Journal of Hazardous Materials 197 (2011) 204–210

Contents lists available at SciVerse ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Use of γ-hexachlorocyclohexane as a terminal electron acceptor by an anaerobic enrichment culture

Vijai Elango[a,*], Harry D. Kurtz Jr.[b], Christina Anderson[c], David L. Freedman[c]

[a] Hazardous Substance Research Center/South and Southwest, Louisiana State University, 3221 Patrick Taylor Hall, Baton Rouge, LA 70803, USA
[b] Department of Genetics and Biochemistry, Clemson University, 100 Jordan Hall, Clemson, SC 29634, USA
[c] Department of Environmental Engineering and Earth Sciences, Box 340919, Clemson University, Clemson, SC 29634-0919, USA

ARTICLE INFO

Article history:
Received 14 July 2011
Received in revised form 5 September 2011
Accepted 20 September 2011
Available online 29 September 2011

Keywords:
Lindane
Hexachlorocyclohexane
Organohalide respiration
Reductive dechlorination

ABSTRACT

The use of γ-hexachlorocyclohexane (HCH) as a terminal electron acceptor via organohalide respiration was demonstrated for the first time with an enrichment culture grown in a sulfate-free HEPES-buffered anaerobic mineral salts medium. The enrichment culture was initially developed with soil and groundwater from an industrial site contaminated with HCH isomers, chlorinated benzenes, and chlorinated ethenes. When hydrogen served as the electron donor, 79–90% of the electron equivalents from hydrogen were used by the enrichment culture for reductive dechlorination of the γ-HCH, which was provided at a saturation concentration of approximately 10 mg/L. Benzene and chlorobenzene were the only volatile transformation products detected, accounting for 25% and 75% of the γ-HCH consumed (on a molar basis), respectively. The enrichment culture remained active with only hydrogen as the electron donor and γ-HCH as the electron acceptor through several transfers to fresh mineral salts medium for more than one year. Addition of vancomycin to the culture significantly slowed the rate of γ-HCH dechlorination, suggesting that a Gram-positive organism is responsible for the reduction of γ-HCH. Analysis of the γ-HCH dechlorinating enrichment culture did not detect any known chlororespiring genera, including *Dehalobacter*. In bicarbonate-buffered medium, reductive dechlorination of γ-HCH was accompanied by significant levels of acetogenesis as well as methanogenesis.

1. Introduction

Use of lindane as an insecticide has resulted in global environmental release of 10 million tons and subsequent contamination of soil, groundwater and the atmosphere [1]. Lindane consists of more than 90% of γ-hexachlorocyclohexane (HCH) plus lower levels of other HCH isomers (α, β and δ), which differ in the orientation of their chlorine atoms (equatorial or axial) [2]. The axially oriented chlorine atoms are known to be active sites for enzymatic degradation and therefore, γ-HCH and α-HCH, each with three axial chlorines, are more easily biodegraded relative to other isomers, including β and δ [2]. Only γ-HCH is known to exhibit insecticidal properties but both lindane and technical grade lindane (10–15% γ-HCH + 85–90% α-, β- and δ-HCH) have been used worldwide as an insecticide. The major isomers in technical grade HCH exhibit different degrees of acute and chronic toxicity [3]. According to the United States Environmental Protection Agency [4], technical grade HCH is a probable human carcinogen and the maximum contaminant level for lindane in drinking water is 0.2 μg/L.

Biodegradation of γ-HCH under anaerobic conditions has been widely reported [2] with pure cultures [5–7], enrichment cultures [5,8,9], and soil slurries [10]. The predominant end products of γ-HCH anaerobic dechlorination are benzene (via three dihaloelimination reactions) and chlorobenzene (CB; via two dihaloelimination reactions and one dehydrohalogenation step) [5,8,11], such that the molar sum of benzene and CB formed is equal to the molar amount of γ-HCH consumed. Other intermediates or products reported from γ-HCH include tetrachlorobenzene, trichlorobenzenes, dichlorobenzenes, tetrachlorocyclohexadiene, and pentachlorocyclohexadiene [12]. The requirement for an exogenous electron donor to sustain γ-HCH dechlorination is well established [2,5,7,12,13].

Pure cultures of the sulfate reducing bacteria *Desulfovibrio gigas*, *D. africanus* and *Desulfococcus multivorans* are able to dechlorinate γ-HCH in the presence of sulfate [5,11]. However, γ-HCH dechlorination was also observed with enrichment cultures in the absence of sulfate [5,8], suggesting that sulfate reduction is not a requirement for anaerobic dechlorination of γ-HCH. A co-culture of *Dehalobacter* and *Sedimentibacter* was shown to reductively dechlorinate β-HCH. The co-culture was maintained through successive transfers over three years with hydrogen as the electron donor and β-HCH as the only terminal electron acceptor, leading van

* Corresponding author. Tel.: +1 864 650 2444; fax: +1 864 656 0672.
E-mail address: velango@g.clemson.edu (V. Elango).

