed with the HOB12 and CNB12 treatments.

Effect redox active vitamin concentrations on CT-bioconversion

The effect of variable CNB12 concentrations on the biological conversion of CT (100 μM) is illustrated in Figure 4A. Significant increases in CT elimination were already evident at the lowest concentration tested, 0.5 μM of CNB12. The CT elimination continued to improve as the CNB12 was increased to 20 μM. A dynamic the pattern of CF accumulation was observed (Figure 4B). In the unamended culture and the culture receiving the lowest CNB12 of 0.5 μM, CF gradually increased with time. However at higher CNB12 concentrations, CF initially accumulated and was later degraded, the effect of which was most pronounced at 20 μM. The k values for the 0.5, 2, 10 and 20 μM CNB12 amended cultures were 2.8-, 4.4-, 5.7 and

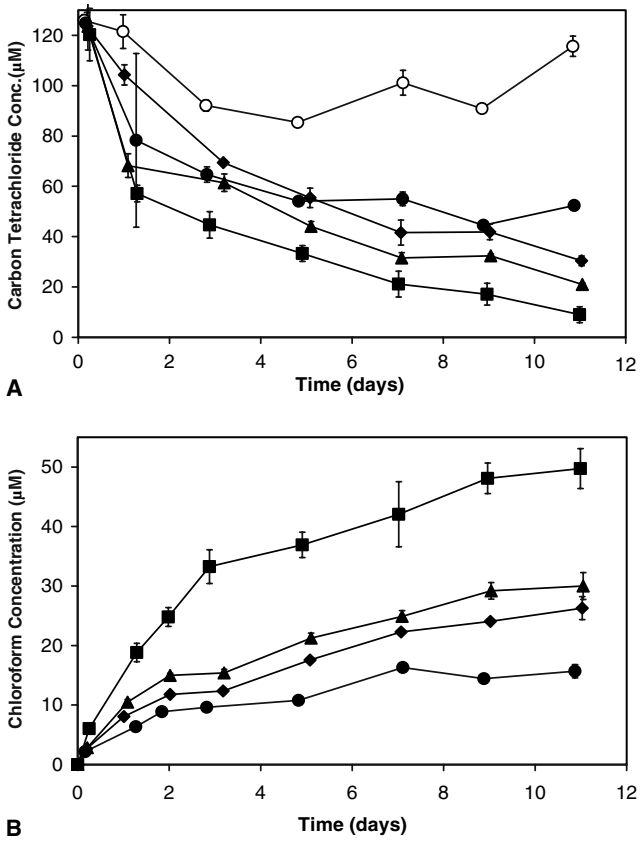

Figure 5. The time course of the anaerobic biotransformation of CT (100 μM) by anaerobic granular sludge in the presence of variable concentrations of riboflavin. *Panel A* shows the disappearance of CT. *Panel B* shows the accumulation of CF. *Legend:* no riboflavin added, (closed circles); 0.5 μM, (closed diamonds); 2.0 μM, (closed triangles); and 20.0 μM, (closed squares) of added riboflavin, respectively; and abiotic medium control (open circles). Error bars indicate standard deviation.

11.5-fold faster compared to that of the unamended culture, respectively.

A chlorine balance was established on day 6 (Table 3). The recovery of inorganic chloride increased with increasing CNB12 concentrations. In contrast the recovery of chlorine in CF decreased with increasing CNB12 concentrations. The combined recovery of chlorine as Cl^- and CF

Table 4. First order rate constant (k) and chlorine balance on day 12 in the experiment evaluating the impact of different concentrations of RF on the biological conversion of CT

Treatments	k (day^{-1})	Chlorine recovery day 12			$\sum$ products[a] (%CT-C1 removal)
		CT-C1	CF-C1	Cl^-	
		% initial CT-Cl			
No RF	0.102 (±0.005)	45.3 (±1.6)	10.2 (±0.7)	35.6 (±0.9)	83.7
RF 0.5 μM	0.145 (±0.016)	26.3 (±1.6)	17.0 (±1.3)	46.4 (±0.2)	86.1
RF 2 μM	0.172 (±0.009)	18.2 (±0.5)	19.5 (±1.5)	46.4 (±2.2)	80.5
RF 10 μM	0.248 (±0.008)	9.1 (±0.2)	30.5 (±5.3)	47.4 (±0.2)	85.7
RF 20 μM	0.245 (±0.044)	7.8 (±2.7)	32.3 (±2.2)	45.0 (±0.5)	83.8

[a]Definition in Table 1 footnote.

accounted for 46–50% of the CT chlorine removed from the various treatments (Table 3).

