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# Enhanced anaerobic biotransformation of carbon tetrachloride with precursors of vitamin $B_{12}$ biosynthesis

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**Abstract**

Relatively low concentrations of Vitamin $B_{12}$ are known to accelerate the anaerobic biotransformation of carbon tetrachloride (CT) and chloroform (CF). However, the addition of vitamin $B_{12}$ for field-scale bioremediation is expected to be costly. The present study considered a strategy to generate vitamin $B_{12}$ by addition of biosynthetic precursors. One of the precursors, porphobilinogen (PB) involved in the formation of the corrin ring, significantly increased the CT biotransformation rates by 2.7–, 8.8- and 10.9-fold when supplemented at 160, 500 and 900 $\mu$M, respectively. A positive control with 10 $\mu$M of vitamin $B_{12}$ resulted in a 5.9-fold increase in the CT-bioconversion rate. PB additions provided high molar yields of inorganic chloride (57% of CT organochlorine), comparable to that obtained with vitamin $B_{12}$ supplemented cultures. The primary substrate, methanol, known to induce vitamin $B_{12}$ production in methanogens and acetogens, was required for PB to have a significant impact on CT conversion. The observation suggests that PB's role was due to stimulating vitamin $B_{12}$ biosynthesis. The present study therefore provides insights on how to achieve vitamin $B_{12}$ enhanced rates of CT bioremediation through the use of less complex compounds that are precursors of vitamin $B_{12}$. Although PB is a costly chemical, its large impact points to corrin ring formation as the rate-limiting step.

*Abbreviations:* $CNB_{12}$ – cyanocobalamin; PB – porphobilinogen; VFA – volatile fatty acids; CT – carbon tetrachloride; CF – chloroform; VSS – volatile suspended solids; COD – chemical oxygen demand

## Introduction

Vitamin $B_{12}$ is an important enzyme cofactor implicated in reductive dechlorination. In abiotic systems, vitamin $B_{12}$ can catalyze the dechlorination of a variety of chlorinated solvents when incubated with an appropriate reducing agent, such as Ti(III)-citrate, cysteine or sulfide (Assafanid et al. 1994; Burris et al. 1996; Gantzzer & Wackett 1991; Glod et al. 1997a, b; Krone et al. 1989; Lesage et al. 1998). Vitamin $B_{12}$ is also known to stimulate reductive dechlorination of chlorinated methanes in mixed and pure microbial cultures. Most studies have evaluated the impact of cyanocobalamin ($CNB_{12}$) or hydroxycobalamin ($HOB_{12}$) on the anaerobic biotransformation of carbon tetrachloride (CT). A 30-fold enhancement in CT degradation rates were observed in pure cultures of the acetogenic bacterium, *Acetobacterium woodii*, when CT was applied a molar ratio of 0.11 $HOB_{12}$:CT (Hashsham & Freedman 1999). CT degradation rates were increased 10-fold by $CNB_{12}$ in a dichloromethane (DCM) enrichment culture with a molar ratio of 0.1 $CNB_{12}$:CT

(Hashsham et a1. 1995). $CNB_{12}$ and $HOB_{12}$, supplied at a molar ratio of 0.1 vitamin:CT, increased CT bioconversion rates by 13.3- and 13.6-fold; respectively, in an unadapted methanogenic consortium (Guerrero-Barajas & Field 2005a). Vitamin $B_{12}$ also stimulated the chloroform (CF) dechlorination rates in an anaerobic DCM-degrading enrichment culture (Becker & Freedman 1994) as well as in an unadapted methanogenic consortium (Guerrero-Barajas & Field 2005b).

The cited studies illustrate that vitamin $B_{12}$ is effective at increasing microbial dechlorination rates when supplied at substoichiometric concentrations. Molar ratios as low as 0.005 $CNB_{12}$:CT had very significant effects on the CT degradation rate (Guerrero-Barajas & Field 2005a). The substoichiometric ratios would suggest redox cycling of vitamin $B_{12}$ between oxidized and reduced forms. Microorganisms are implicated in the reduction of oxidized forms of vitamin $B_{12}$. Oxidized cobalamins occur in the Co(III) state and can potentially 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 was responsible for the reduction (Fonseca & Escalante-Semerena 2000). The second part of the vitamin $B_{12}$ redox cycle would involve the reduction of CT or CF by the reduced cobalamin. Cobalamin Co(II) was shown to effectively reduce CT in cell free systems while concomitantly becoming oxidized to cobalamin Co(III) (Workman et al. 1997).

Potentially vitamin $B_{12}$ could be added to sites contaminated with chloromethanes to improve their bioremediation. However, vitamin $B_{12}$ is a costly biochemical and thus not suited for large scale additions. Bioremediation strategies taking advantage of vitamin $B_{12}$ are thus limited to methods promoting vitamin $B_{12}$ biosynthesis. Certain primary substrates have been shown to favor the biosynthesis of vitamin $B_{12}$. Single carbon substrates such as formate and methanol enhance vitamin $B_{12}$ production by methanogens and acetogens (Lebloas et al. 1994; Mazumder et al. 1987; Toraya et al. 1975; Van Eekert et al. 1998) due to corrinoid dependent methyltransferases involved in Cl metabolism. Evidence for the secretion of extracellular vitamin $B_{12}$ was observed from a culture of the methanogen, *Methanosarcina barkeri*, cultivated on methanol (Lin et al, 1989; Mazumder et al. 1987). Vitamin $B_{12}$ biosynthesis is commonly promoted by utilizing 1,2-propanediol as a growth substrate. The first step in the catabolism is the conversion of 1,2-propanediol to propionaldehyde which is dependent on vitamin $B_{12}$ and thus the presence of 1,2-propanediol induces vitamin $B_{12}$ biosynthesis (Roth et al. 1996). The use of 1,2-propanediol as a growth substrate stimulated vitamin $B_{12}$ production in an enrichment culture cycled between anaerobic/aerobic conditions, The increased vitamin $B_{12}$ production was correlated with increased rates of CT degradation (Zou et al. 2000).