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doi:10.1016/j.jhazmat.2011.09.080

Doesburg et al. [9] to conclude that β-HCH dechlorination occurs via organohalide respiration. The same culture was also able to dechlorinate γ-HCH, although use of γ-HCH as a terminal electron acceptor was not demonstrated. Ohisa et al. [14] observed ATP formation during reductive dechlorination of γ-HCH by *Clostridium rectum* strain S-17. However, this occurred with cells pregrown on a rich medium in the absence of γ-HCH; use of γ-HCH as the sole terminal electron acceptor in successive transfers of the culture was not evaluated.

Numerous anaerobic bacteria have been identified that respire chlorinated aliphatic and aromatic compounds [15–18]. A variety of approaches have been used to demonstrate the occurrence of organohalide respiration. For example, with *Dehalococcoides ethenogenes* strain 195, an increase in cell counts and protein occurred during organohalide respiration of tetrachloroethene [19]. With *Dehalococcoides* sp. strain BAV1, organohalide respiration of vinyl chloride was demonstrated by an increase in 16S rRNA gene copies [20]. In mixed cultures, organohalide respiration of various compounds was demonstrated based on the fraction of electron equivalents from the electron donor used for reductive dechlorination (f_e); f_e values of 0.6–0.7 were indicative of organohalide respiration, while much lower values indicated that dechlorination was cometabolic [21]. To our knowledge, reductive dechlorination of γ-HCH has not yet been linked to organohalide respiration and microbes with such capability have not been identified. The objective of this study was to demonstrate that γ-HCH can be used as a terminal electron acceptor via organohalide respiration.

2. Materials and methods

2.1. Chemicals and media

The sources and purity of chemicals used were: γ-HCH (99%) from Sigma–Aldrich; CB (99.5%) from TCI America; benzene (99%) from Fisher Scientific; anhydrous sodium acetate (99%) from EM Science; sodium lactate from Mallinckrodt Baker, Inc. (60%, w/w, syrup); yeast extract from Difco Laboratories; vancomycin (biotechnology grade) from Sigma–Aldrich; HEPES from VWR; hydrogen (99.99%) and methane (99%) from National Welders. All other chemicals used were reagent grade or equivalent in purity.

Three types of mineral salts medium (MSM) were used. MSM-1 was buffered at pH 6.5–7.2 with sodium bicarbonate in equilibrium with a headspace of 30% CO_2 and 70% N_2 and yeast extract was provided as a source of vitamins and growth factors [22]. MSM-2 was the same as MSM-1 except that $MgSO_4$ was replaced with $MgCl_2$ (in order to remove all sulfate from the medium) and yeast extract was replaced with a defined vitamin mixture [22], to eliminate yeast extract as a potential electron donor; MSM-3 was the same as MSM-2 except it was buffered with 10 mM HEPES rather than bicarbonate [23] and the headspace was purged with high purity N_2 rather than the CO_2/N_2 gas mixture.

2.2. Analytical methods

γ-HCH was analyzed by extracting aliquots of culture twice with hexane [24] and injecting 1 μL of hexane extract onto a Hewlett Packard 5890 Series II Plus gas chromatograph (GC) equipped with an HP 7673 autosampler, ZB 624 capillary column (30 m × 0.53 mm × 3.0 μm film; Zebron) and electron capture detector. Before taking samples, serum bottles were placed upright to allow the particulates to settle and an aliquot of the clarified aqueous phase (200 μL) was transferred to a 2 mL glass vial. Hexane (500 μL) was added and the vial was closed with a screw cap and vortexed (Baxter Scientific Vortex Mixer) for 2 min. The hexane was then transferred to a clean 2 mL glass vial. Additional hexane (500 μL) was added to the first vial and the process was repeated to further extract γ-HCH from the sample. Hexachlorobenzene was added (0.56 ng/sample, dissolved in hexane) to the combined 1 mL of hexane extract, to serve as an internal standard. The injector, oven and detector temperatures were 220, 240 and 250 °C, respectively. Helium (5.5 mL/min) was used as the carrier gas and nitrogen (30 mL/min) was the makeup gas. The detection limit for γ-HCH was 0.2 μg/L.

Methane, benzene and CB were analyzed by injecting headspace samples (500 μL) onto a HP 5890 Series II Plus GC equipped with an RTX 5 column (30 m × 0.53 mm × 1.5 μm film; Restek Corp.) and flame ionization detector. The injector and detector temperatures were 250 and 325 °C, respectively. The oven temperature program was 50 °C for 4 min, increased at 10 °C/min to 80 °C, and hold 2 min. Helium (5 mL/min) was used as the carrier gas and nitrogen (30 mL/min) was the makeup gas. The GC response to a headspace sample was calibrated to give the total mass of compound (M) in that bottle [25]. Assuming the headspace and aqueous phases were in equilibrium, the total mass present was converted to an aqueous phase concentration (Eq. (1)):

$$C_l = \frac{M}{V_l + H_c V_g} \quad (1)$$

where C_l, concentration in the aqueous phase (μM); M, total mass present (μmol/bottle); V_l, volume of the liquid in the bottle (L); V_g, volume of the headspace in the bottle (L); and H_c, Henry's constant ((mol m−3 gas concentration)/(mol m−3 aqueous concentration)) at 23 °C [26]. Aqueous phase detection limits were 1.0 μg/L for benzene and 2.0 μg/L for CB.

