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Reductive dechlorination of hexachlorocyclohexane (HCH) isomers in soil under anaerobic conditions

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Abstract

The biological anaerobic reductive dechlorination of β-hexachlorocyclohexane under methanogenic conditions was tested in a number of contaminated soil samples from two locations in the Netherlands. Soils from a heavily polluted location showed rapid dechlorination of β-hexachlorocyclohexane to benzene and chlorobenzene with lactate as electron donor. Soils from an adjacent slightly polluted location did not show substantial dechlorination of β-hexachlorocyclohexane within 4 months. A heavily polluted sample was selected to optimise the dechlorination. All tested hexachlorocyclohexane isomers (α-, β-, γ-, and δ-), either added separately or simultaneously, were dechlorinated in this soil sample. The most rapid dechlorination was observed at a temperature of 30 °C. Dechlorination of β-hexachlorocyclohexane was observed with acetate, propionate, lactate, methanol, H_2, yeast extract and landfill leachate as electron donors. In a soil percolation column, packed with a selected heavily polluted soil sample, the presence of 10 mM sulphate in the influent led to simultaneous dechlorination of β-hexachlorocyclohexane and sulphate reduction. When the column was fed with 10 mM nitrate instead of sulphate, dechlorination ceased immediately. After omitting nitrate from the influent, dechlorination activity recovered in about 1 month. Also in a separate column, the addition of nitrate from the start of the experiment did not result in dechlorination of β-HCH. The significance of these experiments for *in situ* bioremediation of polluted soils is discussed.

Introduction

During the production of the insecticide γ-hexachlorocyclohexane (γ-HCH, lindane), large amounts of waste HCH-isomers have been stored or discarded in the environment (Bachmann et al. 1988; Hernandez et al. 1991; Oliveira & Brilhante 1996; Willett et al. 1998). The resulting polluted soils are difficult to deal with since no (economically) satisfying treatment is available. In some cases the most heavily contaminated areas have been excavated and stored until appropriate treatment becomes available. One such treatment, incineration, requires high temperatures to avoid formation of highly carcinogenic polychlorinated dioxins, and is therefore very expensive. An alternative method, biological cleanup of with HCH polluted soils appeared to have serious drawbacks since the β-HCH isomer was thought to be persistent under anaerobic and aerobic conditions (Bachmann et al. 1988; Doelman et al. 1990). We have demonstrated that β-HCH can be dechlorinated to benzene and chlorobenzene under methanogenic conditions both in soil columns and batch experiments (Middeldorp et al. 1996). Van Eekert et al. (1998) showed that unadapted anaerobic granular sludge was able to dechlorinate all HCH isomers, including β-HCH. These findings

have led to the development of an *in situ* bioremediation technique (Langenhoff et al. 2002). This technique is similar to the *in situ* techniques which have been developed for the treatment of tetra- and trichloroethene polluted soils (Alphenaar et al. 1995; Gibson & Sewell 1992; Middeldorp et al. 1998). However, little is known about the micro-organisms that perform the reductive dechlorination of HCH, and the conditions under which they perform the dechlorination. In this paper, we report about the presence of HCH dechlorinating bacteria in different polluted soil samples. We have tested the dechlorination activity under different environmental conditions. Additionally, we have simulated *in situ* bioremediation using soil percolation columns.

Materials and methods

Chemicals

All HCH-isomers (>99% purity) used were obtained from C.N. Schmidt B.V. (Amsterdam, The Netherlands). Pentane (≥98% purity) was from Fluka (Zwijndrecht, The Netherlands). All other chemicals were from Merck Nederland B.V. (Amsterdam, The Netherlands). Fermented municipal landfill leachate, containing mainly a mix of different fatty acids was a kind gift from TNO (Apeldoorn, The Netherlands).

