POP-DEHALOGENATING MICROBES IN AN ACTIVE LANDFILL TREATING HERBICIDE- AND DIOXIN-CONTAMINATED SOILS

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Introduction

During the Vietnam War (1961-1971), about 100 million liters of herbicides (most of them were contaminated by dioxin) were used by The US Army in order to remove forest canopies and destroy crops in the middle and the South of Vietnam. The Bien Hoa airbase was the major staging point for the Operation "Ranch Hand" in South Vietnam. Previous studies showed that the dioxin contamination in Bien Hoa was very high, especially in the Z1 zone, where 410,000 ng TEQ/kg (average 18,750 ng TEQ/kg) and 5,470 ng TEQs/kg (average 2,990 ng TEQ/kg) were analyzed in soils and sediments, respectively (Le *et al.,* 2011). Four bioremediation cells were implemented in the Z1 zone in a so-called active landfill, in total containing 3,384 m^3 of soils heavily contaminated with herbicides and dioxins. In these cells, the dioxin concentration declined rapidly (Dang *et al*., 2013). However, an intense chlorophenol smell of the soil indicated that the herbicides are not completely removed after a treatment period of 36 months.

In the Vietnam War, the most widely used herbicide was Agent Orange, herbicide Orange, which is composed of a mixture of 50% 2,4-dichlorophenoxyacetic acid (2,4-D) and 50 % 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Stellman *et al.,* 2003, Young *et al.,* 2004). The broadleaf herbicide 2,4-D has been found to be rapidly degraded by aerobic microorganisms, and these are common in all geographical locations examined (Tiedje *et al.,* 1997). Aerobic biotransformation of 2,4,5-T has been observed in both pure and mixed microbial cultures (e.g. Rice *et al.,* 2005). However, anaerobic biodegradation of these herbicides has not been extensively studied. Under anaerobic conditions, chlorines are removed from chlorinated aromatic compounds by microbial reductive dechlorination. 2,4,5-T reductive dechlorination has been observed in sewage sludge, pond sediment, a methanogenic aquifer and soils (Mikesell and Boyd,1985; Gibson and Suflita, 1986; Gibson and Suflita, 1990; Chang *et al.,* 1998). The first 2,4,5-T dechlorination products were identified as 2,4-D or 2,5-D, but monochlorophenoxyacetic acids, tri-, di- and monochlorophenols as well as phenol were also reported. Several anaerobic bacteria, mostly belonging to the genera *Desulfitobacterium* and *Dehalococcoides,* are reported, which are able to couple reductive dechlorination of chlorophenols to growth by organohalide respiration (e.g. Adrian *et al.,* 2007).

In this study, we examined the potential of anaerobic bacteria to transform the phenoxyacetate herbicide 2,4,5-T and the putative intermediates 2,4,5-trichlorophenol (TCP) and 2,4-dichlorophenol (DCP) in bioremediating soil of the active landfill. In a first attempt we studied the presence of known reductively dechlorinating bacteria using a molecular biology approach. In addition, anaerobic enrichment cultures were established from soil samples of two treatment cells using 2,4,5-T, 2,4,5-TCP and 2,4-DCP as electron acceptors for organohalide respiring bacteria. The microbial transformation and dechlorination was analyzed and pathways for the 2,4,5-T removal were proposed.

Materials and methods

Samples. Soil samples were taken after 18 and 36 months from cell 1 (BH1.1) and cell 4 (BH4.3 and BH4.4). Bien Hoa airbase is located in Dong Nai province at 105°58'30"N, 106°49'10E, 700 meters from Dong Nai River on the West. Collected samples were stored at 4 °C until analyses.

Enrichment cultures. Anaerobic mineral medium "204" (Bunge *et al.,* 2008) was prepared and supplemented with acetate (2.5 mM) as a carbon source and a mixture of pyruvate and lactate (5 mM each) or propionate and butyrate (2.5 mM each) as potential electron donors. For primary enrichment single cultures were established with different combinations of electron donors and chlorinated compounds. The cultures containing 2,4,5-T and those set up with soil from BH1.1 were incubated only with pyruvate/lactate. The enrichment cultures established with soils from BH4.3 and BH4.4 were incubated with pyruvate/lactate and propionate/butyrate each in combination with 2,4-DCP and 2,4,5-TCP. Anaerobic stock solution of 2,4,5-T (5 mM in acetone) was added separately to empty sterile serum bottles to give a final concentration of 100 µM. Acetone was evaporated by a stream of filter-sterilized N_2 gas. Soil samples taken after 36 months of biotreatment were added into empty bottles or 2,4,5-T-supplemented bottles to a final concentration of 20% (w/v) under a stream of filter-sterilized N_2 gas. The bottles were closed with Teflon-coated rubber stoppers. For the 2,4,5-T- and chlorophenol-containing cultures, 40 ml and 20 ml, respectively, of sterile anaerobic medium was added. 2,4-DCP and 2,4,5-TCP were added from anaerobic stock solutions (10 mM each in 0.01 N NaOH) to give a final concentration of 100 µM. The gas headspace of the serum bottles was flushed with a filter-sterilized CO_2/N_2 gas mixture (20%/80%, v/v). The chlorophenol- and the 2,4,5-T-containing cultures were incubated statically or on a shaker, respectively, at 30°C in the dark. Subcultures (50 ml) with 2,4,5-T were prepared in duplicate from the primary enrichment by adding 10% (v/v) to 18 ml of fresh mineral salt medium supplemented with pyruvate and lactate. Medium without inoculum served as abiotic control. Samples (0.6-1 ml) were taken, centrifuged (10 min, 12000 x g) and 2,4,5T and chlorophenols were analyzed in the supernatant.