Hydrogen was analyzed by injecting headspace samples (100 μL) onto a HP 5890 Series II GC equipped with a thermal conductivity detector and Carbosieve SII 100/120 column (1.0 m × 3.1 mm, Supelco). The injector, oven and detector temperatures were maintained at 200, 105 and 200 °C, respectively. High purity nitrogen was used as the carrier gas (30 mL/min) and reference gas (30 mL/min).

Sulfate was quantified on a Dionex AS50 ion chromatography system equipped with a CD25 conductivity detector, Dionex guard column (AG9-HC, 4 mm × 50 mm) and IonPac® AS9-HC anion-exchange column (4 mm × 250 mm) with 9 mM Na_2CO_3 as the eluant (1.0 mL/min). Lactate and acetate were analyzed on a Waters 600E HPLC system composed of an autosampler (Waters 717 plus), pumping system (Waters 600), a UV/Vis detector (Model 490E) set at 210 nm, and an Aminex® HPX-87H ion exclusion column (300 mm × 7.8 mm; BioRad) with 0.01 N H_2SO_4 as eluant (0.6 mL/min).

2.3. Enrichment cultures

Cultures were grown in 160 mL serum bottles with 100 mL of liquid. They were prepared in an anaerobic chamber containing an atmosphere of approximately 98% N_2 and 2% H_2. γ-HCH was added either dissolved in methanol or as neat crystals. The bottles were sealed with Teflon-faced red rubber septa and aluminum crimp caps. Headspaces were purged with either high purity nitrogen or 30% CO_2 and 70% N_2. Bottles were incubated in the anaerobic chamber under quiescent conditions, in an inverted position.

Four types of enrichment cultures were developed, as summarized in Table 1. Enrichment I was started with groundwater from an industrial site contaminated with α- and γ-HCH, benzene, and chlorinated benzenes. We previously performed a microcosm study with groundwater and soil or crushed dolomite from this site, to compare sequential anaerobic/aerobic versus aerobic/anaerobic bioremediation [27]. Several of the anaerobic soil microcosms exhibited high rates of HCH biodegradation; these were used as

Table 1
Summary of enrichment culture conditions.

	Enrichment culture			
	I	II	III	IV
Liquid	Groundwater	MSM-1	MSM-2	MSM-3
Lactate added	Yes	Initially	No	No
Methanol added	Yes	Initially	No	No
Hydrogen added	No	No	Yes	Yes
Nutrients added	No	Yeast extract	Vitamins	Vitamins
Sulfate added	No	Yes	No	No
Buffer added	No	Bicarbonate	Bicarbonate	HEPES
Headspace	N_2	70% N_2, 30% CO_2	70% N_2, 30% CO_2	N_2

the source of inoculum for enrichment culture I in this study. γ-HCH was delivered dissolved in methanol (0.38 g/L) to provide an initial aqueous concentration of 10 mg/L (3.3 μmol/bottle), which is approximately the saturation level [2]. Lactate was also added (500–600 mg/L). The total initial amount of electron donor exceeded the amount needed for complete dechlorination of the γ-HCH by a factor of 375. Samples of the liquid were removed periodically to measure γ-HCH; when the concentration decreased below 1.0 mg/L, more was added.

Enrichment II was initiated by making a 1% transfer (v/v) from enrichment I to MSM-1. In the course of making eight transfers in MSM-1, use of lactate was stopped, leaving methanol as the only available electron donor; it was also used to deliver the γ-HCH. Samples of the liquid were removed periodically to measure γ-HCH; when the concentration decreased below approximately 1.0 mg/L, more was added. At least 40 mg/L of cumulative γ-HCH was consumed (i.e., at least 4 mg/bottle) before making a transfer to fresh MSM-1.

Enrichment III was prepared by making a 1% transfer (v/v) from enrichment II to MSM-2. At this stage, hydrogen was the only electron donor added; γ-HCH was added as neat crystals rather than dissolved in methanol. Hydrogen (5.0 mL/bottle) was added as a neat gas. Initially, 10 mg of γ-HCH was added; when measurement of benzene + CB indicated that 60–80% of the γ-HCH had been consumed, the bottles were opened in a fume hood and, while purging the headspace with 30% CO_2 and 70% N_2, 30–50 mg of additional γ-HCH was provided and then the bottles were resealed; this process took 15–30 s. Enrichment III was transferred to fresh medium four times (1%, v/v). At least 4 mg/L of benzene and 14 mg/L of CB were formed as γ-HCH dechlorination products before making a transfer to fresh MSM-2. Some of the treatments evaluated with enrichment III included addition of acetate (i.e., 500–600 mg/L) without hydrogen, to determine if acetate could serve as an electron donor; addition of acetate (50–100 mg/L) along with hydrogen, to determine if acetate was needed as a carbon source; and the effect on γ-HCH dechlorination rate in the presence of vancomycin (100 mg/L, based on a previous study by DiStefano et al. [28]), which inhibits cell wall synthesis in gram positive bacteria.

