Biodegradation **13:** 411–424, 2002.
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Complete dechlorination of tetrachloroethene to ethene in presence of methanogenesis and acetogenesis by an anaerobic sediment microcosm

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Accepted 19 December 2002

Key words: ethene, homoacetogenesis, reductive dechlorination, sediment microcosm, tetrachloroethene, vinyl chloride

Abstract

An anaerobic consortium taken from brackish sediments, enriched by PCE/CH_3OH sequential feeding, was capable of completely dechlorinating tetrachloroethene (PCE) to ethene (ETH). In batch experiments, PCE (0.5 mM) was dechlorinated to ethene (ETH) in approximately 75 h with either CH_3OH or H_2 as the electron donor. When VC (0.5 mM) was added instead of PCE it was dechlorinated without any initial lag by the PCE/CH_3OH enriched consortium, although at a lower dechlorination rate. In batch tests H_2 could readily replace CH_3OH for supporting PCE dechlorination, with a similar PCE dechlorination rate and product distribution with respect to those observed with methanol. This indicates that H_2 production during CH_3OH fermentation was not the rate-limiting step of PCE or VC dechlorination. Acetogenesis was the predominant activity when methanol was present. A remarkable homoacetogenic activity was also observed when hydrogen was supplied instead of methanol.

Introduction

Tetrachloroethene (PCE) and trichloroethene (TCE) have been widely used in the past for dry cleaning, degreasing operations, and in manufacturing of electronic components. Due to improper use and disposal these toxic compounds are common organic contaminants of soil and groundwater. In Italy, groundwater contamination due to the presence of chlorinated hydrocarbons has become critical especially in the northern part of the country where economical-industrial development has been higher (Berbenni 1994). For the cleanup of contaminated soil and groundwater, the potential of microorganisms to transform toxic chlorinated compounds into harmless products is a major advantage of biological remediation techniques in comparison to chemical-physical techniques where pollutants are often only transferred into another phase. Although aerobic cometabolic degradation of PCE by toluene-*o*-xylene monooxygenase of *Pseudomonas stutzeri* OX1 has been recently reported (Ryoo et al. 2000), this compound appears to be resistant to metabolism by aerobic microorganisms due to its oxidized nature. However, PCE can be transformed via step-wise reductive dechlorination (RD) to less chlorinated ethenes (Vogel et al. 1987). Sequential reductive dechlorination of PCE proceeds via TCE, DCEs (dichloroethenes), VC (vinyl chloride), ETH (ethene), and even ethane (Middeldorp et al. 1999). Although complete dechlorination of PCE or TCE to ETH or ethane has been observed in the field and in laboratory experiments involving microcosms, reactors, and enrichment cultures (Freedman & Gossett 1989; DiStefano et al. 1991; Carter & Jewell 1993; de Bruin et al. 1992; Komatsu et al. 1997), VC often accumulates. This is often because VC reduction appears to be the rate limiting step in the RD of PCE to ETH (Freedman & Gossett 1989; Vogel et al. 1987; Tandoi et al. 1994; Haston & McCarty 1999). Since VC is more toxic than its parent compounds and is a known human carcinogen, the factors controlling its accumulation and persistence are of special concern.

Several investigators have shown that many different electron donors (e.g., glucose, formate, methanol, lactate) can sustain RD of PCE to ETH. However, there is growing evidence that hydrogen is in many cases the actual electron donor for dechlorination and that organic electron donors serve mainly as primary precursors that supply the hydrogen via fermentation (DiStefano et al. 1992; Maymò-Gatell et al. 1995; Tandoi et al. 1994; Ballapragada et al. 1997). In a methanol fed culture, methanogens and acetogens both compete for the available methanol. Competition for the primary electron donor can affect dechlorination by determining the portion of electrons from methanol converted to hydrogen. There is also competition for hydrogen by, dechlorinators, hydrogenophilic methanogens and homoacetogens (Smatlak et al. 1996; Ballapragada et al. 1997; Fennel et al. 1997; Yang & McCarty 1998; Mazur & Jones 2001).

In this study, microbial activities including acetogenesis, methanogenesis, and dechlorination, were investigated within an anaerobic methylotrophic consortium enriched from sediments from an industrial canal. The consortium was capable of completely dechlorinating high concentrations of PCE to ETH. Characterization of the microbial activities was performed by using a set of simultaneous batch tests with several combinations of different electron donors and acceptors.

Materials and methods

Development of the anaerobic dechlorinating culture

The source of the inoculum was a brackish sediment that was collected in an industrial canal in the North-East of Italy (Aulenta et al. 2001). Total solids (TS) in the sediment were 0.57 g TS g^{-1} wet sediment, and volatile solids accounted for 8.5% of TS. The microbial consortium was initially prepared by dispensing a known amount (about 20 g) of wet sediment into a 120-ml serum bottle with basal medium to a final liquid volume of 75 ml (Tandoi et al. 1994). The basal medium contained: (final concentration in g l^{-1}): NH_4Cl, 0.5; K_2HPO_4, 0.4; $MgCl_2{\cdot}6H_2O$, 0.1; $CaCl_2{\cdot}H_2O$, 0.05; resazurin (redox indicator) 0.001; and 10 ml of a trace metal solution. The serum bottle was then sealed with Teflon coated stoppers (Wheaton, Millville, NJ) and the headspace was flushed with a 70% N_2–30% CO_2 gas mixture. The bottle received 1 ml vitamin solution, 0.5 ml $Na_2S{\cdot}9H_2O$ 5% w/v, 2 ml $NaHCO_3$ 10% w/v, and 0.1 ml yeast extract 10% w/v (per 100 ml medium). The final chloride concentration in the serum bottle was 2.50 g l^{-1}. PCE at concentration 0.4 mM (considering the PCE completely dissolved in the liquid) and methanol at 2.3 mM (as main electron donor, about 4 times in excess with respect to PCE) were then added to the serum bottle. The serum bottle was quiescently incubated at 20 °C.

During acclimation and enrichment of the microbial community, PCE and methanol were monitored twice a week, and supplied again when exhausted. Before each PCE and methanol re-addition the bottle headspace was flushed with 70% N_2–30% CO_2 in order to remove residual volatile compounds. Liquid samples (1 ml) were drawn twice a week for pH measurement. When necessary $NaHCO_3$ 10% solution was added to keep the pH in the range 6.6–7.2. An average of 0.28 ml d^{-1} of microbial suspension was wasted to keep the hydraulic retention time (HRT) and the sludge age (Θ_c) at 280 days.