The effect of variable RF concentrations on the biological conversion of CT (100 μM) was also investigated. Increasing RF concentrations corresponded to an initial increase in the rate of CT conversion (Figure 5A). Based on the calculated k values, as little as little as 0.5 μM RF had a significant impact on the rate of CT degradation (Table 4). The k values the 0.5, 2, 10 and 20 μM RF amended cultures were 1.4-, 1.7-, 2.4- and 2.4-fold faster compared to that of the unamended culture, respectively. The effect of RF was also reflected in an initial increase in the CF formation rate (Figure 5B). CF formation was also significantly impacted by the addition of as little as 0.5 μM RF.

A chlorine balance was established on day 12 (Table 4). The recovery of chlorine as CF increased progressively with increasing concentrations of RF. On the other hand, all the RF amended treatments significantly increased the recovery of Cl^- to the same extent. The combined recovery of chlorine as Cl^- and CF was highest in this experiment and accounted for and accounted for 81–86% of the CT chlorine removed from the various treatments (Table 4).

Other biotransformation products

CF and Cl^- were routinely monitored as major products. Occasionally the presence of DCM and PCE was additionally monitored. DCM concentrations were low, generally ranging in values from 0.5 to 0.9 μM in all treatments when detected. Higher concentrations of up to 5.9 μM were only observed after prolonged incubations of 18 days in control cultures lacking redox active vitamin additions. Previously we have reported that PCE is a minor product of anaerobic CT biotransformation (Cervantes et al. 2004). In this study, PCE was also found but at very low levels. In RF and CNB12 amended cultures, PCE was detected at concentrations less than 0.1 μM. In control cultures control cultures lacking redox active vitamin additions, PCE was detected at concentrations up to 0.84 μM, occurring after 18 days of incubation.

Biodegradation of RF and CNB12

RF and CNB12 supplied at 100 μM were incubated under similar conditions as those used in the CT-bioconversion assays. After a 7-day incubation period, no loss in RF and or CNB12 was observed based on absorbance measurements at the absorbence maxima (results not shown). The results indicate that these redox active vitamins resisted anaerobic biodegradation in short-term assays.

Discussion

The results from this study indicate that several redox-mediating compounds served to increase rates of CT bioconversion rates. The effective redox mediators included: RF, AQDS and two types of cobalamins, HOB12 and CNB12. To the best of our knowledge this constitutes the first report in which RF was considered as a redox mediator to stimulate reductive dehalogenation. When supplied at 10 μM, the rates of RF stimulated CT biotransformation were similar to those of AQDS which was approximately 4-fold faster than cultures lacking redox mediators. 30 However, the cobalamins at 10 μM were more effective, enabling CT-conversion rates greater than 13-fold faster than cultures lacking redox mediators.

Abiotic versus biotic conversion

The mechanism of CT-conversion enhancement by the redox mediators is largely due to 5 biologically catalyzed reactions. However, RF and cobalamins were also shown to support slow catalysis of CT in heat killed sludge at rates approximately 4-fold slower than corresponding rates in RF and cobalamin supplemented living sludge cultures. The activity of redox mediators in heat killed sludge is in keeping with previous observations that cobalamins supported the abiotic conversion of CT in cell-free anaerobic medium containing sulfide as bulk reducing agent 10 (Tanaka 1997). Additionally it was shown that CNB12 supported chemical reduction of CT with Fe^{2+}, S^{2-} and FeS as reducing agents (Assaf-Anid & Lin 2002). Previously numerous studies indicated that cobalamins catalyzed abiotic CT conversions with stronger reducing agents such as titanium(III) citrate, dithiothreitol or cysteine (Assafanid et al. 1994; Chiu & Reinhard 1996; Gantzer & Wackett 1991; Krone et al. 1991). Reduced compounds in the heat killed sludge 15 therefore probably supported CT-

conversion catalyzed by the added redox mediators. In this study, the cobalamins dramatically accelerated CT-conversion in heat killed sludge by over 70-fold. This observation starkly contrasted the minor effects of CNB12 on heat killed cells in a previous study with a DCM degrading enrichment culture (Hashsham et al. 1995).