Enrichment IV was prepared by making a 1% transfer (v/v) from enrichment III to MSM-3. This enrichment was transferred to fresh medium (1%, v/v) three times. It was maintained in the same manner as enrichment III; i.e., γ-HCH was added as neat crystals (although N_2 was used to purge the headspace, rather than 30% CO_2 and 70% N_2); hydrogen was the only electron donor added; some of the treatments received acetate as a potential carbon and energy source; and the effect of vancomycin was evaluated. At least 8 mg/L of benzene and 34 mg/L of CB were formed as γ-HCH dechlorination products before making a transfer to fresh MSM-3.

2.4. Electron equivalents for γ-HCH dechlorination

For enrichments III and IV, the fraction of hydrogen consumed for γ-HCH dechlorination was calculated on an electron equivalent (eq) basis, as described by Löffler et al. [21]. The net cumulative amount of hydrogen consumed (i.e., total minus hydrogen loss from autoclaved controls) was plotted against the cumulative amount of benzene + CB formed, using the conversions: 2 μeq/μmol H_2; 4 μeq/μmol CB; and 6 μeq/μmol benzene. The slope of the linear regression provided the fraction of hydrogen used for γ-HCH dechlorination.

2.5. DGGE and cloning analysis

Denaturing gradient gel electrophoresis (DGGE) was performed with samples from several of the enrichment cultures. Descriptions of the methods used for DNA extraction, amplification, DGGE, and sequencing of several of the excised DGGE bands are provided in the Supplementary data. Procedures used to develop clone libraries for several of the enrichment III and IV treatments are also presented in the Supplementary data.

3. Results

3.1. Enrichment in groundwater and MSM-1

Following a lag of several weeks, γ-HCH dechlorination started in enrichment culture I (i.e., in groundwater) and rates increased with subsequent additions of γ-HCH (Fig. 1). Decreases in γ-HCH coincided with increases in benzene and CB; no other volatile

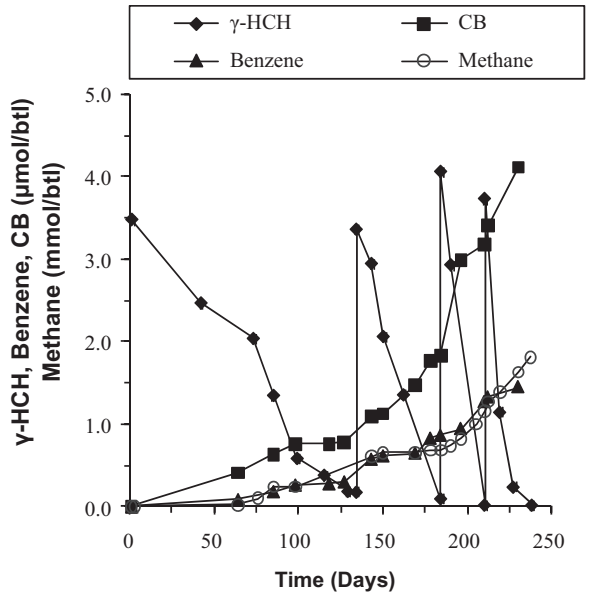

Fig. 1. γ-HCH dechlorination by enrichment culture I (in groundwater) with methanol and lactate as electron donors; each data point represents the average of triplicate bottles; standard deviations for each compound are shown in Supplementary data; btl = bottle.

products were detected, including di- and trichlorobenzenes. Concurrent with dechlorination of γ-HCH was a significant level of methanogenesis. Approximately 10% of the electron equivalents of methanol and lactate added was used for methane formation, versus only 0.06% for γ-HCH dechlorination.

Following transfer (1%, v/v) to MSM-1, a robust level of γ-HCH dechlorination continued in enrichment culture II (i.e., in MSM-1). When methanol was added as the electron donor without lactate, there was no noticeable decrease in the rate of γ-HCH dechlorination, so lactate additions were stopped. During successive transfers, enrichment culture I dechlorinated a total of 80–90 mg/L of γ-HCH at the rate of 1.0–1.5 mg/L/d. Benzene and CB were the only volatile daughter products detected. Methanogenesis continued to be a prominent activity, with greater than 90% of the methanol added as electron equivalents recovered as methane. The sulfate present in MSM-1 (0.50 mM) was also consumed, suggesting the occurrence of sulfidogenesis.