Preliminary batch tests to characterize RD activity during this acclimation and enrichment period were performed at 3, 4, and 7 months. After 8 months, the enriched consortium was anaerobically transferred into a 2-l serum bottle and diluted to 1.25 l with basal medium (containing 10 mg l^{-1} yeast extract). So the ratio between liquid and headspace volume in the reactor was maintained identical to that of the originally inoculated 120-ml serum bottle. PCE was added at higher concentration (0.5 mM) to the 2-l serum bottle, while maintaining methanol at 2.3 mM. Enrichment procedure was maintained as reported above. The long term operation of the 120-ml and 2-l serum bottles, with respect to PCE, is reported in Figure 1.

After about 11 months a set of batch experiments (hereafter described) was carried out, aimed at characterizing the main microbial activities (namely dechlorination, acetogenesis, and methanogenesis) in the developed dechlorinating culture. At the time of the experiments detailed here, the culture was removing a dose of PCE in about 10 days under quiescent conditions.

Batch studies

Batch studies were conducted at 20 °C using 120-ml serum bottles. For these tests 75 ml of culture from the 2-l serum bottle were transferred into 120-ml serum bottles. These were sealed with Teflon coated rubber stoppers and aluminum crimps to maintain anaerobic conditions, and flushed with 70% N_2–30% CO_2 to remove residual volatile compounds. Adsorption of chlorinated compounds on the rubber stoppers was minimal ensuring the capacity for good mass balances. Electron donors (methanol or H_2) and acceptors (PCE or VC) were added at the chosen concentrations. The contents of the bottles were mixed via a magnetic stirrer at 150 rpm. The headspace was analyzed for PCE, TCE, DCE, VC, ETH, H_2 and CH_4. Aqueous samples were analyzed for methanol and acetate. Each experiment was carried out in duplicate to test reproducibility. Maximum rates of utilization or formation were calculated from linear time profiles of concentrations. All given rates refer to an average of the two replicate batch tests (but where differently specified). All the rates have not been referred to volatile solids (VSS $= 1.03$ g l^{-1} at the time of batch tests), since the presence of the original sediment would have not allowed a reliable estimation of biomass.

The percentage of reducing equivalents from the electron donor used in methanogenesis, acetogenesis and dechlorination was calculated at the end of each batch experiment from the measured levels of methane, acetate, and dechlorination products formed and the electron donor consumed. Molar equivalents factors used were: 8 eq mol^{-1} for methane, 8 eq mol^{-1} for acetate, 2 eq mol^{-1} for each chlorine removed from chlorinated ethenes, 6 eq mol^{-1} for methanol, and 2 eq mol^{-1} for hydrogen, as previously reported (DiStefano et al. 1991; Gao et al. 1997).

Analytical procedures

Volatile components (PCE, TCE, DCE, VC, ETH, CH_4) were quantified by injecting 50 μl of serum bottle headspace (with a gas-tight Hamilton syringe) into a Perkin Elmer GC 8500 gas chromatograph (2 m × 2 mm glass column packed with 60/80 mesh Carbopak B/1% SP-1000 Supelco; N_2 carrier gas 18 ml min^{-1}; oven temperature from 190 °C to 210 °C; flame ionisation detector temperature 260 °C). Methanol was quantified by injecting 1 μl of aqueous sample into the GC (2 m × 2 mm glass column packed with 60/80 mesh Carbopak B/1% SP-1000 Supelco). Acetate was determined by injecting 1 μl of aqueous sample into the GC (2 m × 2 mm glass column packed with 80/120 mesh Carbograph 1AL, Alltech). H_2 was determined by injecting 0.5 ml of headspace gas into a GC Autosystem Perkin Elmer, equipped with a molecular sieve column and a thermal conductivity detector (TCD). Standards for PCE, TCE, *trans*-DCE, VC, ETH, CH_4, and H_2 were prepared by adding a known amount of each compound to a serum bottle with the same headspace to liquid ratio as the samples being analyzed (Gossett 1987).

Total solids (TS) and volatile solids (VS) were determined according to Standard Methods (APHA 1994) using a GF/C filter and triplicate controls. Chloride determination was performed by ion-chromatography (Dionex DX-100, Ionpac As9-Sc column).

Chemicals

All the chemicals used to prepare analytical standards and feed solutions were of analytical grade. Liquid PCE, TCE, and *trans*-DCE were purchased from Aldrich. VC, ETH, hydrogen and methane gases (99+%) were purchased from Scott Speciality Gases.

Results

Enrichment of the microbial consortium

Evidence of RD activity by the microbial consortium was detected 2 months after inoculation. After 3 months of enrichment, the consortium was dechlorinating PCE mainly to TCE and to DCE, with more reduced end products being absent (data not shown). The rate of PCE dechlorination gradually increased with time and shifted toward more reduced end-products: after 4 months of enrichment the consortium was completely dechlorinating a dose of PCE (0.4 mM) to ETH in approximately 70 h; after 7 months of enrichment, complete transformation of the added PCE (0.4 mM) to ETH was achieved in 45 h (data not shown).

Once stable RD to ETH was established, the microcosm was transferred into a 2-l serum bottle reactor thus making a larger volume of culture available for performing parallel batch experiments. This set of experiments consisted of 16 parallel batch tests (see materials and methods) comprising duplicates of 8 different combinations of added electron donors (CH_3OH or H_2 or none) and acceptors (PCE or VC or none) so that different microbial activities (i.e., dechlorinating, methylotrophic, hydrogenophilic or acetoclastic) were allowed or excluded in the consortium.

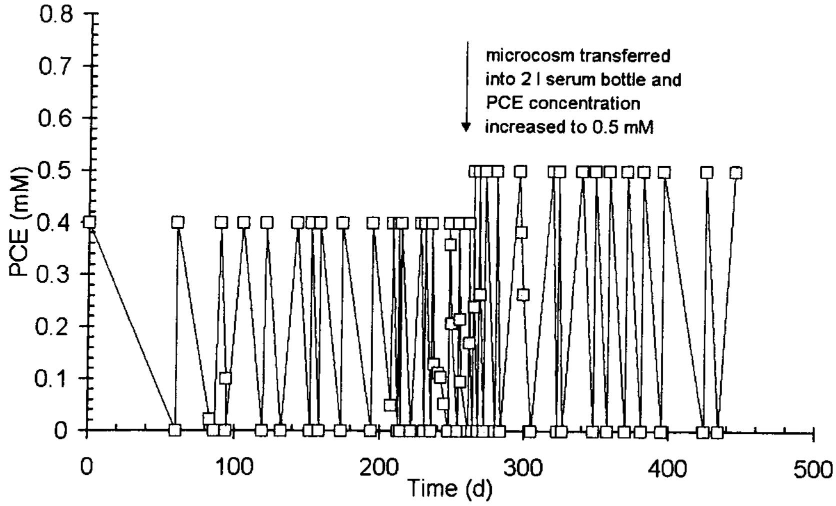

Figure 1. Long-term operation of the consortium with respect to PCE.

