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

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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.

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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

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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)):

$$C_l = \frac{M}{V_l + H_c V_g} \tag{1}$$

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

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