Screening of soil samples for β-HCH dechlorination

Ten soil samples were taken anaerobically at different depths from two HCH-polluted locations in Hengelo (Akzo Nobel Chemicals site (ANC, heavily polluted, four samples)) and Oude Hengelose Dijk (OHD, slightly polluted, six samples), The Netherlands, and stored under nitrogen at 4 °C until use. In an anaerobic glove box, ca. 4 g wet weight from each sample was added to 120 ml serum flasks containing 20 ml reduced anaerobic mineral medium (Holliger et al. 1992), favouring methanogenic conditions. The bottles were closed with viton stoppers (Maag Technik, Dübendorf, Switzerland) and the headspace was replaced with 80% N_2/20% CO_2. Approximately 0.1 ml of a β-HCH solution (1 g l^{-1}, dissolved in acetone) was added to the bottles resulting in an average final HCH concentration of 17 μM. Sodium lactate (20 mM) was added as electron donor. The bottles were incubated statically at 30 °C in the dark. Benzene and chlorobenzene production in the headspace was followed as indication for β-HCH dechlorination activity. All soil samples have been tested in duplicate bottles.

Temperature influence

From the screening experiment, one soil sample from the ANC location was selected for further research. About 4 g of the soil sample was added under anoxic conditions to the same medium and supplements (lactate, β-HCH) as described above, and incubated at 4, 10, 20, and 30 °C statically in the dark. Benzene and chlorobenzene production in the headspace were followed during the incubation. Duplicate bottles were prepared for each temperature.

Dechlorination of different HCH-isomers

The above mentioned soil sample (about 4 g) was incubated as described above (lactate 20 mM) and different HCH-isomers were tested separately. From acetone stock solutions (2 g l^{-1} α-, β-, γ-, δ-HCH and a mixture of these isomers, 0.5 g l^{-1} each) 0.1 ml was added to duplicate 120-ml serum bottles. Parallel to this, 27 ml glass tubes were prepared similarly (ca. 1 g of soil, 5 ml medium + amendments) in series of 10 tubes per tested isomer or isomer mixture. This allowed total extraction at different time intervals for HCH analysis. Autoclaved bottles and tubes were prepared as negative controls. All bottles and tubes were incubated statically at 30 °C in the dark.

Electron donors

About 4 g of soil sample was incubated similar to the above described incubations, but besides lactate (20 mM), also other electron donors were tested. Acetate (50 mM), propionate (25 mM), methanol (100 mM), hydrogen (80% H_2/20% CO_2 in the headspace), yeast extract (0.5 g l^{-1}) or fermented landfill leachate (0.1 ml) were added separately to the bottles, which were subsequently incubated statically at 30 °C in the dark. Benzene and chlorobenzene production in the headspace were followed in time. Duplicate tests were performed for each electron donor.

Soil percolation column experiments

Three glass columns (15 cm × 2.3 cm i.d.) were packed with material from the same soil sample as used in the above described batch tests and percolated under up-flow conditions at 20 °C with anaerobic medium by using a cassette peristaltic pump (Watson Marlow model 502S). The medium, which favours methanogenic conditions (Langenhoff et al. 1996), was saturated with β-HCH before entering the column by leading it through a small column containing Chromosorb G AW particles (60–80 mesh) (Chrompack, Bergen op Zoom, The Netherlands) that had been coated with β-HCH. The coating was made by mixing the Chromosorb with an 80 g l^{-1} β-HCH in acetone solution and evaporating off the acetone. Samples for analysis were taken from the influent and the effluent of the columns with a glass syringe as described previously (Bosma et al. 1988). The columns were fed lactate (2 mM) as electron donor and in two of the columns sulphate-reducing or denitrifying conditions were created by the addition of 10 mM Na_2SO_4 or 10 mM $NaNO_3$, respectively. Electron donors and acceptors were aseptically added together with Na_2S (1 mM) to the influent using a multiple syringe pump (Harvard Apparatus model 22) with stock solutions in 25 ml syringes. Diagrams of the column systems have been published previously (Middeldorp et al. 1996, 1998).