Simultaneous characterization of microbial activities (11 months enriched consortium)

Reductive dechlorination

PCE and VC dechlorination with methanol

Figure 2a and b shows a typical batch test where PCE was added in the presence of methanol as electron donor (CH_3OH/PCE batch test), as in the acclimation/enrichment procedure. PCE was mainly dechlorinated to ETH passing through intermediate accumulation of VC, and TCE and DCE remained at a low concentration during the whole experiment. ETH formation started from the beginning of the test, and in the presence of PCE (3.7 ± 2.4 μmol l^{-1} h^{-1}), even though ETH formation rate increased (6.6 ± 4.5 μmol l^{-1} h^{-1}) when PCE was depleted (70 h). At the end of the experiment (150 h), the mass balance (on molar basis) showed that approximately 90% of the initial PCE was recovered in form of ETH and 10% in form of VC. Little fraction of methanol was used for RD (5% on reducing equivalent basis), this result being expected since methanol was added in excess of the amount required for complete dechlorination of PCE.

When VC (0.5 mM) was added in place of PCE (CH_3OH/VC batch tests) to the PCE/methanol enriched consortium, it was dechlorinated to ETH without an initial lag period (Figure 2c and d). After 20 h, a dose of PCE (0.5 mM) was added and a decrease in ETH formation rate was observed: The maximum ETH formation rate was 7.6 ± 1.2 μmol l^{-1} h^{-1} before PCE spike then dropped to 2.2 ± 0.1 μmol l^{-1} h^{-1}. These values are similar to those reported above for the CH_3OH/PCE batch tests, in absence or in presence of PCE respectively (Table 1). These results confirm that ETH formation was possible, though partially inhibited, in the presence of PCE. Alternatively, the VC formation rate from PCE (methanol/PCE tests) was higher than VC consumption rate even in the absence of PCE (methanol/VC tests). Therefore, VC will accumulate, independently from further negative effect due to PCE inhibition on VC RD.

Maximum utilization and formation rates for the different compounds in CH_3OH/PCE and CH_3OH/VC batch tests are summarized in Table 1.

PCE and VC dechlorination with H_2 external addition

In order to evaluate if H_2 was the true electron donor for dechlorination in the PCE/methanol enriched culture, H_2/PCE batch tests were performed. In the H_2 batch tests 15% of the bottle headspace was replaced by molecular hydrogen, resulting in an initial liquid concentration of approximately 120 μM. This value is 3 orders of magnitude greater than the K_S value for PCE dechlorinaton with molecular hydrogen (140 nM), reported by Smatlak et al. (1996), and 4 orders of magnitude greater than the range of K_S values for PCE dechlorinaton with molecular hydrogen (9–14 nM) reported by Ballapragada et al. (1997). Therefore, it was not expected that H_2 would limit dechlorination rate.

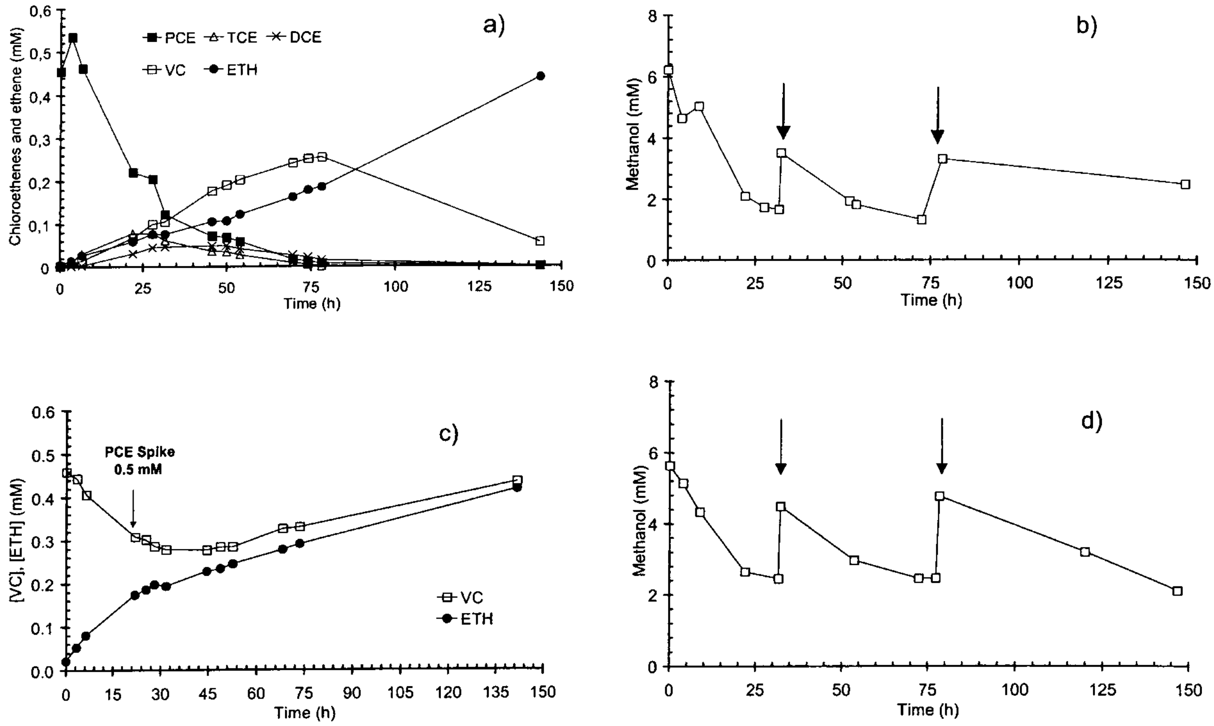

Figure 2. Reductive dechlorination of PCE and VC with methanol as electron donor. Time course of PCE dechlorination (a), and methanol consumption (b) during the CH_3OH/PCE batch test (the arrows indicate re-addition of methanol to maintain it at non limiting concentration). Time course of VC dechlorination (c), and methanol consumption (d) during the CH_3OH/VC batch test (the arrows indicate re-addition of methanol to maintain it at non limiting concentration). PCE was added after 20 h, to study the interaction between the presence of PCE and VC dechlorination.

The time course of PCE dechlorination with H_2 is reported in Figure 3a and b. Similarly to what was observed with methanol, PCE was mainly converted to ETH, passing through intermediate accumulation of VC, and TCE and DCE concentration remained below 0.1 mM during the test. Also in this case ETH formation was observed from the beginning of the experiment at a rate of 5.8 ± 4.9 μmol l^{-1} h^{-1}. This rate increased to 11.8 ± 5.1 μmol l^{-1} h^{-1} after PCE depletion. At the end of the experiment (150 h) mass balance showed that the initial PCE was stoichiometrically dehalogenated to ETH. In the H_2/PCE batch experiments, about 25% of the reducing equivalents from hydrogen were channelled to dechlorination products. Though quite higher than those seen in methanol/PCE tests, this percentage clearly shows that other microbial activities are involved in H_2 consumption.