Certain precursors of vitamin $B_{12}$ synthesis are also known to increase vitamin $B_{12}$ production in bacterial strains used for the industrial production of vitamin $B_{12}$ by the fermentation industry. For example, vitamin $B_{12}$ biosynthesis in one of the best industrial strains, *Propionibacterium shermanii*, is enhanced by addition of the precursor, 5,6-dimethylbenzimidazole (DMDI) (Martens et al. 2002).

The objectives of this study were to determine if CT degradation in an unadapted methanogenic consortium can be stimulated by addition of vitamin $B_{12}$ precursors and by the use of primary substrates known to stimulate vitamin $B_{12}$ production. The most important precursors of vitamin $B_{12}$ are presented in Table 1. Substrates implicated in an enhanced production of vitamin $B_{12}$ are 1,2-propanediol, glycerol and ethanolamine (Martens et al. 2002; Roth et al. 1996) as well as methanol (Mazumder et al. 1987; Nishio et al. 1975).

## Material and methods

### *Chemicals*

Carbon tetrachloride (CT), Chloroform (CF), dichloromethane (DCM), perchloroethylene (PCB), methanol HPLC grade were purchased at

*Table 1*. Precursors of vitamin $B_{12}$ biosynthesis (Battersby & Leeper 1998; Martens et al. 2002; Roth et a1.1996; Warren et al. 2002)

| Part of vitamin $B_{12}$ | Immediate precursors | Earlier precursors | Earliest precursors |
|---|---|---|---|
| Corrin Ring | Porphobilinogen cobalt SAM[a] | $\delta$-aminolevulinate | succinyl-CoA Glycine |
| Lower ($\alpha$) Ligand | DMBI[a] | Riboflavin | |
| Link $\alpha$ Ligand to Corrin | Aminoisopropanol Threonine[b] | | |
| Upper Ligands | | | |
| 5′-Deoxyadenosine | ATP[a] | | |
| Methyl group | SAM | | |

[a]SAM = *S*-adenosylmethionine; DMBI = 5,6-dimethyl-benzimidazole; ATP = adenosine-triphosphate.
[b]Threonine required for the synthesis of aminoisopropanol but it is not incorporated into vitamin $B_{12}$.

Sigma–Aldrich (St. Louis, MO, USA). Cyanocobalamin ($CNB_{12}$), adenine, 5,6-dimethylbenzimidazole (DMBI), $\beta$-aminolevulinic acid, L-Threonine, L-Methionine, glycerol, polyethylene glycol (PEG), and ethanolamine, were all purchased at Sigma–Aldrich (St. Louis, MO, USA). Porphobilinogen (PB) source l was purchased at Fluka Chemical Corp. (Milwaukee WI, USA) and source 2 at Frontier Scientific Inc. (Logan, UT, USA); 1,2-propanediol was purchased at EM Science (Gibbstown, NJ, USA). Acetic acid glacial, 98% purity and sodium hydroxide solution (50% w/w) were purchased at Spectrum Chemicals (New Brunswick, NJ, USA); propionic acid (99% purity) and butyric acid (99% purity) were purchased at Sigma–Aldrich (St. Louis, MO, USA).

*Microorganisms*

Methanogenic granular sludge was obtained from an industrial anaerobic treatment plant treating distillery waste waters (Nedalco BV, Bergen op Zoom, The Netherlands). The content of volatile suspended solids (VSS) in the Nedalco sludge was 10.0%. The maximum specific acetoclastic methanogenic activity of the Nedalco sludge was 361 ml $CH_4$ $g^{-1}$ volatile suspended solids (VSS) $day^{-1}$. The Nedalco sludge was stored under nitrogen gas at 4 °C.

*Basal media*

The basal media utilized contained (g $l^{-1}$): 0.3 $NH_4C_2H_3O_2$, 0.08 $K_2HPO_4$, 0.05 $MgSO_4{\cdot}7H_2O$, 2.015 $NaHCO_3$, 0.035 $Ca(OH)_2$, and 1 ml $l^{-1}$ trace elements solution excluding chloride and cobalt salts. The trace element solution contained (mg $l^{-1}$) 50 $H_3BO_3$, 2800 $FeSO_4{\cdot}7H_2O$, 106 $ZnSO_4{\cdot}7H_20$, 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 $NiSO_4{\cdot}6H_2O$, 100 $NaSeO_3{\cdot}5H_2O$, 157 $CuSO_4{\cdot}5H_2O$, 1000 EDTA, and 200 resazurin. The cobalt, when required, was added to the medium at a final concentration of 0.05 mg $l^{-1}$. The medium was buffered at pH 7.

*Cyanocobalamin precursors screening assay*

All the treatments and controls were prepared in duplicate in 120 ml serum bottles with a liquid volume of 50 ml (70 ml headspace). The assays were composed of basal medium, sludge (0.5 g VSS $l^{-1}$) and methanol supplied at 20.83 mM. The different vitamin $B_{12}$ precursors were supplied at a final concentration of 1 mM each. The vitamin $B_{12}$ precursors tested were porphobilinogen (PB) from source 1, adenine, 5,6-dimethylbenzimidazole (DMBI), $\delta$-aminolevulinic acid, L-Threonine, L-Methionine, and a treatment containing a mixture of all of them (each at 1 mM). Four different controls were prepared. The chemical, (abiotic control) was not inoculated with sludge but contained buffered basal medium with cobalt and CT. The positive control contained sludge and basal medium with cobalt and 10 $\mu$M $CNB_{12}$. Two negative controls were the same as the positive control but lacked $CNB_{12}$ or any precursor amendments. One negative control contained cobalt the other did not. Treatments and controls were flushed with a mixture of

$N_2/CO_2$ (80:20) and sealed with Viton stoppers (Maag Technic AG, Dubendorf, Switzerland). Treatments and controls were incubated for 48 h at 29 °C at which time CT was added to provide 100 $\mu$mol $1^{-1}$ of liquid. Bottles were gently shaken (120 strokes/min) at 29 °C during the entire experiment.

*Porphobilinogen concentration gradient assay*

Experimental conditions were identical to that described for the precursor screening experiment. In this experiment, porphobilinogen from source 2 was added to provide several different assay concentrations (40, 160, 500 and 900 $\mu$M), Three different controls were prepared according to the previous descriptions: (1) abiotic control; (2) positive control; and (3) negative control with cobalt.