3.2. γ-HCH dechlorination in bicarbonate buffered MSM-2

γ-HCH was rapidly dechlorinated to benzene and CB by enrichment culture III (i.e., in MSM-2) (Fig. 2a). In this version of the media, vitamins replaced the yeast extract, there was no sulfate present and γ-HCH was delivered as neat compound. Benzene and CB were the only volatile daughter products detected, accounting for 87 ± 8.6% of the γ-HCH consumed (25% benzene + 62% CB). The addition of acetate along with hydrogen did not increase the rate of benzene and CB accumulation. Addition of vancomycin strongly inhibited dechlorination of γ-HCH (Fig. 2a). Hydrogen consumption was greatest in the treatments without γ-HCH present, indicating that another use for hydrogen was significant. Acetate accumulated as hydrogen was consumed in all of the treatments without vancomycin. Based on the amount of acetate formed, acetogenesis was the dominant process in enrichment culture III (Table 2). Higher hydrogen consumption in the treatments that did not receive γ-HCH suggested that γ-HCH was at least partially inhibitory to acetogenesis (Fig. 2b; Table 2). Vancomycin significantly inhibited hydrogen consumption, in addition to inhibiting γ-HCH dechlorination; however, the treatments with vancomycin produced methane and consumed acetate, presumptively for methanogenesis (Table 2). The fraction of hydrogen electron equivalents consumed relative to the equivalents needed to form benzene and CB was 0.052 for the treatment with H_2 as electron donor and 0.056 for the treatment with H_2 and acetate added (Fig. 2c); the majority of hydrogen consumption in enrichment culture III was associated with acetogenesis and methanogenesis. Dechlorination of γ-HCH did not occur in uninoculated MSM-3 controls, based on a lack of any accumulation of benzene or CB.

3.3. γ-HCH dechlorination in MSM-3

In order to prevent hydrogen use for acetogenesis, enrichment culture III in MSM-2 (bicarbonate buffered) was used to initiate enrichment culture IV in MSM-3 (HEPES buffered). Following a lag period, γ-HCH dechlorination to benzene + CB occurred at a higher overall rate in the treatments with hydrogen available (Fig. 3a) versus the companion enrichment III treatments in MSM-2 (Fig. 2a). Accumulation of benzene and CB reached 17 and 51 μmol/bottle (Table 2), or 26 mg/L and 76 mg/L, respectively, when taking partitioning between the headspace and liquid into account. In spite of these high concentrations, there was no apparent inhibition of γ-HCH dechlorination. Without hydrogen added, little or no γ-HCH dechlorination to benzene and CB occurred (Fig. 3a). As with enrichment III, vancomycin strongly inhibited γ-HCH dechlorination (Supplementary data). None of the treatments in MSM-3 yielded any accumulation of acetate or methane (Table 2).

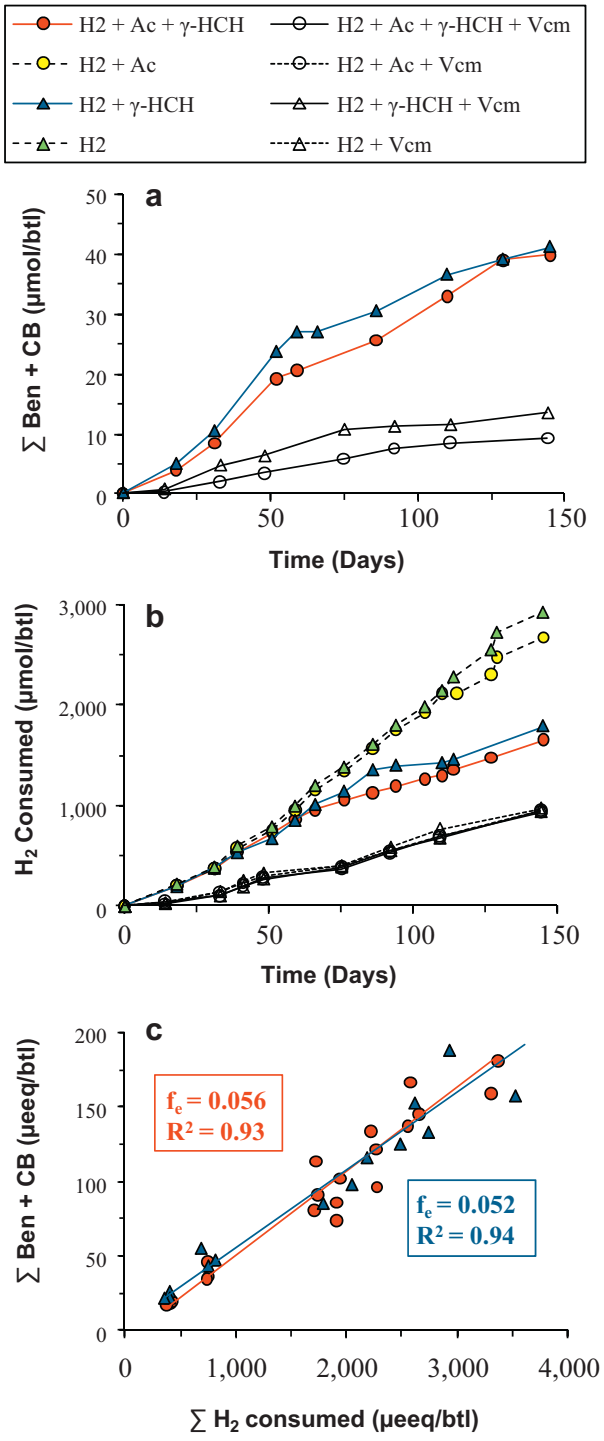

Fig. 2. γ-HCH dechlorination by enrichment culture III: (a) cumulative benzene and CB formation; (b) cumulative H_2 consumption; and (c) determination of f_e. In panels a and b, each data point represents the average of triplicate bottles; results for individual bottles are shown in Supplementary data. In panel c, data from all of the bottles are shown. Ac, acetate; Ben, benzene; btl, bottle; Vcm, vancomycin.