The results of H_2/VC dechlorination batch tests (Figure 3c and d) were similar to the CH_3OH/VC batch tests where VC was rapidly dechlorinated to ETH, and ETH formation was partially inhibited after PCE addition. Maximum utilization and formation rates of different compounds for H_2/PCE and H_2/VC batch tests are summarized in Table 1. RD maximum rates were very similar independently from the electron donor used (CH_3OH or H_2).

PCE dechlorination with neither methanol nor H_2

PCE batch tests with neither methanol nor H_2 added were performed in order to verify whether PCE RD could be sustained by acetate, which was already present in the medium at initial concentration of about 1.5 mM. These tests showed that after 150 h of incubation less than 10% of initial PCE was dechlorinated as determined by daughter products formation (Table 1). Other than acetate, yeast extract (10 mg l^{-1}) or H_2 produced from endogenous fermentation could also have contributed to provide the reducing equivalents used for partial PCE dechlorination. Overall, this PCE RD was quite slower than with the presence of CH_3OH or H_2.

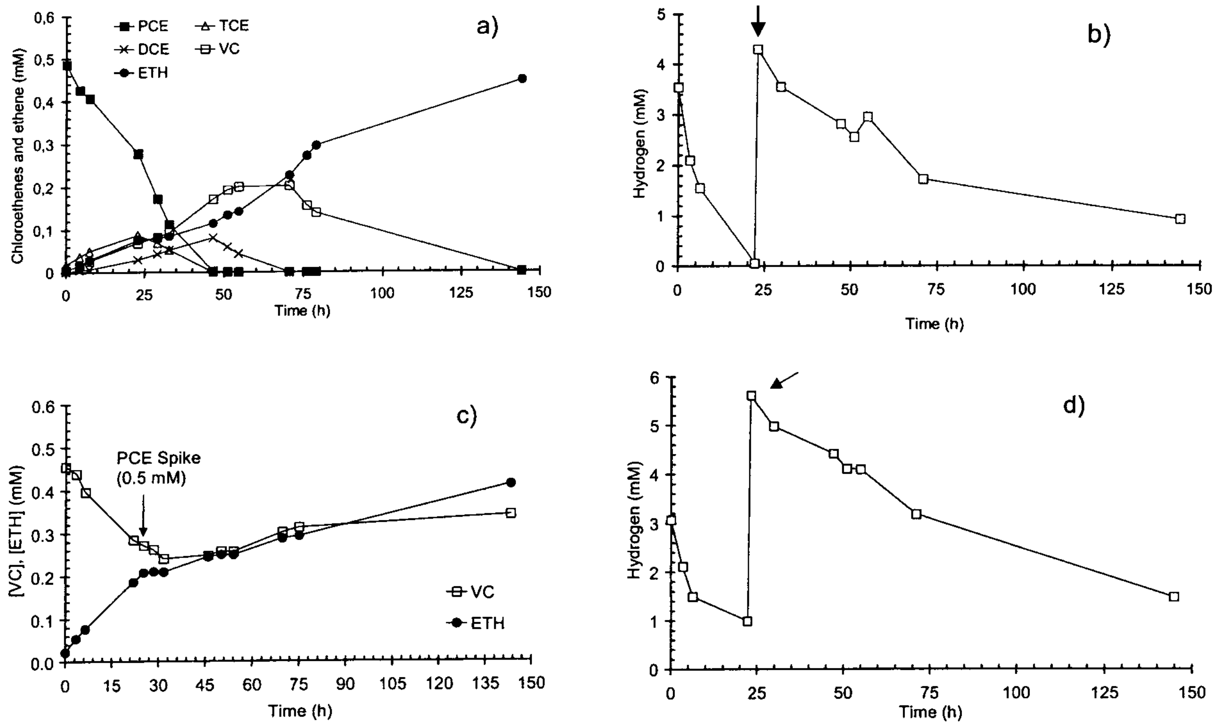

Figure 3. Reductive dechlorination of PCE and VC with H_2 as electron donor. Time course of PCE dechlorination (a), and H_2 consumption (b) during the H_2/PCE batch test (the arrows indicate re-addition of H_2 to maintain it at non limiting concentration). Time course of VC dechlorination (c), and H_2 consumption (d) during the H_2/VC batch test (the arrows indicate re-addition of H_2 to maintain it at non limiting concentration). PCE was added after 20 h, to study the interaction between the presence of PCE and VC dechlorination.

Methanogenic activity

Methane formation from acetate

Figure 4 shows the concentration of acetate and methane over time in a typical acetate batch test. In these tests neither electron acceptors (PCE or VC) nor electron donors (methanol or H_2) were added into the medium, and acetate was already present in the medium at residual concentration of about 1.5 mM.

Under these conditions it is not likely that methane formation rates were at the maximum (as also shown from the observed decrease of rate during time), being acetate concentration in the range of K_S value reported for acetoclastic methanogens (Pavlostathis & Giraldo-Gomez 1991). Accordingly, the acetate was utilized very slowly (initial degradation rate 0.057 ± 0.029 μmol l^{-1} h^{-1}) and little methane was formed (initial formation rate 0.055 ± 0.035 μmol l^{-1} h^{-1}).

In the presence of PCE (acetate/PCE batch tests), acetate degradation and methane formation were completely inhibited, with an average acetate degradation rate of 0.0 ± 0.1 μmol l^{-1} h^{-1} and an average methane formation rate of 0.0 ± 0.1 μmol l^{-1} h^{-1}.

Methane formation with H_2 external addition

In the H_2 batch tests (no PCE or VC added to the medium), H_2 was externally added to the acetate which was already present in the medium. Therefore methane formation was due to both substrates. Figure 5a shows the time course of methane and hydrogen concentrations in a typical H_2 batch test. Methane was formed only during the first 25 h. Although hydrogen was still present in the serum bottle, methane concentration did not increase after hour 25.

On the other hand, hydrogen continued to be consumed even after methane formation stopped, indicating the presence of another H_2-utilizing process (other than RD due to the absence of chlorinated substrates). This hydrogen-consuming metabolism was later identified as homoacetogenesis (discussed in the following paragraph). Initial methane formation rate (calculated during the first 25 h of the test) was higher than that observed in the presence of the sole acetate (average value of duplicate experiments 6.7 ± 3.4 μmol h^{-1} l^{-1}). This increased methanogenesis could be due to the presence of hydrogenotrophic methanogens. However, it is possible that an in-

Table 1. Summary of maximum utilization or formation rates in batch tests (average values and related standard deviation for duplicate experiments.