*Substrates and cyanocobalamin production stimulation screening assay*

Experimental conditions were identical to that described for the precursor screening experiment except that different primary substrates were tested. The primary substrates tested in the treatments were: methanol (20.83 mM); 1,2-propanediol (7.81 mM); glycerol (8.93 mM); ethanolamine (12.5 mM); polyethylene glycol (PEG) (0.775 g $1^{-1}$) and a mixture volatile fatty acids (VFA) (5.2 mM of acetate, 3.0 mM of propionate and 2.1 mM of butyrate). All of the alternative primary substrates were supplied at the same electron equivalents as 20.83 mM of methanol, corresponding to 1 g chemical oxygen demand $1^{-1}$. Two different controls were prepared. One was the abiotic control, prepared as indicated in the former sections. The other was the biological control including sludge, basal medium, and the VFA mixture as substrate supplemented with $CNB_{12}$10 $\mu$M.

*Analytical methods*

Analysis for CT, CF, DCM and PCE in the headspace was performed by a gas chromatograph (GC Hewlett Packard 5890 series) equipped with an ECD detector and a GS-GASPRO column (30 m×0.317 mm, J&W Scientific). Standards for CT, CF, DCM and PCE were prepared in serum bottles maintaining the same conditions established for the treatments for temperature, headspace and liquid volume. The injector temperature was 200 °C, the detector temperature was 275 °C, the oven temperature was 200 °C, the gas carrier was He and the injection volume was. 100 $\mu$l. The retention times for CT, CF, DCM and PCE were 5.2, 5.0, 4.7 and 7.1 min, respectively. Analysis for chloride in the liquid phase was performed by liquid chromatography, (Ion Chromatograph equipped with Dionex IP25 isocratic pump, Dionex EG40 eluent generator, Dionex CD 20 conductivity detector and a LC20 chromatography enclosure in which a Dionex IonPac AS11-HC 4×250 mm analytical column was installed.) The eluent solution was KOH at a 1.5 mM concentration for 5 min, followed by 20 mM for 10 min and 1.5 mM for another 5 min in order to separate the chloride peak. The retention time for the chloride peak was 3.2 min.

## Results

*Screening of vitamin $B_{12}$ precursors*

Table 1 indicates the roles of various precursors in vitamin $B_{12}$ biosynthesis. Most of the precursors tested are implicated in the biosynthesis of the corrin ring; such as, porphobilinogen (PB), $\delta$-aminolevulinic acid, methionine (as a precursor of S-adenosylmethionine) and cobalt. 5,6-Dimethylbenzimidazole (DMBI) is a precursor to the lower ligand and has been used in the microbial production of vitamin $B_{12}$ (Martens et al. 2002). Threonine is a required cofactor in the synthesis of aminoisopropanol which links the lower ligand to the corrin ring. Adenine (as a precursors of adenosine-triphosphate) is a base required for the upper ligand in adenosylcobalamin. Methionine is a potential source of the methyl group in methylcobalamin.

The impact of each precursor (1 mM) as well as a precursor mixture (1 mM each) on CT (100 $\mu$M) degradation kinetics in an unadapted methanogenic consortium was evaluated with methanol as the primary substrate. The results were compared with negative controls (not containing precursors) and a positive control (containing 10 $\mu$M $CNB_{12}$). The first order rate constants and the residual concentration of CT on day 8 and day 18 are shown in Table 2. The Table illustrates that CT

*Table 2.* Impact of vitamin $B_{12}$ precursors on the first order rate constant ($k$) of carbon tetrachloride (CT) conversion

| Treatment | $k$ ($day^{-1}$) | | CT day 8 ($\mu$M) | | CT day 18 ($\mu$M) | | CF day 8 ($\mu$M) | | CF day 18 ($\mu$M) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Avg[a] | SD[b] | Avg | SD | Avg | SD | Avg | SD | Avg | SD |
| Abiotic control | | | 95.8 | 6.9 | 96.8 | 6.5 | 0.0 | 0.0 | 0.0 | 0.0 |
| Negative control (no Co) | 0.096 | 0.002 | 50.4 | 7.3 | 17.0 | 0.7 | 14.1 | 0.4 | 16.0 | 2.1 |
| Negative control (with Co) | 0.160 | 0.004 | 43.7 | 2.7 | 5.3 | 0.1 | 16.1 | 3.2 | 3.9 | 2.7 |
| Positive control ($CNB_{12}$, 10 $\mu$M) | 1.847 | 0.014 | 0.0 | 0.0 | NM[c] | | 0.6 | 0.1 | NM | |
| Porphobilinogen (PB) | 0.472 | 0.005 | 1.8 | 0.1 | 0.0 | 0.0 | 16.3 | 2.5 | 7.6 | 0.8 |
| Adenine | 0.245 | 0.004 | 40.0 | 2.0 | 1.0 | 0.0 | 15.9 | 0.2 | 20.4 | 0.4 |
| 5,6-Dimethylbenzimidazole (DMBI) | 0.119 | 0.004 | 44.1 | 5.6 | 10.2 | 0.8 | 17.8 | 0.8 | 23.0 | 1.2 |
| $\delta$-Aminolevulinic acid | 0.305 | 0.002 | 40.4 | 7.7 | 0.3 | 0.0 | 18.2 | 1.2 | 19.2 | 0.2 |
| L-Threonine | 0.407 | 0.008 | 33.1 | 0.2 | 0.1 | 0.0 | 19.0 | 1.2 | 20.0 | 0.2 |
| L-Methionine | 0.219 | 0.004 | 34.9 | 3.2 | 1.6 | 0.0 | 16.8 | 0.1 | 20.3 | 0.6 |
| Mixture precursors | 0.398 | 0.009 | 12.8 | 5.2 | 0.1 | 0.0 | 30.9 | 1.4 | 24.7 | 0.4 |

CT was initially supplied at 100 $\mu$M. Also shown is the residual CT concentrations and concentrations of intermediate, chloroform (CF), on days 8 and 18.
[a]Avg = average.
[b]SD = standard deviation.
[c]NM = not measured.

degradation occurred slowly in the negative control; while the rate was 11.5-fold faster in the positive control in accordance with the previous study (Guerrero-Barajas & Field 2005a). Of all the precursors tested, the best response was obtained with PB, providing a rate constant that was 3-fold greater than that of the negative control with cobalt (Table 2, Figure 1). CT concentrations were also significantly lower compared than those of the negative controls at all time points during the experiment (Table 2, Figure 1). Significant increases in the first order rate constants for CT bioconversion by 1.4 to 2.5-fold were also observed for adenine, $\delta$-aminolevulinic acid, threonine, methione and the mixture of precursors (Table 2). The two negative controls, compared the presence and absence of a cobalt addition (50 $\mu g\ l^{-1}$) on CT conversion. The presence of cobalt increased the first order rate constant by 67% and CT concentrations were consistently lower in the cobalt-amended control, although the differences were modest.