Enrichment culture IV with hydrogen as the electron donor (with and without acetate added) was successfully maintained for one year through three transfers (1%, v/v), suggesting that γ-HCH dechlorination is linked to the growth of microbes mediating this reaction.

Consistent with the extent of benzene and CB accumulation, the highest level of hydrogen consumption occurred in enrichment culture IV treatments with γ-HCH added (Fig. 3b). A low level

Table 2
Mass balance between hydrogen consumed and benzene and CB produced from γ-HCH dechlorination.

Enrichment culture (medium)	Treatment[a]	H_2 consumed (μmol/btl)[b]	Benzene formed (μmol/btl)	CB formed (μmol/btl)	CH_4 formed (μmol/btl)	Acetate (μmol/btl)[c]
III (MSM-2)	H_2 + Ac + γ-HCH	1654 ± 29[d]	11 ± 0.90	28 ± 4.5	51 ± 21	325 ± 34
III (MSM-2)	H_2 + Ac + γ-HCH + Vcm	936 ± 80	3 ± 0.61	6 ± 1.4	125 ± 36	−54 ± 26
IV (MSM-3)	H_2 + Ac + γ-HCH	192 ± 11	17 ± 0.32	51 ± 2.6	0	0
III (MSM-2)	H_2 + γ-HCH	1786 ± 28	11 ± 3.3	30 ± 5.6	58 ± 81	458 ± 52
III (MSM-2)	H_2 + γ-HCH + Vcm	940 ± 17	5 ± 1.3	9 ± 2.1	98 ± 33	46 ± 16
IV (MSM-3)	H_2 + γ-HCH	178 ± 7.9	13 ± 0.87	40 ± 4.1	0	0
III (MSM-2)	H_2 + Ac	2669 ± 26	NA[e]	NA	NM[f]	506 ± 90
III (MSM-2)	H_2 + Ac + Vcm	953 ± 6.5	NA	NA	NM	−94 ± 13
IV (MSM-3)	H_2 + Ac	31 ± 15	NA	NA	NM	0
III (MSM-2)	H_2	2920 ± 127	NA	NA	NM	502 ± 104
III (MSM-2)	H_2 + Vcm	967 ± 16	NA	NA	NM	21 ± 6.3
IV (MSM-3)	H_2	21 ± 5.2	NA	NA	NM	0

[a] Ac, acetate; Vcm, vancomycin.
[b] H_2 consumed in each treatment after subtracting for H_2 consumed in media controls; btl, bottle.
[c] A positive number indicates acetate accumulation, a negative number indicates net consumption.
[d] Average and standard deviation of triplicate bottles.
[e] NA, not applicable, since γ-HCH was not added.
[f] NM, not measured; headspace samples were evaluated for hydrogen; however, methane was not quantified.

of hydrogen consumption occurred in the inoculated treatments without γ-HCH added, which was only slightly greater than the hydrogen consumption in uninoculated sterile controls. Hydrogen consumption in the latter was likely due to diffusive losses.

The fraction of hydrogen electron equivalents consumed relative to the equivalents needed to form benzene and CB was 0.79 for the treatment with H_2 as electron donor and 0.90 for the treatment with H_2 + acetate added (Fig. 3c). These values are considerably higher than for enrichment culture III in MSM-2 (Fig. 2c), consistent with the lack of acetogenesis and methanogenesis in HEPES-buffered MSM-3 (Table 2).

A transfer was made from the MSM-3 enrichment IV that received γ-HCH + hydrogen (with and without acetate) back to MSM-2. The rate of benzene and CB formation was very similar to the rate shown in Fig. 2a (Supplementary data); however, the amount of hydrogen consumed decreased by one order of magnitude to approximately 140 μmol/bottle (versus 1654–1786 in enrichments always maintained in MSM-2; Table 2), and no methane was produced (versus 51–58 μmol/bottle; Table 2). Thus, it was possible to achieve γ-HCH dechlorination in MSM-2 at much lower levels of acetogenesis and no methanogenesis.