Analytical methods

HCH isomers and polychlorinated benzenes
Amounts of 1–5 ml of liquid sample were extracted in 1 ml of pentane and the organic phase was transferred to autosampler vials. The extracts were analysed using gas chromatography coupled to a mass selective detector (GC-MSD) as described previously (Middeldorp et al. 1996) using external standards for quantification.

Benzene and chlorobenzene
A sample of 0.4 ml was taken from the headspace with a 1 ml syringe. After drawing the needle tip back into the stopper, a vacuum was created by drawing the plunger up to 0.8 ml. The needle was pulled out and the sample was injected on a Chrompack 436 gas chromatograph (Chrompack, Bergen op Zoom, The Netherlands) equipped with a flame ionisation detector (FID) and a Sil 5CB capillary column (25 m × 0.32 mm, 1.2 μm film). Operation temperatures were 60 °C for 4 min, raised by 15 °C min^{-1} to 105 °C, and held at 105 °C for 1 min. External standards were used for quantification. The influence of the presence of soil in the medium on the water/air distribution of benzene and chlorobenzene (Henry constant) due to e.g. sorption was determined but no noteworthy shifts were observed (calculations not shown).

Chlorinated phenols
The whole content of the 27 ml tubes was acidified to pH 1 with concentrated hydrochloric acid and shaken for 20 min with 10 ml of acetone. Then, the pH was brought up to alkalinity by using a 40% KOH solution and acetylation buffer (0.15 M NaOH; 42.3 mM $NaHCO_3$, pH 10) and 0.1 ml of acetic anhybrid was added. Then the whole content was extracted with 4 ml of hexane. The hexane extract was analysed using GC-MSD as described above for HCH analysis.

Nitrate and sulphate anions
Samples of 1 ml were centrifuged in Eppendorf tubes at 13,000 rpm for 10 min. Then, 50 μl of the sample was diluted with 450 μl deionised and filtrated water and transferred to autosampler vials containing 0.5 ml of a 20 mM mannitol solution containing 2 mM KF as internal standard. The samples were analysed by anion exchange high performance liquid chromatography coupled to a conductivity detector as described previously (Scholten & Stams 1995).

Results

Screening of soil samples for β-HCH dechlorination

Ten soil samples from the polluted locations ANC and OHD were screened for microbial β-HCH dechlorinating activity. The results of the screening experiment are shown in Table 1. In the six soil samples from location OHD and sample ANC3 only little production of benzene or chlorobenzene from β-HCH was observed after 28 days of incubation (Table 1). These values had not increased after 80 days. In the soil samples ANC1, ANC2 and ANC4, high levels of benzene and chlorobenzene were found.

Table 1. Benzene and chlorobenzene production from β-HCH added to different soil samples after 28 days of incubation

Soil sample	Dechlorination products formed		% β-HCH dechlorinated
	Benzene (μM)	Chlorobenzene (μM)	
6 OHD samples	n.a.	n.a.	<3.5%
ANC1	3.3 ± 0.1	10.9 ± 2.5	83%
ANC2	4.4	13.5	100%
ANC3	0.3 ± 0.01	0.2 ± 0.01	2.9%
ANC4	13.5	4.7	100%

In all batches, methane formation occurred, which indicates anaerobic microbial activity in all samples.

Influence of temperature on the dechlorination

Sample ANC2 was tested for β-HCH dechlorination at different temperatures under methanogenic conditions. Figure 1 shows that at 30 °C β-HCH dechlorination started after 12 days. At 20 °C, dechlorination started after 34 days of incubation. Once dechlorination had started, the dechlorination rates were comparable at both temperatures. At 4 and 10 °C, no measurable dechlorination took place within 100 days. However, after 248 days, when all cultures were analysed again, all β-HCH had been dechlorinated to benzene and chlorobenzene in the batches at 10 °C, but not at 4 °C.

On day 69, the batches incubated at 30 °C were spiked with an additional 20 μM β-HCH and transferred to a 10 °C incubator. In contrast to the batches incubated at 10 °C, the dechlorination started within 20 days, although at a lower rate than at 20 and 30 °C. In all batches where dechlorination was observed, all β-HCH was dechlorinated.