The formation of the intermediate, CF, was also measured. In the positive control, CF was formed and subsequently degraded after reaching a maximum of 6.8 $\mu$M on day 4. CF degradation by this methanogenic consortium is known to be stimulated by vitamin $B_{12}$ (Guerrero-Barajas & Field 2005b), which accounts for the subsequent decline. The PB amended treatment followed the pattern observed in the positive control but CF reached a maximum of 16.3 $\mu$M on day 8 (Figure 1). The CF results for the other precursor amended treatments reached values of 15–20 $\mu$M and remained more or less constant thereafter. In the negative controls, CF also accumulated to similar levels but CF declined again in the control supplemented with cobalt. The treatment receiving the mixture of precursors accumulated CF up to 31 $\mu$M and thereafter the concentration declined to 25 $\mu$M by day 18. The precursor mixture treatment also accumulated dichloromethane (DCM) up to 83 $\mu$M on day 18. The highest DCM levels in the other treatments ranged from 2 to 26 $\mu$M. Perchloroethylene (PCE) was also observed at low concentrations as a CT-degradation side product in agreement with earlier observations (Cervantes et al. 2004; Guerrero-Barajas & Field 2005a). Concentrations of PCE ranged from 0.05 to 2.53 $\mu$M on day 18.

### *Effect of porphobilinogen concentration*

Since PB was found to be the most effective precursor of vitamin $B_{12}$ in stimulating the anaerobic conversion of CT, the impact of different levels of

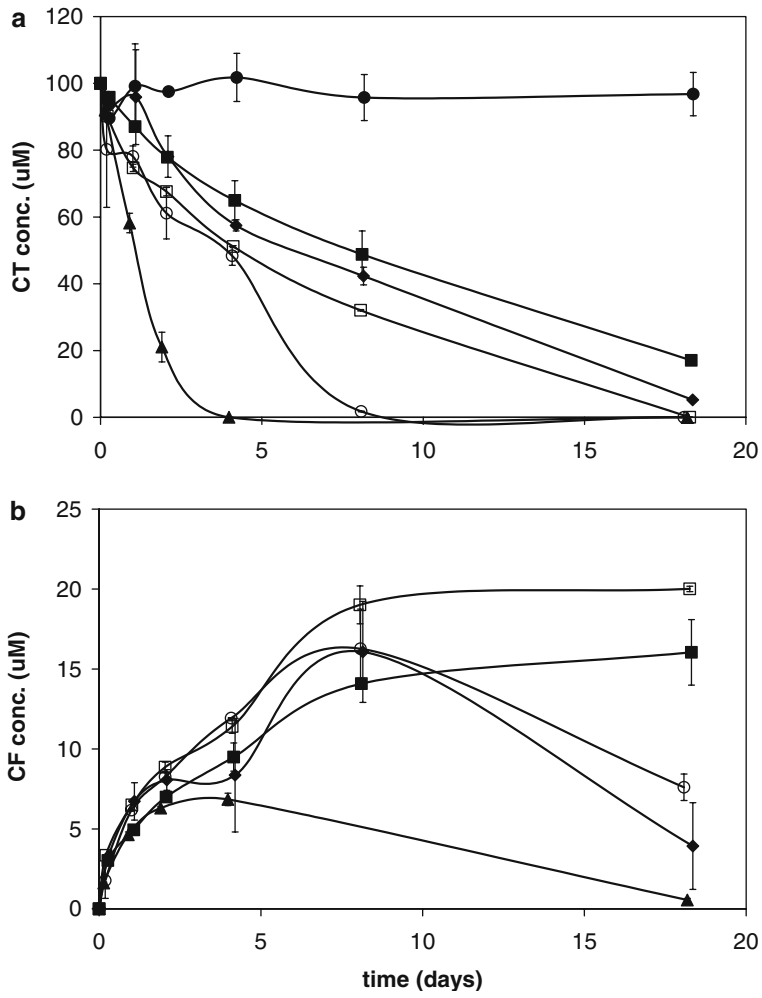


*Figure 1.* The effect of vitamin $B_{12}$ precurors on CT depletion (a) and intermediate formation of CF (b) in the methanogenic consortium fed with methanol as primary substrate. Legend: abiotic control (filled bullets); no precursor and no cobalt control (filled squares); no precursor control with cobalt (filled diamonds); 1000 $\mu$M threonine (empty squares); 880 $\mu$M PB (empty bullets); positive control with 10 $\mu$M $CNB_{12}$ (filled triangles). PB was from source 1.

PB was evaluated. The results shown in Figure 2 show that increasing concentrations of PB resulted in increasing rates of CT-bioconversion. The highest two PB concentrations tested, 500 and 900 $\mu$M, provided results that were similar to those of the positive control (with 10 $\mu$M $CNB_{12}$).The first order rate constants in treatments with 500 and 900 $\mu$M PB were 8.8 and 10.9-fold higher compared to the negative control (Table 3), respectively. PB at 160 $\mu$M also significantly increased the first order rate constants by 2.7-fold.

Concentrations of the intermediate CF accumulated to levels of 11–14.8 $\mu$M on day 10 in treatments with PB and 7.4 $\mu$M in the treatment with $CNB_{12}$. DCM accumulated up to levels of 0.6–1.0 $\mu$M in treatments with PB and 0.3 $\mu$M in the treatment with $CNB_{12}$. PCE was also detected in trace amounts around 0.07 to 0.08 $\mu$M on day 10.