3.4. DGGE and cloning analysis

DGGE analysis of PCR-amplified DNA from the MSM-1 enrichment cultures with and without γ-HCH added did not reveal major differences, with methanol serving as the sole electron donor in both treatments (Supplementary data). Sequencing of bands excised from the DGGE gel revealed the presence of a *Desulfomicrobium* sp. and several uncultured bacteria. In the same enrichment cultures, the absence of a *Dehalobacter* sp. was indicated by a lack of DNA amplification using *Dehalobacter* specific primers. *Dehalobacter* sp. have previously been shown to anaerobically dechlorinate β-HCH in other enrichment cultures [9].

DGGE analysis of samples from several of the enrichment III and IV treatments, as well as sequencing of 12 of the excised bands, also did not reveal apparent differences in the microbes present in treatments with and without γ-HCH. Furthermore, clone libraries for several of the enrichment III and IV treatments did not yield any of the microbes previously associated with organohalide respiration, including *Dehalobacter* (Supplementary data).

4. Discussion

Based on the fraction of electron equivalents used for γ-HCH dechlorination in HEPES-buffered enrichment culture IV and the ability to transfer this culture with γ-HCH as the sole terminal electron acceptor, this study is the first to demonstrate organohalide respiration of γ-HCH. The fraction of electron equivalents used for γ-HCH dechlorination to a mixture of benzene and CB ranged from 79 to 90%, confirming that γ-HCH dechlorination was the major sink for H_2 consumption. This range for f_e values is slightly higher than ones reported for other chlororespiratory processes [21,29]. The HEPES-buffered enrichment culture was maintained on H_2 as the sole electron donor and γ-HCH as the sole electron acceptor through three transfers (1%, v/v) to fresh MSM-3 over a one year period, without loss of dechlorination activity. These results are consistent with other reports of growth associated with the use of chlorinated compounds as the terminal electron acceptor [9,30].

The source of carbon used by the HEPES-buffered enrichment culture IV during organohalide respiration of γ-HCH is not yet clear. Acetate is a common carbon source for many chlororespiring cultures that use hydrogen as an electron donor. In the treatments that received acetate along with hydrogen and γ-HCH, the rate of γ-HCH dechlorination (based on the rate of benzene and CB accumulation) became higher beyond day 60 (Fig. 3a). However, there was no detectable decrease in acetate (Table 2 and Supplementary data), although the amount of acetate used for biomass synthesis may have been within the error of the HPLC method used. Since we were able to make several transfers of the treatment that received only hydrogen and γ-HCH, the possibility of HEPES serving as the carbon source cannot be ruled out. Enrichment cultures that reductively dechlorinate trichlorobenzene to dichlorobenzene with only H_2 additions have been reported, although activity was eventually lost over successive transfers [31]. Additional studies are needed to conclusively identify the carbon source(s) used during organohalide respiration of γ-HCH.

γ-HCH dechlorination rates were notably higher in the HEPES-buffered enrichment culture (Fig. 3a) compared to the bicarbonate-buffered enrichment culture (Fig. 2a). Competing demands for hydrogen is a likely explanation. In the bicarbonate-buffered enrichment culture, acetogenesis and methanogenesis were the dominant uses for hydrogen (Table 2), while both of these processes were absent in the HEPES-buffered enrichment culture. The effect of these competing hydrogenotrophic processes on organohalide respiration is widely reported [32–34]. By eliminating sulfate from MSM-2 and MSM-3, it was possible to rule out cometabolic reductive dechlorination of γ-HCH by sulfate reducing microbes, as described previously [5,11]. Although acetate was added in considerable excess to both HEPES and bicarbonate-buffered treatments, the results indicate that acetate was not