Cobalamins as redox mediators during CT-bioconversion

Experiments evaluating variable CNB12 and RF concentrations on the biological conversion of CT suggested that very low concentrations of these redox-mediating vitamins had significantly impacts. Similar finding were observed with AQDS in a previous publication (Cervantes et al. 2004). A molar ratio of only 0.005 CNB12: CT significantly increased the first order rate constant (k) of CT removal by 2.7-fold. Rates continued to increase up to 11.2-fold with the highest molar ratio tested of 0.2 CNB12: CT in the concentration gradient experiment (Table 3). The molar ratios used in this study were far lower than those evaluated previously. Zou et al. (2000) observed 3- to 7-fold enhancement of CT degradation kinetics in enrichment cultures developed from anaerobic digester sludge with a molar ratio 2.3 CNB12:CT. A 10-fold increase in CT degradation rates was observed in DCM-enrichment cultures when a molar ratio of 0.1 CNB12:CT was applied (Hashsham et al. 1995). A 30-fold enhancement in CT degradation rates were observed in pure cultures of the acetogenic bacterium, *Acetobacterium woodii*, when a molar ratio of 0.11 HOB12:CT was applied (Hashsham & Freedman 1999).

The results from this study and those of Hashsham et al. (1995; Hashsham & Freedman 1999), clearly suggest that substoichiometric concentrations of cobalamins can sustain enhanced rates of CT degradation. The substoichiometric relationship would suggest a cycling between oxidized and reduced forms of cobalamins. In abiotic systems, the reduction of cobalamins is carried out by bulk reducing agents. However, biological reactions in the living cultures far exceeded the rates observed in heat killed cultures. This observation implies that microorganisms in the sludge consortium were probably responsible for the reduction of cobalamins. Oxidized cobalamins occur in the Co(III) state and can be reduced to the Co(II) and Co(I) states with standard reduction potentials at pH 7 (E'^0) versus the standard hydrogen electrode (SHE) of 0.20 and -0.61 V for the couples Co(III)/Co(II) and Co(II)/Co(I), respectively (Lexa & Saveant 1983). The iron reducing bacterium, *Shewanella alga* strain BrY was shown to reduce cobalamin Co(III) to cobalamin Co(II) when provided with an adequate electron donor such as lactate or H_2 (Workman et al. 1997). Similarly the reduction was also catalyzed by *Salmonella enterica* strain serovar Typhimurium LT2. An NAD(P)H-dependent flavin oxidoreductase (FRE) was isolated from the latter organisms that was capable of reducing cobalaminCo (III) to cobalamin Co(II) (Fonseca & Escalante-Semerena 2000).

The second part of the cobalamin cycling would involve the reduction of CT by the reduced cobalamin with concomitant oxidation of cobalamin. Cobalamin Co(II) prepared biologically with *S. alga* strain BrY was shown to effectively reduce CT in cell free systemswhile becoming oxidized to cobalamin Co(III) (Workman et al. 1997). A reaction proceeding via CF would be energetically favorable with E'^0 values 0.20 and 0.67 for the redox couples cobalamin Co(III)/cobalamin Co(II) and CF/CT (McCormick et al. 2002), respectively. These observations provide evidence for a biologically fueled redox cycling of cobalamins that could account for significant impacts observed in this study at molar CNB12:CT ratios as low as 0.005.

Riboflavin as redox mediators during CT-bioconversion

RF was also observed to significantly enhance CT-bioconversion at very low molar ratios. The formation rate of the major product, CF, was significantly enhanced at molar ratios as 5 low as 0.005 RF:CT and the k of CT removal was also significantly increased by 42%. As the RF concentration was increased to 10 μM, the rate enhancements increased to 2.4-fold but with diminishing returns for each additional increment in the concentration gradient experiment (Table 4). The maximum rate was already obtained at 10 μM since no further rate enhancements were observed at 20 μM. The increment of k versus RF concentration followed Michaelis–Menton kinetics with a K_m of approximately 1 μM RF. A similar kinetic

pattern was observed for RF-mediated reduction of an azo dye by methanogenic sludge (Field & Brady 2003). As was argued above for the cobalamins, the significant impact on CT degradation obtained with substoichiometric concentrations suggests redox cycling of RF. The first half of the cycle would be the biological reduction of RF. RF is the redox active moiety in ubiquitous enzyme cofactors such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) and as such should be susceptible to reduction by a wide variety of flavin reductases (Tu 2001). Riboflavin reductases have been implicated in the mediation of azo dye reduction by bacteria (Gingell & Walker 1971; Roxon et al. 1967). During the incubations conducted in this study, visual observations clearly indicated reduction of yellow oxidized riboflavin to colorless riboflavin, which upon sampling were rapidly reoxidized to the yellow oxidized form due to contact with air.