In this experiment $Cl^-$ measurements were conducted, enabling the presentation of a mass balance of chlorine on day 10 as shown in Table 3. The table shows that PB at 500 and 900 $\mu$M enabled the mineralization of CT-Cl to $Cl^-$ by 56%, close to the value of 66% observed in the positive control. The net production of detectable products (CF, DCM, and $Cl^-$) as a percentage of CT-Cl converted reached 65% at high PB levels similar to the value of 72% obtained in the positive control. It is interesting to note that PB treatments as low

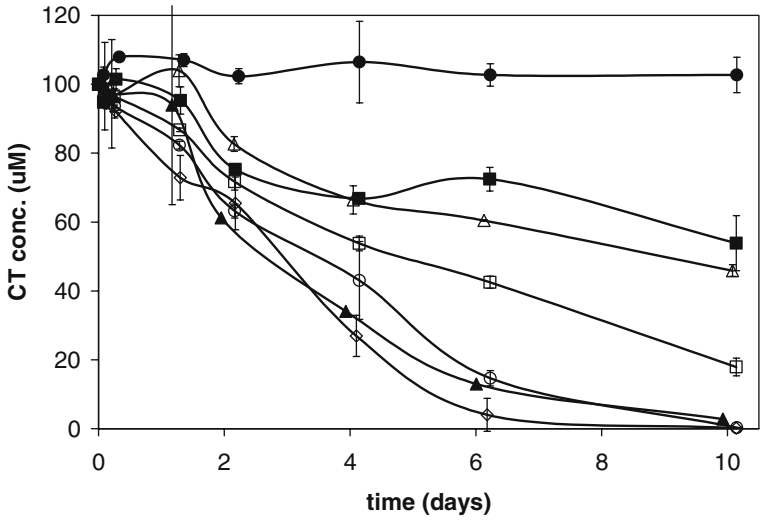


*Figure 2*. The effect PB concentration on CT depletion in the methanogenic consortium fed with methanol as primary substrate. Legend: abiotic control (filled bullets); 0 $\mu$M PB (filled squares); 40 $\mu$M PB (empty triangles); 160 $\mu$M PB (empty squares); 500 $\mu$M PB (empty bullets); 900 $\mu$M PB (empty diamonds); positive control with 10 $\mu$M $CNB_{12}$ (filled triangles). PB was from source 2.

*Table 3*. First order rate constant ($k$) and chlorine balance on day 10 in the experiment evaluating different concentrations of porphobilinogen (PB) during the biodegradation of CT

| Additions | $k$ ($day^{-1}$) | CT-Cl | CF-Cl | DCM-Cl | $Cl^-$ | Σ products (% CT-Cl converted)[a] |
|---|---|---|---|---|---|---|
| | | % Initial CT-CI | | | | |
| None | 0.061 (±0.013) | 44.96 (±6.68) | 6.19 (±0.52) | 0.26 (±0.01) | 14.94 (±0.66) | 38.86 |
| PB (40 $\mu$M) | 0.082 (±0.003) | 38.31 (±1.40) | 9.23 (±4.40) | 0.41 (±0.24) | 21.04 (±2.08) | 49.74 |
| PB (160 $\mu$M) | 0.166 (±0.011) | 14.95 (±2.12) | 7.57 (±0.10) | 0.26 (±0.00) | 38.64 (±0.10) | 54.63 |
| PB (500 $\mu$M) | 0.536 (±0.004) | 0.24 (±0.04) | 7.11 (±0.11) | 0.25 (±0.01) | 56.99 (±2.92) | 64.50 |
| PB (900 $\mu$M) | 0.665 (±0.090) | 0.11 (±0.04) | 6.89 (±0.24) | 0.30 (±0.09) | 55.96 (±1.08) | 63.23 |
| $CNB_{12}$ (10 $\mu$M) | 0.353 (±0.000) | 2.30 (±0.00) | 4.45 (±0.16) | 0.12 (±0.18) | 65.70 (±11.25) | 72.13 |

Values in parenthesis correspond to standard deviations of duplicate treatments.
[a]$100\times(\text{CF-Cl} + \text{DCM-Cl} + Cl^-)/\text{CT-Cl}_{converted}$.

as 40 $\mu$M significantly increased the recovery of $Cl^-$ and other products from CT-Cl (Table 3).

*Effect of primary substrates*

Several primary substrates were evaluated for their ability to stimulate CT conversion based on previous works implicating the primary substrates as inducers of vitamin $B_{12}$ biosynthesis. These substrates included: 1,2-propanediol, glycerol and ethdnolamine (Martens et al. 2002) or methanol (Nishio et al. 1975). CT degradation with these substrates was compared with that obtained with volatile fatty acid mixture (VFA), polyethylene glycol (PEG) and no added substrate (endogenous substrate) as shown in Figure 3. The first order rate ($k$) constants estimated for all primary substrate tested are given in Table 4. Compared to VFA, only glycerol and 1,2-propanediol caused a noteworthy increase in the rate of CT degradation by a factor of 2.5 and 2.9, respectively. The increases were substantially lower compared to 10 $\mu$M to $CNB_{12}$ in the VFA fed culture, causing a 7.4-fold increase in the rate. In a separate experiment, ethanolamine was also tested, but it had no significant effect on increasing the rate of CT degradation compared to the VFA control (results not shown).