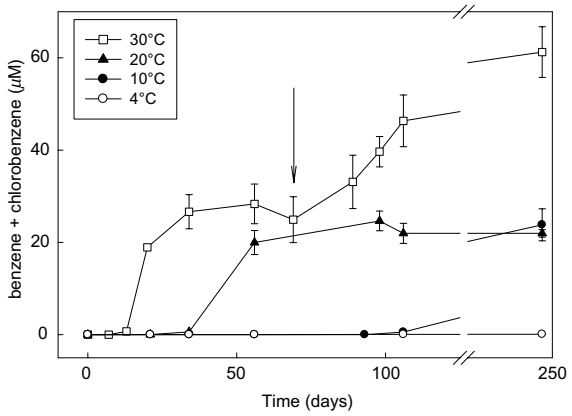

Figure 1. Influence of temperature on the dechlorination of β-HCH. The arrow on day 62 indicates when the culture incubated at 30 °C was refed with β-HCH and further incubated at 10 °C.

Isomer spectrum

Soil sample ANC2 was incubated separately with different HCH-isomers and with a mixture of α-, β-, γ-, and δ-HCH. The benzene and chlorobenzene formation is shown in Figure 2. Dechlorination of γ-HCH started within 10 days. Dechlorination of α- and δ-HCH also started within 10 days, but was substantially slower. The dechlorination of β-HCH only started after 20 days of incubation, but had a higher rate than all other isomers. HCH analyses in the parallel tubes were consistent with the observations for the production of benzene and chlorobenzene (data not shown). In the batches incubated with a mixture of α-, β-, γ-, and δ-HCH, dechlorination started within 10 days and benzene and chlorobenzene were produced almost linearly. Specific HCH isomer analysis in the parallel tubes revealed that the γ-HCH dechlorination started within 10 days, whereas the dechlorination of the other three isomers started only after 10 days of incubation. After 10 days, a simultaneous dechlorination of all tested isomers took place at a similar rate. The overall dechlorination rate of the mixture of isomers is approximately in the range of the separately incubated α-, β-, and δ-isomer, but lower than the γ-HCH dechlorination rate.

Electron donors

Soil sample ANC2 was tested for β-HCH dechlorination with different electron donors. A compa-

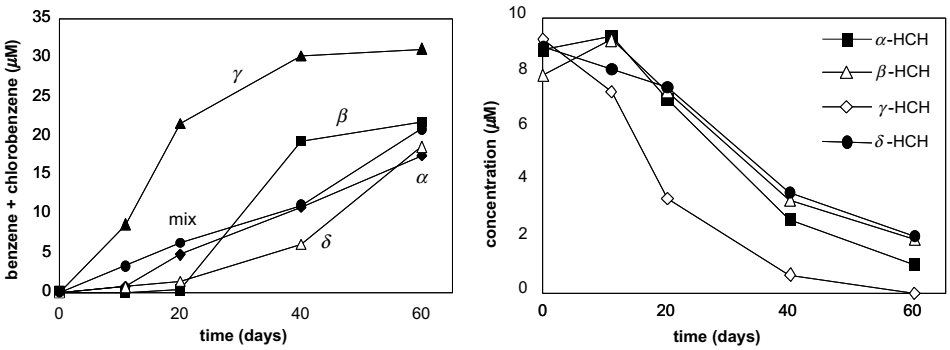

Figure 2. Reductive dechlorination of different HCH isomers in the soil slurry when applied both separately, and as a mixture (top panel, measured as benzene and CB production), and disappearance of the separate HCH-isomers in the mixture (bottom panel).

rable dechlorination rate was observed with the electron donors lactate, yeast extract, and fermented landfill leachate (FLL) (Figure 3). With these electron donors, dechlorination started within 20 days of incubation.