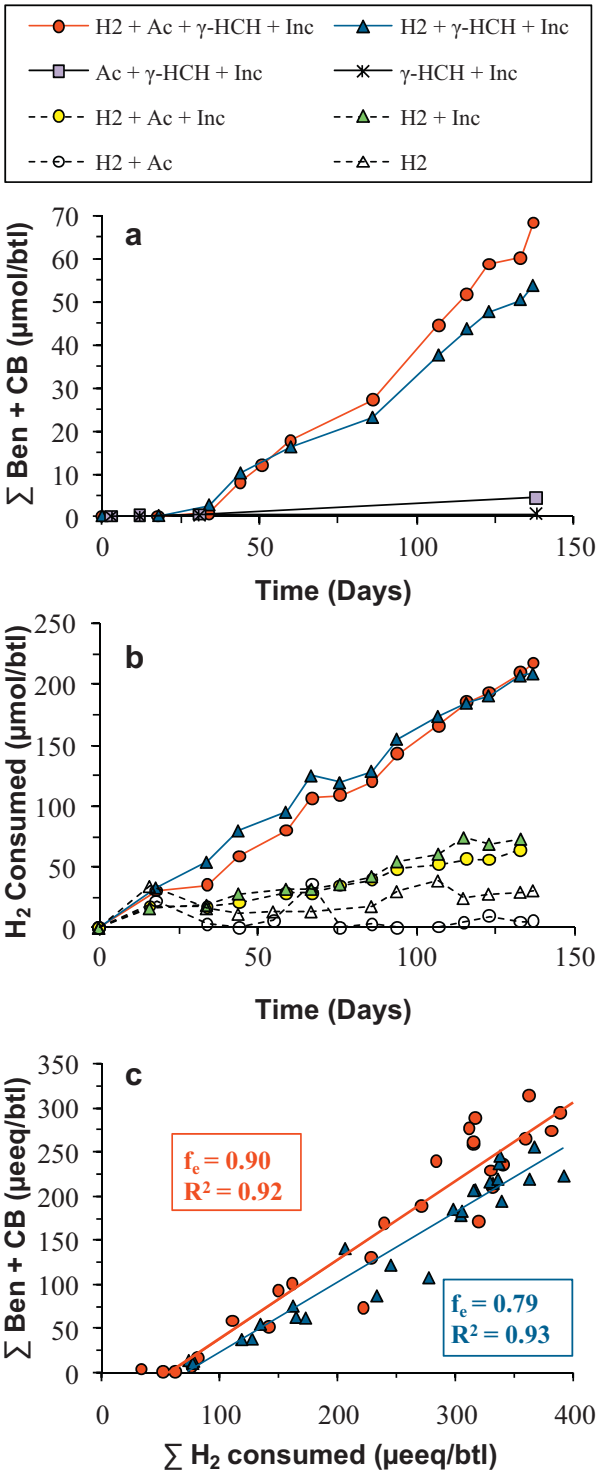

Fig. 3. γ-HCH dechlorination by enrichment culture IV: (a) cumulative benzene and CB formation; (b) cumulative H_2 consumption; and (c) determination of f_e. In panels a and b, each data point represents the average of triplicate microcosms; results for individual bottles are shown in Supplementary data. In panel c, data from all of the bottles are shown. Ac, acetate; Ben, benzene; btl, bottle; Inc, inoculated with enrichment culture IV.

used as an electron donor. This was most evident in the HEPES-buffered treatment that received acetate without hydrogen; only a minor amount of γ-HCH dechlorination occurred (Fig. 3a). Among chlororespiring mixed and pure cultures, use of hydrogen as an electron donor is more common than acetate [9,21,35].

Addition of vancomycin (100 mg/L) to the enrichment culture III and IV treatments with H_2 as the electron donor strongly inhibited γ-HCH dechlorination. Similar results were reported with an enrichment culture that reductively dechlorinates β-HCH [8] and another that dechlorinates 4,5,6,7-tetrachlorophthalide [36]; both enrichments contained *Dehalobacter* spp. In other cases, addition of vancomycin (100–500 mg/L) has not inhibited reductive dechlorination. For example, 100 mg/L of vancomycin did not impact reductive dechlorination of tetrachloroethene to ethene by an enrichment culture later shown to contain *D. ethenogenes* [28]; and 500 mg/L did not inhibit organohalide respiration of polychlorinated benzenes, phenols and dibenzodioxins [31]. The effect of vancomycin on γ-HCH dechlorination in this study suggests that the microbe responsible is likely non-spore forming and gram positive. Additional studies are needed to identify which microbe is responsible.

Advances in understanding the physiology and phylogeny of organohalide respiration of chlorinated organic compounds have facilitated improvements in anaerobic bioremediation of chlorinated organic compounds. Nevertheless, with γ-HCH, the main products of dechlorination (benzene and CB) are not environmentally acceptable endpoints. In order for complete anaerobic bioremediation of γ-HCH to become feasible, improvements are also needed in anaerobic biodegradation of benzene and CB. Reductive dechlorination of CB to benzene has recently been demonstrated [30] and cultures that anaerobically oxidize benzene have been reported [37,38], although further advances will be needed before a fully anaerobic approach is feasible in situ. An alternative approach is to follow reductive dechlorination of γ-HCH with aerobic biodegradation of benzene and CB.

5. Conclusions

The use of γ-HCH as a terminal electron acceptor via organohalide respiration was demonstrated for the first time in a HEPES-buffered enrichment culture that was initially developed with anaerobic soil and groundwater from an industrial site. When hydrogen served as the electron donor, 79–90% of the electron equivalents from hydrogen was used to reduce γ-HCH to a mixture of benzene and CB. The enrichment culture remained active with only hydrogen as the electron donor and γ-HCH as the electron acceptor through several transfers to HEPES-buffered mineral salts medium for more than one year. Addition of vancomycin to the culture significantly slowed the rate of γ-HCH dechlorination, suggesting that a Gram-positive organism is responsible for the anaerobic dechlorination of γ-HCH. Microbial community analysis of the γ-HCH dechlorinating enrichment culture, including sequencing of DGGE bands and clone libraries, did not reveal any of the known chlororespiring genera, including *Dehalobacter*. In bicarbonate-buffered medium, reductive dechlorination of γ-HCH was accompanied by significant levels of acetogenesis as well as methanogenesis.

Acknowledgements

We gratefully acknowledge Dr. Frank Löffler for providing cloned 16S rRNA genes of *Dehalobacter* sp. We also thank Susan M. Fredendall for assistance with sample preparation and analyses.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.09.080.

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