The formation of CF is shown in Figure 3b. CF concentrations continually increased in the treatment with glycerol reaching 43 $\mu$M by the end of the experiment. In treatments with 1,2-propanediol, the CF concentration leveled off at 28 $\mu$M on

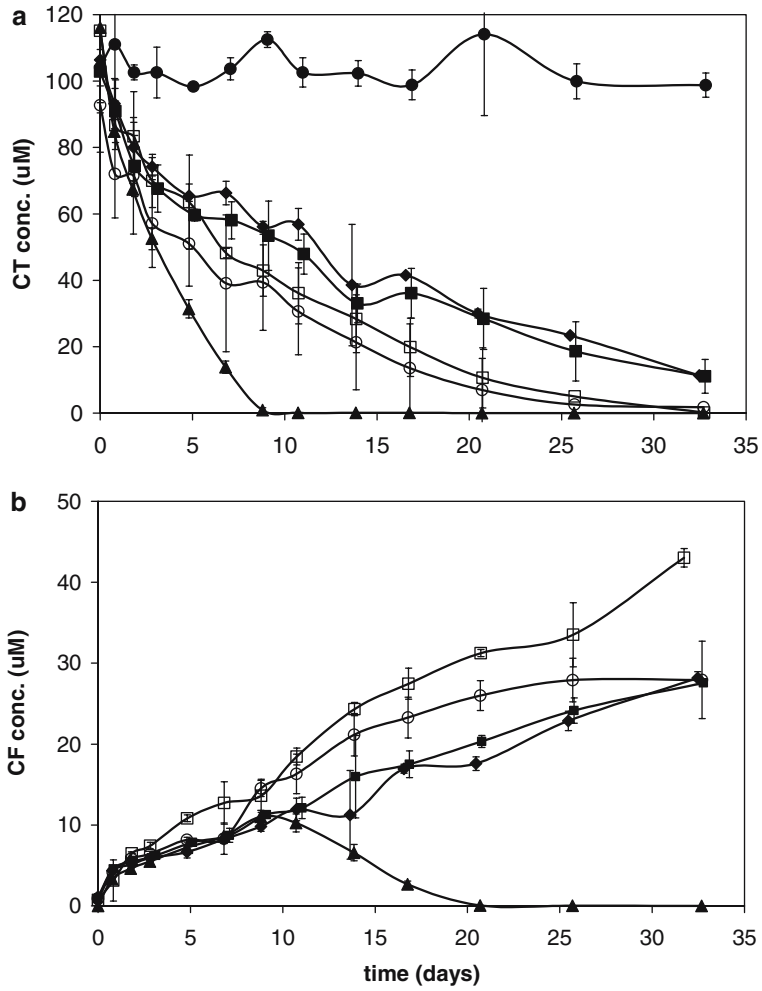


*Figure 3*. The effect of different primary substrates on CT depletion (a) and intermediate formation of CF (b) in the methanogenic consortium. Legend: abiotic control (filled bullets); no primary substrate control (filled squares); VFA (filled diamonds); glycerol (empty squares); 1,2-propanediol (empty bullets); VFA with 10 $\mu$M $CNB_{12}$ (filled triangles).

day 21. CF accumulated in the positive control (VFA and $CNB_{12}$) to a level of 11 $\mu$M on day 9 and thereafter was completely consumed. DCM and PCE were also detected. The highest concentration of DCM (6.7 $\mu$M) was observed in the 1,2-propanediol treatment on day 33. The highest concentration of PCE (1.2 $\mu$M) was detected in the PEG treatment on day 26.

*Effect of primary substrate on effectiveness of porphobilinogen*

The effectiveness of PB in stimulating CT-conversion was found to be limited to experimental conditions in which methanol was used as a primary substrate. The impact of PB supplied at 160 $\mu$M on the CT concentration after 8 days of incubation at 24 °C was evaluated with two primary substrates: either a mixture of volatile fatty acids (VFA) or methanol. The results are presented in Table 5 and are compared with a negative control (no precursors) and a positive control (10 $\mu$M $CNB_{12}$) for each primary substrate. The data clearly illustrates that PB only significantly decreased the CT concentration when incubated with methanol as the primary substrate. In contrast to methanol, PB had no significant impact VFA as primary substrate. In similar experiments conducted with glycerol as primary substrate, PB had only a small impact, which was relatively modest compared to that obtained with methanol (results not shown).

*Table 4*. Effect of primary substrate on the first order rate constant ($k$) and concentrations of carbon tetrachloride (CT) and chloroform on day 11

| Treatments | $k$ ($day^{-1}$) | | CT day 11 ($\mu$M) | | CF day 11 ($\mu$M) | |
|---|---|---|---|---|---|---|
| | Avg[a] | SD[b] | Avg | SD | Avg | SD |
| Abiotic control | | | 102.60 | 4.41 | 0.00 | 0.00 |
| No substrate | 0.063 | 0.014 | 47.90 | 6.03 | 12.11 | 1.32 |
| VFA[c] | 0.060 | 0.001 | 56.87 | 4.80 | 11.92 | 1.39 |
| VFA and $CNB_{12}$ (10 $\mu$M) | 0.445 | 0.016 | 0.08 | 0.00 | 10.26 | 1.11 |
| Methanol | 0.076 | 0.005 | 45.82 | 4.28 | 12.17 | 0.15 |
| 1,2-Propanediol | 0.176 | 0.076 | 30.63 | 13.06 | 16.32 | 2.45 |
| Glycerol | 0.148 | 0.021 | 36.09 | 9.26 | 18.46 | 1.03 |
| Polyethylene glycol | 0.051 | 0.001 | 58.51 | 0.87 | 9.87 | 0.69 |

The initial concentration of CT was 106 $\mu$M.
[a]Avg = average.
[b]SD = standard deviation.
[c]VFA = volatile fatty acid mixture.

*Table 5*. Influence of primary substrate on the impact of PB on CT bioconversion (incubation temperature 24 °C)

| Treatment | | CT day 8 ($\mu$M) | | CF day 8 ($\mu$M) | |
|---|---|---|---|---|---|
| Primary substrate | $CNB_{12}$ or PB additions | Avg[a] | SD[b] | Avg | SD |
| VFA[c] | None | 98.26 | 3.50 | 3.74 | 0.15 |
| VFA | PB (160 $\mu$M) | 95.10 | 1.19 | 3.62 | 0.0 |
| VFA | $CNB_{12}$ (10 $\mu$M) | 25.72 | 1.06 | 4.94 | 0.86 |
| Methanol | None | 100.21 | 7.70 | 4.57 | 0.79 |
| Methanol | PB (160 $\mu$M) | 55.20 | 3.40 | 7.96 | 0.20 |
| Methanol | $CNB_{12}$ (10 $\mu$M) | 39.92 | 0.13 | 0.0 | |

The initial CT concentration was 100 $\mu$M.
[a]avg = average.
[b]SD = standard deviation.
[c]VFA = volatile fatty acid mixture.