Batch tests	Maximum utilization rate of (μmol l^{-1} h^{-1})		Maximum formation rate[f] of (μmol l^{-1} h^{-1})	
	Electron donor[a]	Electron acceptor[b]	VC	ETH[c]
PCE + CH_3OH	150.2 ± 21.6	13.1 ± 0.2	5.0 ± 1.9	3.7 ± 2.4 (6.6 ± 4.5)
VC + CH_3OH	147.5 ± 14.8	6.0 ± 1.5	–	2.2 ± 0.1 (7.6 ± 1.2)
CH_3OH	146.5 ± 7.8	–	–	–
PCE + H_2	279.0 ± 53.7	14.2 ± 5.2	5.0 ± 1.8	5.8 ± 4.9 (11.8 ± 5.1)
VC + H_2	268.0 ± 26.9	7.9 ± 0.1	–	2.5 ± 0.8 (7.7 ± 0.6)
H_2	230.0[e]	–	–	–
PCE + Acetate[d]	0.0 ± 0.1	1.4 ± 0.1	0.0 ± 0.1	0.4 ± 0.1
Acetate[d]	2.4 ± 3.8	–	–	–

[a] The electron donor is methanol, hydrogen, or acetate as specified in the first column.

[b] The electron acceptor is PCE or VC as specified in the first column.

[c] The values in brackets are the maximum formation rates of ethene in the absence of PCE.

[d] In acetate and acetate/PCE batch tests, acetate was not added but was already present in the medium since it had accumulated during culture enrichment.

[e] A technical problem occurred in the measurement of H_2 in one of the two duplicate tests, therefore a single value without standard deviation is reported.

[f] Maximum formation rate of methane was not calculated, as specified in the Subsection 2.2.

crease in acetate concentration due to homoacetogenesis eventually caused an increase in acetoclastic methanogenic activity.

Figure 5b shows the hydrogen and methane concentration over time in a typical H_2/PCE batch test. Again methane formation occurred only during the first 25 h and then declined (methane formation in H_2/VC tests had a similar profile, data not shown). Alternatively, H_2/PCE and H_2/VC batch tests results show methanogenesis was partially inhibited with respect to H_2 batch tests (methane formation rate in H_2/PCE and H_2/VC batch tests were 2.1 ± 0.8 and 2.0 ± 1.9 μmol l^{-1} respectively).

Methane formation with methanol

Figure 6a and b shows the time course of methanol and methane concentration during a CH_3OH and CH_3OH/PCE batch test respectively. As for acetate or H_2 tests, methane formation rate steadily decreased during the test. However, when methanol was added (CH_3OH batch test with no PCE or VC), the initial methane formation rate (8.4 ± 1.1) was higher than in corresponding tests in the presence of acetate (acetate batch test with no PCE or VC) or acetate and hydrogen (H_2 batch test with no PCE or VC) (Figure 7). Moreover, methanogenesis from methanol did not appear to be inhibited by RD as similar methane formation was observed both in the presence and in absence of chlorinated ethenes (Figure 6a and b). This indicates that the microorganisms involved in methane formation from methanol were different from those involved in methane formation from acetate or H_2.

Acetogenic activity

Acetate was present in the medium after each methanol/PCE feeding during consortium acclimation/enrichment. Therefore, it was initially assumed that methanol was partially converted to acetate by methanol utilizing acetogens. In the first set of batch tests acetate was not measured, in order to minimize sampling of the liquid phase. However, tests with molecular hydrogen had shown that most H_2 consumption could not be explained either in terms of dechlorination or of hydrogenotrophic methanogenesis. This could indicate the presence of another H_2-utilizing process (i.e., homoacetogenesis) besides

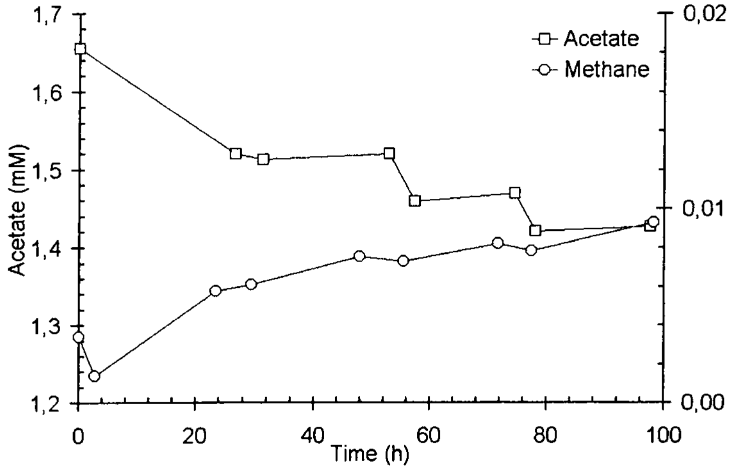

Figure 4. Time course of acetate and methane concentration in a typical acetate batch test.

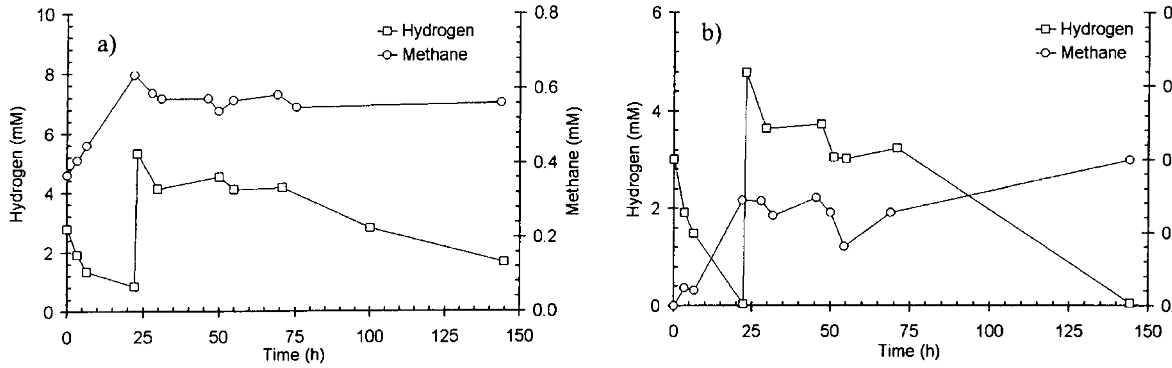

Figure 5. Methane production in a typical H_2 batch test (a) and in typical H_2/PCE batch test (b).

methanogenesis and dechlorination. In order to confirm the presence of homoacetogenic activity, the H_2 batch tests were repeated, and acetate was measured (Table 2). As a control experiment, a CH_3OH/PCE batch test was conducted in parallel, to verify that the microcosm behaved similarly to the previous set of experiments.