Dechlorination with methanol and hydrogen commenced between day 20 and 35, after which in the case of methanol the dechlorination was rapidly completed. With hydrogen, dechlorination ceased after approximately 25% of the β-HCH had been dechlorinated. With acetate and propionate as electron donor, dechlorination started after 35 days of incubation and proceeded at a lower rate than with the other electron donors. The ratio benzene:chlorobenzene produced with different electron donors was stable during the dechlorination and was between 0.2 and 0.5 for all electron donors tested, except in fermented landfill leachate (0.8) and acetate (1.3).

Column experiments

Flowthrough columns filled with polluted soil (sample ANC2) were operated with β-HCH and lactate in mineral medium under different redox conditions. Complete breakthrough of the β-HCH was achieved after 1 month of operation.

In the column fed with nitrate, initially only 70% of total breakthrough was observed (Figure 4), but no degradation products were detected in the effluent. This column reached near complete breakthrough of β-HCH after 90 days, and no disappearance of the β-HCH was observed thereafter. Anion analysis revealed that nitrate was

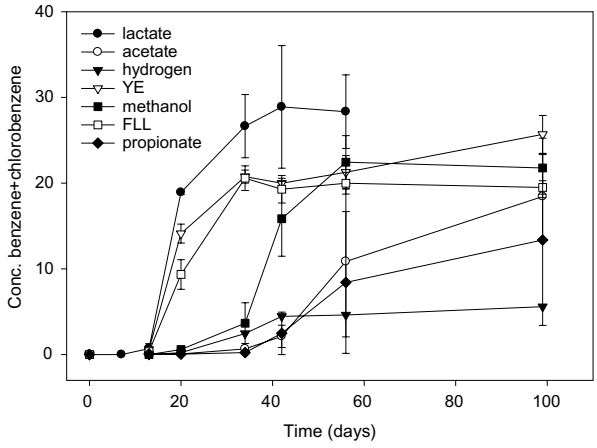

Figure 3. Benzene and chlorobenzene production during dechlorination of β-HCH in soil sample ANC2, stimulated by different electron donors. YE – yeast extract, FLL – fermented landfill leachate.

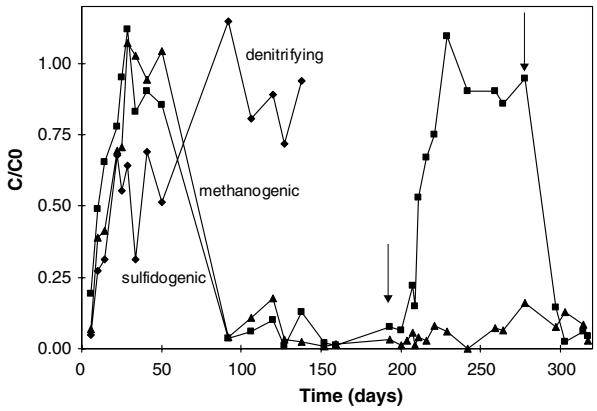

Figure 4. Dechlorination of ß-HCH in soil columns under different redox conditions. The HCH concentration is plotted as the concentration in the effluent over the concentration in the influent. The arrow on day 193 marks the replacement of 10 mM sulphate with 10 mM nitrate in the influent; the arrow on day 278 marks the restoration of the original setup.

completely removed from the column. After 140 days, operation of this column was discontinued.

In the third month after the start of the experiment, disappearance of β-HCH in the column was observed under both methanogenic and sulphate reducing conditions (Figure 4). Both benzene and chlorobenzene were found in the effluents (not shown). Anion analysis showed that approximately 50% of the influent sulphate concentration was removed during column passage. After the replacement of the sulphate with 10 mM nitrate on day 193, the dechlorination ceased immediately. When, on day 278, the nitrate was replaced by sulphate again, the dechlorinating activity in the column was fully recovered within 1 month.