## Discussion

The present study considered two strategies known to increase vitamin $B_{12}$ biosynthesis as a means of increasing CT biotransformation rates in a methanogenic consortium. One approach evaluated potential precursors of vitamin $B_{12}$ biosynthesis. The other approach evaluated primary substrates that induce vitamin $B_{12}$ biosynthesis.

### *Precursors of vitamin $B_{12}$*

A screening of potential vitamin $B_{12}$ precursors revealed that one precursor, porphobilinogen (PB) had the greatest impact in significantly increasing CT bioconversion by the unadapted methanogenic consortium utilized in this study. The first order rate constant of CT bioconversion was increased by a factor 3 compared to a control lacking precursors. Several of the other precursors screened (adenine, $\delta$-aminolevulinic acid, threonine and methionine) had small but significant effects in stimulating CT bioconversion. The impacts of adenine, methionine and threonine are not necessarily related to vitamin $B_{12}$ biosynthesis, since such compounds have multiple biochemical roles. One precursors, DMBI, which is often used as a stimulant for the industrial microbial production of vitamin $B_{12}$ (Martens et al. 2002), had no effect in increasing CT bioconversion. The concentration of DMBI used may have been too high and thus inhibitory. There is at least one report in which

DMBI additions reduced vitamin $B_{12}$ production in methanogenic enrichments under certain culture conditions (Neujahr & Callieri 1959). Incorporation of DMBI in the vitamin $B_{12}$ structure by the most widely used *Propionibacterium* generally requires microaerophilic conditions (Martens et al. 2002).

PB is the direct precursor of the tetrapyrrole, uroporphyrinogen III, which becomes the corrin ring structure (Martens et al. 2002; Raux et al. 2000; Roth et al. 1996; Warren et al. 2002). Because of the large significant impact of PB, we conclude that the biosynthesis of the corrin ring is a limiting factor in the methanogenic consortium. The first precursor prior to PB is $\delta$-aminolevulinic acid, which also had an effect, albeit much lower compared to PB. The lower impact of $\delta$-aminolevulinic acid can be due to two possibilities. The first possibility is that the conversion of $\delta$-aminolevulinic acid to PB is the rate-limiting step in biosynthesis of the corrin ring. The second possibility is that $\delta$-aminolevulinic acid is more rapidly biodegraded in the methanogenic consortium or it is not as readily taken up by cells compared to PB. Both methionine and adenosine which also had small impacts on increasing CT-bioconversion, may have also played a role *via* the synthesis of the corrin ring since s-adenosylmethionine is a methyl donor to uroporphyrinogen III during the biosynthesis of the corrin ring structure (Roth et al. 1996; Warren et al. 2002). However, adenosine and the methyl group of methionine might also be incorporated as upper ligands of adenosylcobinamine or methylcobalamin, respectively (Martens et al. 2002). Threonine is involved as a cofactor transferring a phosphate group during the incorporation of aminoisopropanol (Warren et al. 2002).

The addition of cobalt had a small but significant impact on the first order rate constant of CT conversion as well as the final concentration of CT after prolonged incubations. Thus cobalt supplementation may have contributed to an increase in vitamin $B_{12}$ synthesis as was observed in early research with a methanogenic enrichment culture derived from sewage sludge (Neujahr & Callieri 1959). Another study indicated that addition of cobalt did not stimulate CT bioconversion in a methanogenic consortium (Van Eekert et al. 1998). The differences in response may be due to differences in sulfur concentrations in the basal media, resulting in sulfide that could sequester cobalt.

A second experiment evaluated the effect of PB at different concentrations. The first order rate constant of CT-bioconversion was significantly increased and the residual CT concentrations were significantly decreased at concentrations as low as 160 $\mu$M PB. PB added at concentrations as low as 40 $\mu$M had significant impacts on the mineralization of CT-Cl to $Cl^-$. The fact that such low concentrations of PB had significant effects agrees with a role of PB as a precursor of vitamin $B_{12}$ as opposed to a substrate effect. Concentrations of 500–900 $\mu$M PB provided conversions of CT that were comparable or better than that achieved in positive controls supplemented with vitamin $B_{12}$ at 10 $\mu$M. Based on stoichiometry, 40 $\mu$M of PB is required to synthesize 10 $\mu$M of vitamin $B_{12}$. The fact that at least 500 $\mu$M PB is required to get a similar effect on CT-biotransformation as 10 $\mu$M of vitamin $B_{12}$ could be due to several factors. First, there could be an incomplete uptake of PB. Second, not all of the PB taken up would necessarily by directed to vitamin $B_{12}$ biosynthesis since PB is a precursor of other tetrapyrroles. Third, not all of the vitamin $B_{12}$ produced would necessarily be secreted. In order to convey a redox-mediating role, vitamin $B_{12}$ would need to occur extracellularly. There is at least evidence that some vitamin $B_{12}$ synthesized by methanol metabolizing methanogens is secreted into the extracellular environment (Mazumder et al. 1987).

### *Effect of PB on the mineralization of CT*

In methanogenic consortia, reductive dechlorination of CT proceeds *via* one of two major pathways (Egli et al. 1990; Egli et al. 1988; Field & Sierra-Alvarez 2004; Krone et al. 1991; Van Eekert et al. 1998). In one pathway, reductive hydrogenolysis results in the replacement of chlorine atoms by hydrogen atoms, accounting for the formation of CF and DCM. In the other pathway, two reducing equivalents result in the initial formation formation of an unstable dichlorocarbene intermediate. The chlorine to carbon bonds of the dichlorocarbene intermediate are readily hydrolyzed by water or $H_2S$, forming CO or $CS_2$. In an active methanogenic consortium, CO would be further converted to $CH_4$, acetate and $CO_2$ (Sipma et al. 2003). Radiolabeled [$^{14}$C]CT was shown to

be converted to $^{14}CO_2$ as a major product in an unadapted methanogenic consortium (Van Eekert et al. 1998). In our previous work, we have demonstrated that vitamin $B_{12}$ additions to a methanogenic consortium dramatically increased the mineralization of CT accounting for an increased molar recovery of inorganic chloride per CT converted (Guerrero-Barajas & Field 2005a). The increased molar recovery of chloride would indicate that vitamin $B_{12}$ promotes the reductive hydrolysis reactions. In support of this hypothesis is the observation that the chemical reaction of biologically reduced $CNB_{12}$ with CT resulted in CO as the major product (92%); whereas CF was a minor product (1.4%) (Workman et al. 1997). The ability of cobalamins to form complexes with halomethanes (Krone et al. 1989) may account for the dramatic alteration in the spectrum of products formed during halomethane biotransformation. PB additions influenced CT biotransformation in a pattern similar to that expected with vitamin $B_{12}$. In this study, the molar ratio of inorganic chloride formed to CT converted increased greatly with low level supplementations of PB.