The direct chemical reaction of reduced riboflavin with CT has not yet been documented in the literature. Nonetheless there are several indications that this reaction was occurring. Firstly riboflavin significantly stimulated abiotic CT reduction in heat killed sludge (Table 2). Secondly, reduced FMN is known to catalyze the reductive dechlorination of *p*,*p*′-dichlorodiphenyltrichloroethane (DDT) in the presence of heme cofactors (Sugihara et al. 1998). Porphorinogen-type molecules, similar to hemes, are known to occur in methanogenic cultures (Koons et al. 2001). Lastly the E'^0 value of riboflavin/riboflavin-H_2 of -0.21 V (Roxon et al. 1967) is sufficiently low to allow for a thermodynamically favorable reaction with CT.

Role of redox active vitamins on biotransformation products

The redox mediating vitamins impacted the spectrum of products obtained from the anaerobic bioconversion of CT. Compared to the cultures lacking redox mediating compounds, the cobalamins increased the yield of inorganic chloride and lowered the yield of CF as a fraction of the CT-removed (Tables 1 and 3). CF formation in cobalamin supplemented cultures was dynamic. The initial accumulation of CF was subsequently subjected to a much more extensive degradation compared to CF accumulating in other treatments. The observation suggested that cobalamins also enhanced rates of CF conversion. The suggestion was shown to be correct in a separate experiment in which 100 μM of CF was incubated with 10 μM of CNB12 under the same experimental conditions used for the CT incubations. After a 2-week incubation period, essentially no CF was removed in the unsupplemented culture; whereas CF removal was complete in the culture receiving CNB12 (results not shown). Additionally, a previous study had already demonstrated that a DCM-degrading methanogenic enrichment culture degraded CF approximately 10-fold faster in the presence of cobalamin (Decker & Freedman 1994). It should be noted that CF was never the most important product from cobalamin assisted CT removal. Even at the height of CF accumulation on day 2 in the experiment shown in Figure 1, CF-C1 accounted for only 7.5% of the CT-C1 removed; whereas inorganic Cl^- accounted for 60%. The results clearly suggest cobalamin supported a high degree of organic chlorine mineralization, far exceeding the release of chlorine from the conversion of CT to CF. Furthermore, in experiments where DCM was monitored, DCM never accumulated beyond 1 μM with 10 μM CNB12.

During the anaerobic biodegradation of CT, two types of reductive dechlorination are recognized (Egli et al. 1990, 1988; Krone et al. 1991; Van Eekert et al. 1998). On the one hand, reductive hydrogenolysis occurs in which hydrogen atoms replace chlorine atoms in sequential reduction steps leading to CF and DCM. On the other hand, CT can be reduced to a dichlorocarbene radical, which is subsequently subject to substitution reactions with H_2O and H_2S leading to the formation of CO and CS_2. The results taken as a whole from this study, indicate that reductive hydrogenolysis was a minor pathway; whereas substitutive reactions was the major pathway in cobalamin supplemented cultures. To support this hypothesis, the chemical reaction of biologically reduced CNB12 with CT resulted in CO as the major product (92%); whereas CF was a minor product (1.4%) (Workman et al. 1997). In active methanogenic consortia CO would be further converted to CH_4, acetate and CO_2 (Sipma et al. 2003). Even in unsupplemented methanogenic consortia, CO_2 is a major product from CT conversion (Van Eekert et al. 1998).

In contrast to the results obtained with cobalamins, RF significantly increased the yield of CF as a

fraction of the CT-removed (Tables 1 and 4), compared to the cultures lacking redox mediating compound. The recovery of CF-C1 as percentage of CT-C1 consumed was 35–40% in cultures receiving 10–20 μM RF; whereas the value ranged from 15% to 29% in cultures lacking redox mediators. The results indicate that RF enhances the reductive hydrogenolysis of CT in methanogenic sludge.

Conclusions

Taken as a whole the results of this study clearly demonstrate that very low concentrations of redox active vitamins could potentially play an important role in accelerating the anaerobic CT bioremediation. Additionally, the study reveals a large impact of redox active vitamins on the spectrum of biotransformation products formed.

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