Discussion

We have screened several polluted soil samples for the presence of biological reductive dechlorination activity of β-hexachlorocyclohexane (HCH). This compound was selected since it is the most persistent HCH isomer to bacterial degradation under aerobic conditions (Bachmann et al. 1988; Doelman et al. 1988, 1990; Lang et al. 1992). We have shown that the heavily polluted samples ANC1, ANC2 and ANC4 showed extensive dechlorination of β-HCH, while in the slightly polluted OHD samples, which were taken from the periphery of the HCH plume (no HCH present), no substantial dechlorination was observed (Table 1). This suggests that the presence of the HCH contamination for tens of years in the ANC soil has led to the establishment of a β-HCH dechlorinating microbial community. This is confirmed by the enrichment of an anaerobic β-HCH dechlorinating consortium, containing an organism that uses β-HCH as the only terminal electron acceptor (P.J.M. Middeldorp, unpublished data.).

Under methanogenic conditions, the ANC2 soil sample has the capacity to dechlorinate all tested HCH isomers (α-, β-, γ-, and δ-HCH) to benzene and chlorobenzene as the only products. These products were not further degraded under the conditions applied. The dechlorination of β-HCH occurs also at relatively high rates at a temperature of 10 °C, which is the average soil temperature at the polluted site (Figure 1). Groundwater analyses from the anaerobic zone show the presence of benzene and chlorobenzenes (not shown), which indicates the occurrence of natural attenuation *in situ*. However, this natural attenuation is probably limited by the absence of readily available electron donors to support the dechlorination, since control soil incubations without lactate showed only very slow dechlorination. The lack of electron donors is a typical limiting factor for natural attenuation by means of reductive dehalogenation of compounds, such as chlorinated ethenes and polychlorinated aromatics (Middeldorp et al. 1998; Sewell & Gibson 1991; Van Aalst-Van Leeuwen et al. 1998; Wiedemeier et al. 1997). Several electron donors, including

complex mixtures of organic compounds such as yeast extract and landfill leachate, supported dechlorination of β-HCH. Particularly the latter mixture may be an economic alternative for more defined electron donors.

In percolation column experiments, partial (ca. 50%) reduction of 10 mM sulphate and β-HCH dechlorination both took place. Possibly, these processes occur simultaneously, but it may also be that, due to lack of electron donor (2 mM lactate corresponds to 3 mM sulphate reduced), the sulphate is partially consumed in the first part of the column, after which the relatively small amount of β-HCH is dechlorinated in the second part of the column. If the dechlorinating bacteria have a higher affinity for lactate or hydrogen than the sulphate reducing bacteria, low concentrations of remaining electron donor could then be used for the dechlorination. A similar phenomenon has also been found for chlorinated ethenes (Smatlak et al. 1996).

The dechlorination of β-HCH is completely inhibited by the presence of 10 mM nitrate in the influent. This is confirmed by the fact that the replacement of sulphate with nitrate in the influent of the sulphate reducing column led to complete inhibition of the dechlorination (Figure 4).

Degradation of the products benzene and chlorobenzene was never observed in our experiments. Although anaerobic benzene degradation has been abundantly shown in different soil samples (Anderson et al. 1998; Grbic-Galic & Vogel 1987; Kazumi et al. 1997; Lovley 1997; Lovley et al. 1995; Weiner & Lovley 1998), our experiments confirm that this is not a common feature for anaerobic soils (Baten et al. 1997; Griffioen et al. 1999; Langenhoff et al. 1999, 1996). As far as chlorobenzene is concerned, significant anaerobic degradation of chlorobenzene has not been reported. In our experiments, we have successfully demonstrated *in situ* anaerobic reductive dechlorination of HCH-isomers. Both benzene and chlorobenzene are rapidly degraded in the presence of oxygen (Haider et al. 1974; Nishino et al. 1992; Pettigrew et al. 1991; Reineke & Knackmuss 1984). Therefore, to apply the dechlorination of HCH as a soil remediation technique, the anaerobic phase should be followed by a second phase in which aerobic conditions prevail, in order to allow further mineralisation of the remaining benzene and chlorobenzene. A pilot scale bioscreen to eliminate HCH from the groundwater is currently running at the ANC site according the abovementioned principle (Langenhoff et al. 2002).

Acknowledgements

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