In this study we have also observed perchloroethylene (PCE) as a minor biotransformation product of CT bioconversions as was witnessed in the previous studies (Cervantes et al. 2004; Guerrero-Barajas & Field 2005a). The formation of PCE is hypothesized to occur *via* the coupling of two chloroform radicals, or from reaction of a dichlorocarbene intermediate with CT, forming hexachloroethane. Hexachloroethane is unstable in reducing environments and would readily be subjected to reductive dichloroelimination to PCE (van Eekert et al. 1999).

*Effect of primary substrates*

Several primary substrates may be involved in stimulating vitamin $B_{12}$ production such as 1,2-propanediol, glycerol and ethanolamine (Martens et al. 2002; Roth et al. 1996). The first steps in the metabolism of these substrates in many enteric bacteria involve, propanediol dehydratase, glycerol dehydratase and ethanolamine ammonia lyase; respectively, which are all adenosylcobalamin-dependent enzymes. The substrate, 1,2-propanediol has been used to stimulate vitamin $B_{12}$ production in methanogenic enrichment cultures (Zou et al. 2000). These enrichment cultures were able to degrade CT, and the rates of CT-degradation rates were found to be proportional to intracellular levels of vitamin $B_{12}$. Based on these finding we explored the possibility of 1,2-propanediol, glycerol and ethanolamine in stimulating CT-degradation. Both 1,2-propanediol and glycerol significantly increased rates of CT degradation, while ethanolamine did not. The stimulating impact of 1,2-propanediol and glycerol may be due to the reported increased vitamin $B_{12}$ biosynthesis; however, these primary substrates may have also been superior electron donors compared to the reference VFA substrate.

The stimulating effect of PB depended on the type of primary substrate supplied to the assay. When volatile fatty acids were utilized as the primary substrate, PB had no noteworthy effect. However, when methanol was utilized, the impact of PB was significant. This phenomenon can be rationalized by the induction of vitamin $B_{12}$ synthesis. The anaerobic metabolism of C1 substrates such as methanol by methanogens and acetogens are known to require vitamin $B_{12}$ for methyl transferase reactions (Harms & Thauer 1996; Stupperich et al. 1992; Van Der Meijden et al. 1984a,b). Likewise the production of cobalamin containing proteins are induced by methanol (Harms & Thauer 1996; Stupperich et al. 1992). Vitamin $B_{12}$ production is known to be increased under conditions in which the medium contains Cl substrates such as methanol (Lebloas et al. 1994; Mazumder et al. 1987; Neujahr & Callieri 1959; Van Eekert et al. 1998). Therefore when vitamin $B_{12}$ production is induced, a shortage in precursors is expected to be most noticeable, accounting for a larger impact of PB additions under conditions when methanol is supplied as the primary substrate.

*Mechanisms*

Based on the evidence presented, the hypothesis is that PB enhanced the production of vitamin $B_{12}$, which subsequently acted as an electron shuttle to enhance the conversion rate and mineralization of CT. Since vitamin $B_{12}$ was not measured, the proposed mechanism is based on the following circumstantial evidence. First, the pattern of degradation is first order without any lag phase, which indicates that PB was not acting as a vitamin enriching the growth of any specific microorganisms

capable of utilizing CT. Second, the effect of PB was very specific to methanol, an observation consistent with vitamin $B_{12}$ biosynthesis in methanogenic consortia (Mazumder et a1.1987; Van Eekert et al. 1998). Third, a significant fraction of the vitamin $B_{12}$ produced by methanogens is known to be extracellular (Mazumder et al. 1987). Fourth, extracellular vitamin $B_{12}$ can be reduced by anaerobic microorganisms and reduced vitamin $B_{12}$ can dechlorinate CT *in vitro* (Workman et al. 1997). Lastly, the high degree of mineralization of CT-Cl to inorganic $Cl^-$ obtained with PB was consistent with the product spectrum obtained with vitamin $B_{12}$.

*Applicability*

The objective of this work was to obtain an inexpensive precursor to replace vitamin $B_{12}$. Unfortunately PB is a costly biochemical and as such would not likely offer any economic advantage over vitamin $B_{12}$. Nonetheless, the insights gained from this study, clearly indicate that the synthesis of the corrinoid ring is rate limiting. This information can be used to come up with inexpensive alternatives. An example could potentially be the use of blood byproducts (from slaughter houses) which upon the decay of the heme group could supply precursors (uroporphyrinogen III and porphobilinogen) for the corrin ring.

## Conclusion

The goal of the study was to determine if vitamin $B_{12}$ enhanced rates of anaerobic CT bioconversion could be achieved by use of vitamin $B_{12}$ precursors. The present study indicates that one precursor, PB, significantly increased the CT biotransformation rates and molar yields of inorganic chloride comparable to vitamin $B_{12}$. The large response afforded by PB suggests that the biosynthesis of vitamin $B_{12}$ in methanogenic consortia is limited by the formation of thetetrapyrrole ring. The primary substrate, methanol, known to induce vitamin $B_{12}$ production in methanogens and acetogens was required for PB to have a significant impact. This observation would suggest that PB's role was indeed due to stimulate vitamin $B_{12}$ biosynthesis. The present study therefore provides insights on how to achieve vitamin $B_{12}$ enhanced rates of CT bioremediation through the use of less complex compounds that are precursors or inducers of vitamin $B_{12}$. Although PB is a costly chemical, its large impact points to corrin ring formation as the rate-limiting step, providing the clue that inexpensive heme degradation byproducts could potentially be considered as inexpensive sources of vitamin $B_{12}$ precursors.

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