Acetogenesis from methanol

Figure 8 shows acetate accumulation and methanol consumption in a CH_3OH/PCE batch test. Acetogenesis accounted for about 72% of reducing equivalents from methanol (calculated from amount of acetate formed and methanol consumed at the end of the test). Therefore it was confirmed the initial assumption that acetogenesis was the main microbial activity in the consortium.

These methanol/PCE batch tests made it possible to elucidate the flow of reducing equivalents from methanol to end products (methane, ETH, and acetate). Figure 9 shows that, at the end of the CH_3OH/PCE batch test, reducing equivalents from methanol were channelled toward ETH (4%) and methane (15%). By noting that 72% of reducing equivalents were directed toward acetate, an almost complete recovery was obtained, with the residual reducing equivalents probably being used for cell growth.

Acetogenesis from H_2

Figure 10 shows acetate production and hydrogen consumption during the H_2 batch test. The test confirmed the presence of acetetogenesis from hydrogen: acetate formed accounted for about 60% of reducing equivalents from hydrogen.

As reported above, for H_2 batch tests, the initial H_2 concentration in the liquid phase was 120 μM which is in great excess with respect to K_s values

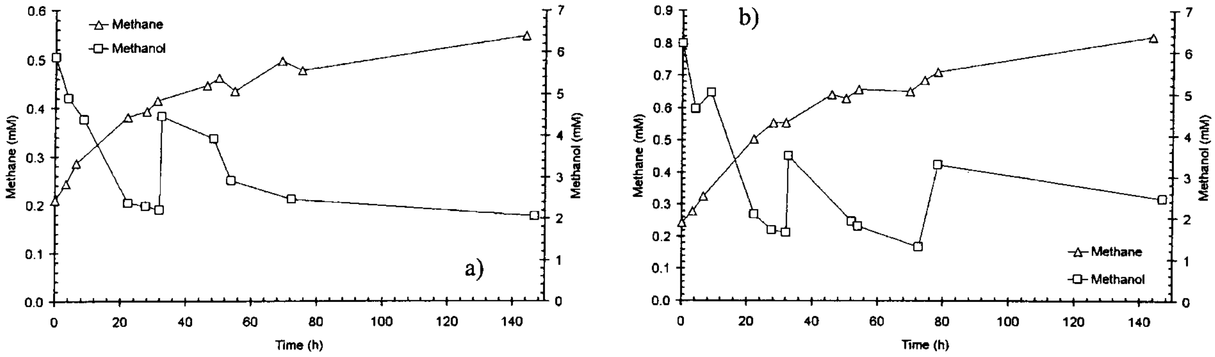

Figure 6. (a) Methane formation and methanol consumption during a CH_3OH batch test. (b) Methane formation and methanol consumption during a CH_3OH/PCE batch test.

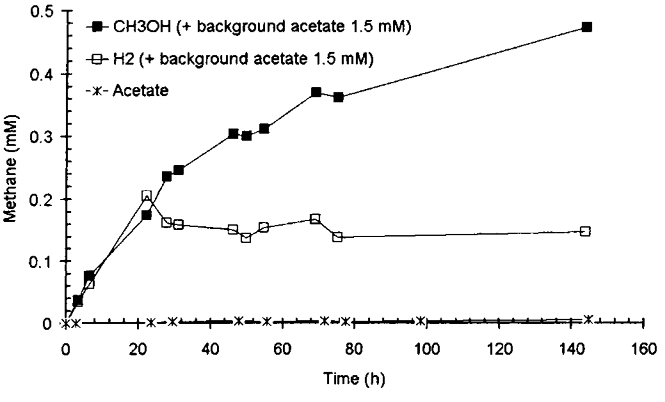

Figure 7. Methane formation in acetate, H_2, and methanol batch tests (average value of duplicate experiments).

of 1400–4000 nM as reported for homoacetogenesis (Kotsyurbenko et al. 2001). Thus, H_2 concentration was not rate limiting and the obtained acetate formation rate was the maximum, though not necessarily corresponding to the actual rate of homocetogenesis when the microbial consortium is fed with methanol.

It is also noteworthy that the maximum acetate formation rate from H_2 was quite lower than acetate formation rate in the presence of methanol (28.1 vs 102.2 μmol l^{-1} h^{-1}, respectively). Thus, in methanol/PCE batch tests most acetate was formed from methanol fermentation.

Discussion

Tables 1 and 2 show a summary of the results obtained in the parallel batch tests aimed at characterizing the main microbial activities in the sediment microcosm.

The PCE/CH_3OH fed consortium originating from sediments was able to completely dechlorinate PCE to ETH by using either methanol or H_2 as electron donor. As commonly reported (Vogel et al. 1987), VC was the slowest to be degraded and therefore the main intermediate that accumulated in PCE dechlorination to ETH. ETH formation from VC in absence of PCE was faster (6.6 ÷ 7.6 μmol l^{-1} h^{-1} with methanol and 7.7 ÷ 11.8 μmol l^{-1} h^{-1} with hydrogen) than when PCE was present (2.2 ÷ 3.7 μmol l^{-1} h^{-1} with methanol and 2.5 ÷ 5.8 μmol l^{-1} h^{-1} with hydrogen). However, the RD of VC to ethene started immediately in the presence of PCE so VC accumulation never reached more than 50% of the initial PCE concentration. VC accumulation in contaminated groundwater as a result of reductive dechlorination of PCE is of concern due to the toxic nature of this compound. Thus, a high VC dechlorination rate is a prerequisite for the use of RD as a bioremediation process.

In the CH_3OH fed enriched consortium, H_2 could readily replace methanol for PCE or VC dechlorination with no lag and similar rate. It is noteworthy that the methanol/PCE enriched consortium had never been fed before with molecular hydrogen, thus this

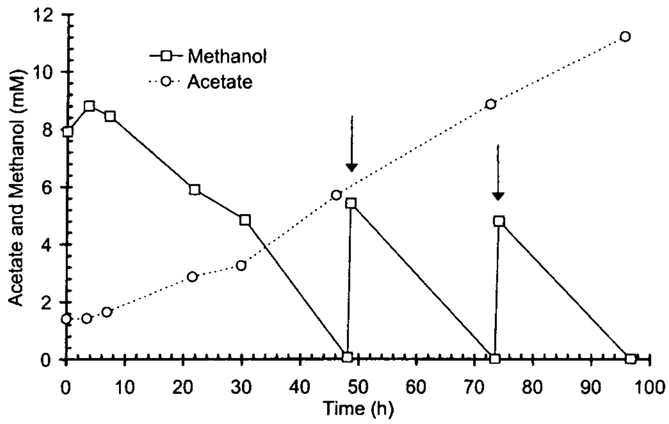

Figure 8. Methanol consumption and acetate accumulation in a CH_3OH/PCE batch test. The arrows indicate re-addition of methanol.

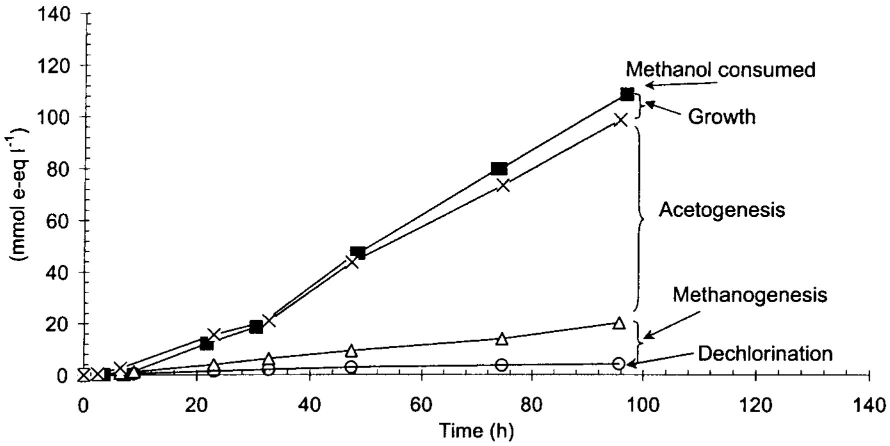

Figure 9. Flow of reducing equivalents towards different metabolisms, in a CH_3OH/PCE batch test.

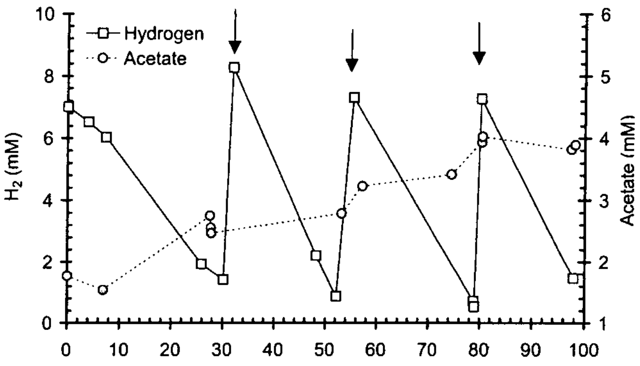

Figure 10. H_2 consumption and acetate accumulation in a H_2 batch test. The arrows indicate re-addition of H_2.

Table 2. Homoacetogenic activity in H_2 batch test (the control CH_3OH/PCE batch test was performed in parallel to verify that the culture behaved similarly to the previous set of experiments.

Batch tests	Maximum utilization rate of (μmol l^{-1} h^{-1})		Maximum formation rate of (μmol l^{-1} h^{-1})		
	Electron donor	Electron acceptor	VC	ETH	Acetate
PCE + CH_3OH (control test)	153.6	13.4	4.8	2.4 (4.2)	102.2
H_2	197.0	–	–	–	28.1

observation supports the hypothesis that in the methanol/PCE enriched consortium, the actual source of reducing equivalents for RD is the H_2. This finding is consistent with the model of DiStefano et al. (1992), in which H_2 is the primary electron donor for reductive dechlorination of PCE in a methanol/PCE fed culture. Since methanol was the only electron donor (with the exception of little yeast extract) given to the culture from the start of the acclimation, it is likely that H_2 was produced from methanol utilising microorganisms.

The formation of H_2 by the methanol-utilising acetogen *Sporomusa acidovorans* has already been reported in combination with H_2 interspecies transfer to different hydrogenophilic microorganisms (i.e., sulfate reducers, and hydrogenophilic methanogens) (Cord-Ruwisch & Ollivier 1986).

Once the role of H_2 as the actual electron donor for RD is assumed, it has also to be pointed out that, PCE or VC dechlorination rates with H_2 (14.2 and 7.9 μmol l^{-1} h^{-1} respectively) were very similar to those observed with methanol (13.1 and 6.0 μmol l^{-1} h^{-1}). This indicates that hydrogen production during methanol fermentation was not rate-limiting to PCE or VC dechlorination. It also suggests that methylotrophic H_2-producing microorganisms were able to produce hydrogen at partial pressure quite higher than RD Ks (ranging from 9 to 140 nM).

Similar results have been reported by Carr & Hughes (1998) who did not observe changes in the extent and rate of PCE dechlorination by adding H_2 to methanol or lactate-fed columns. Different results have been reported by Rossetti et al. (2003), who observed a threefold increase in PCE dechlorination rate by a metylotrophic consortium when molecular hydrogen was supplied instead of methanol for PCE dechlorination. Their observation indicates that the hydrogen formation from methanol was the rate limiting step in the overall reaction.

Acetate did not sustain PCE or VC dechlorination, confirming that hydrogen or a hydrogen- generating substrate is required to maintain activity. Because the methanol utilization rate was approximately the same (with or without chlorinated compounds), methanol-utilizing acetogens were probably not inhibited by the presence of PCE or VC.

Homoacetogenesis was the predominant pathway of hydrogen utilization in H_2 batch tests (up to 60% on reducing equivalent basis). As for dechlorinating microorganisms, the presence of homoacetogens in the methanol/PCE enriched consortium (with no previous addition of hydrogen), confirms that H_2 is usually present in the medium, and is likely produced from methanol. Hydrogen threshold concentration for homoacetogenesis has been reported as 400 nM (Yang & McCarty 1998), such a hydrogen liquid phase concentration is then required to sustain the growth of homoacetogenic microorganisms. This confirms that there was an intense H_2 production in the PCE/CH_3OH enriched consortium to allow both PCE RD at non-limiting concentration (much above 140 nM, the highest K_S value on hydrogen reported for PCE RD) and homoacetogenesis (at least above 400 nM). Table 3 shows the proposed catabolic H_2-releasing reaction of methanol (He et al. 2002) and indicates the free energy change associated with this reaction under standard conditions. Also indicated is the free energy change which was calculated at H_2 concentration of 400 nM (i.e., H_2 threshold concentration for homoacetogenesis). The reaction is energetically favorable at that H_2 concentration, which confirms that methanol metabolism, with resulting hydrogen production, can sustain homoacetogenic activity and to a greater reason the RD.

Table 3. Catabolic H_2-releasing reactions of methanol (adapted from He et al. 2002).

H_2-releasing reaction	ΔG^{0a} (kJ/rxn)	$\Delta G'^{b}$ (kJ/rxn)
$CH_3OH + 2H_2O \rightarrow HCO_3^- + H^+ + 3H_2$	+23.03	−31.74

[a] Gibbs free energy changes under standard conditions (25 °C, concentrations of reactants at 1 M or 1 atm) at pH 7.0.

[b] Calculations of free energy changes at 25 °C and pH 7.0 according to equation $\Delta G' = \Delta G^0 + \mathrm{RT}\ln([\text{products}]/[\text{reactants}])$ and based on the following concentrations: methanol, 6 mM; HCO_3^-, 30 mM; H_2, 400 nM (i.e., hydrogen threshold concentration for homoacetogenesis).

Because both RD in H_2/PCE batch tests and homoacetogenesis in H_2 batch tests were at their maximum rates, the concentration of responsible microbial groups in the overall microbial consortium can be (roughly) estimated, as follows, from reported maximum rates of pure cultures.

In regard to RD, the dechlorination rate from PCE to VC and ETH has been reported to be equal to 69.0 ± 10.5 nmol of chloride released min^{-1} mg^{-1} of protein in *D. ethenogenes* strain 195 (Maymò-Gatell et al. 1997). If it is assumed that the main conversion of PCE is to VC and the TSS is 55% protein, this dechlorination rate would correspond to 18.2 ± 2.7 μmol VC mg^{-1} TSS d^{-1}. Assuming that the dechlorinating microorganisms in our culture have similar kinetics to strain 195, we roughly estimated that the dechlorinators responsible for the observed PCE reduction would be about 6.6 mg TSS/l.

Regarding homoacetogenesis, an acetate formation rate has been reported to be equal to 74.8 μmol acetate mg^{-1} TSS d^{-1} for *Acetobacterium woodii* (Peters et al. 1998). Assuming that the homoacetogens in our culture have similar kinetics to *Acetobacterium woodii*, we roughly estimated that the homoacetogens responsible for the observed acetate production would be about 9.0 mg TSS/l.

Though assumptions have been made and extrapolation of kinetic data from pure to mixed cultures is always dubious, this estimation suggests that PCE dechlorinating microorganisms and homoacetogens have comparable concentration in the microbial consortium. This indicates that homoacetogens, similar to dechlorinating microorganisms, are also using hydrogen at high rate during methanol fermentation.

The presence of homoacetogenic activity has also been reported in a dechlorinating consortium enriched on benzoate as the main electron donor (Yang & McCarty 1998). When hydrogen was added as electron donor for *cis*-DCE dechlorination, 88% of reducing equivalents from H_2 was used for acetate production. In contrast, when the H_2 level dropped below 11 nM, dechlorination was the main hydrogen consuming process, this finding is expected as the hydrogen threshold concentration for *cis*-DCE dechlorination is lower than that for homoacetogenesis.

Methanogenesis was observed under most conditions in this study, though at variable extent and rate. Under all conditions a time decrease of methane formation rate was observed which could be due to the high sensitivity of methanogens to slight fluctuations of pH following acetate accumulation (from either hydrogen or methanol conversion). The highest methane formation rates were observed in the presence of methanol where possible contributions could be more directly due to methylotrophic microorganisms than to acetoclastic and hydrogenophilic microorganisms. Indeed, both methanogenesis from H_2 and acetate (at least at low concentration) were inhibited from PCE or VC whereas no inhibition was observed in the presence of methanol, and supporting the idea that most methane was directly produced from methanol by methylotrophic microorganisms in the methanol/PCE enriched consortium. However, the comparison of methane production in batch tests with or without methanol did not allow for a clear picture of the system (also due to non linear profile of methane during most of the tests), thus requiring further experimentation.

In conclusion, the Figure 11 shows an estimation of overall microbial activities (in terms of electron flow) in the methanol/PCE enriched consortium, also including flux to and from the key-intermediate, hydrogen. Based on this picture, most methanol (72%) was converted into acetate, both directly (not less than 52.2%) and through hydrogen (not more than 19.8% based on H_2 batch tests, at not-limiting H_2 concentration). Hydrogen formation accounted for no more than 25.4% of electron flow from methanol. Main competition for hydrogen was exerted by homoacetogenesis

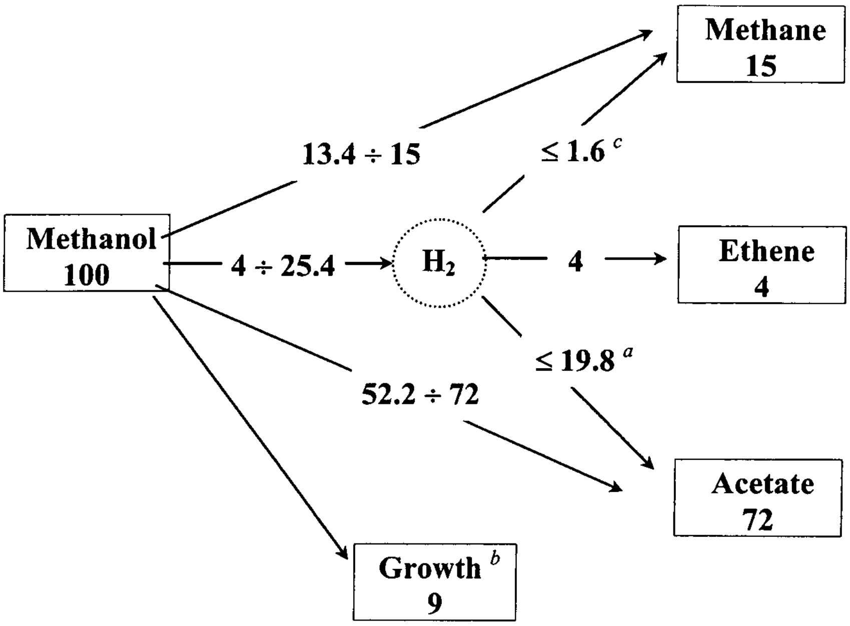

Figure 11. Distribution (%) of reducing equivalents from methanol toward different metabolic activities in the methanol/PCE enriched consortium, also including flows toward and from its key-intermediate, the hydrogen. [a] Upper value (19.8%) is the maximum H_2 flow toward acetate (at not-limiting H_2 concentration) as determined from H_2 batch tests. [b] Growth was indirectly estimated as the lacking fraction in the electron balance. [c] Upper value (1.6%) is the maximum H_2 flow toward methane (at not-limiting H_2 concentration) as determined from H_2 batch tests.

(up to 19.8% based on H_2 batch tests, at no limiting H_2 concentration) and RD (4%), being hydrogenophilic methanogenesis quite less important (no more than 1.6%, based on H_2 batch tests).

From a practical point of view, it is noteworthy that the enriched consortium was able to degrade VC into ETH at a rate high enough with respect to VC formation rate from PCE, so allowing that VC never accumulated at more than 50% of PCE initial concentration and that ETH was the only end product of RD. This behaviour is promising for the application of RD to remediation of PCE contaminated sites since allow to achieve complete detoxification without accumulation of the harmful intermediate VC.

Acknowledgments

This research was financially supported by the Ministry of the University and Research (MIUR) (COFIN 1999) and the National Research Council, CNR, (Progetto Strategico Ambiente e Territorio).

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