Extraction of DNA, amplification and sequencing of 16S rRNA genes. Total DNA was extracted from 250 mg of soil samples using the PowerSoil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) and served as template (1 µl) for PCR using the HotStarTaq Plus DNA polymerase (Qiagen, Hilden, Germany) and the conditions recommended by the manufacturers. The following primers were used targeting the 16S rRNA genes of *Dehalococcoides mccartyi*, DET730f/DET1350r (Ballerstedt *et al.,* 2004); *Dehalogenimonas*, BL-DC-142f/BL-DC-1243r (Yan *et al.,* 2009); representatives of both, *Dehalococcoides* and *Dehalogenimonas*, Fp DHC 1/Rp DHC 1377 (Hendrickson *et al.,* 2002); *Dehalobacter restrictus*, DRE445f (Ballerstedt *et al.,* 2004)/DRE1248r (5´-GGCTTCCGTTCCCCGTCTG-3´); *Desulfitobacterium,* DES436f/DES1027r (5´-TGTCTTCAGGGGACGAACG-3´/5´-CTCATAGCTCCCCGAAGG-3´); *Desulfuromonas chloroethenica*, DCH205f/DCH1015r (Ballerstedt *et al.,* 2004). Genomic DNA from pure cultures of *D. mccartyi* strain CBDB1 and *Desulfitobacterium hafniense* strain TCP-A were used as positive control, if appropriate. The PCR product obtained with primers Fp DHC 1/Rp DHC 1377 was cloned using the GeneJet PCR Cloning Kit (Fermentas, St. Leon-Roth, Germany). The inserts of isolated plasmids were sequenced (Seqlab, Göttingen, Germany).

Analytical methods. 2,4,5-T and the chlorophenols were analyzed by HPLC (VWR-Hitachi) using a C18-column (LiChrospher, 250 x 4 mm, 5 µm), an oven temperature of 30 °C, a flow rate of 1 ml min^{-1}. 2,4,5-T and 2,4,5-TCP were analyzed in a water-acetonitrile gradient and detected at 210 nm as described by Rice *et al.* (2005). 2,4,5-TCP, 2,4-DCP, 3,4-DCP, 2,5-DCP, 3- and 4-monochlorophenol (MCP) were analyzed using isocratic elution in methanol:water (65:35 or 60:40; v/v) at 220 nm. The compounds were quantified based on a four-level calibration curve (12.5-100 µM). 2,4-DCP and 3,4-DCP were not separated under these conditions. Therefore, respective samples were additionally analyzed by gas chromatography after acetylation and hexane extraction as described by Adrian *et al* (2007).

Results and discussion

Identification of dehalogenating bacteria in bioremediation cells

Samples were taken from treatment cells 1 (BH1.1) and 4 (BH4.3 and BH 4.4) after 18 months. DNA was extracted and subjected to PCR with primers targeting the 16S rRNA genes of (i) *Dehalococcoides mccartyi* or (ii) *D. mccartyi* and members of the phylogenetically related *Dehalogenimonas* (Hendrickson *et al.,* 2002). Both genera are known to rely on organohalide respiration for growth. A product of the expected size was obtained from BH1.1, but not from BH4.3 and BH4.4 with the *D. mccartyi*-specific primers. In contrast, the second primer pair yielded a high amount of product only from the BH4.4 extract. Three sequences (800 bp) were obtained from BH4.4, which were all most closely related to sequences from uncultured Chloroflexi. However, the most closely related 16S rRNA gene sequences of cultivated bacteria belonged to *Dehalogenimonas* and *Dehalococcoides* species (86-87 % sequence identity). An extended primer set was used to analyze samples

taken after 36 months. No products were obtained with *D. mccartyi*- or *Dehalogenimonas*-specific primers, but high amounts of products were obtained from BH1.1 and BH4.3 samples with the primer pair targeting both, *Dehalococcoides* and *Dehalogenimonas,* suggesting that relatives of these bacteria are common in the treatment cells, although their detectability changed over time at the different sampling sites. Further studies should address the dehalogenation potential of this so far unexplored bacterial group. Whereas no PCR products were obtained with *Dehalobacter* and *Desulfuromonas*-specific primers, amplicons were obtained from BH1.1 and BH4.3 with primers specific for *Desulfitobacterium* known as a chlorophenol respirer. The results of this screening approach strongly suggested the presence of anaerobic bacteria capable to reductively dechlorinate chlorinated pollutants in the treatment cells.

Anaerobic transformation of 2,4,5-T, 2,4,5-trichlorophenol and 2,4-dichlorophenol in enrichment cultures

After 2 weeks, the initial concentration of 2,4-DCP had decreased by 95-99% in all enrichment cultures. 4-CP was formed as the main product. A second dose of 100 µM of 2,4-DCP was fed and decreased within eight days below the detection limit in most of the cultures. 4-CP was formed in all cultures but did not accumulate stoichiometrically suggesting that 4-CP was further transformed. In contrast, dechlorination of 2,4,5-TCP was generally slower. Only one enrichment culture, BH4.3 fed with propionate/butyrate, exhibited a complete transformation after 2 weeks, whereas in all other cultures an almost complete removal of 2,4,5-TCP was observed only after 3 or 4 weeks. 3,4-DCP was detected as main dechlorination product by HPLC and gas chromatography. These results demonstrate a high capability of anaerobic bacteria in the contaminated soil to dechlorinate the tested chlorophenols at the *ortho*-position regardless of the added electron donor combination.

Interestingly, the added herbicide 2,4,5-T was completely removed within 2 weeks in all three enrichment cultures. Kinetics and pathway of transformation were studied in greater detail in duplicate subcultures supplemented with pyruvate/lactate. The 2,4,5-T concentration decreased most rapidly in the BH4.3 subculture (Fig. 1A) followed by the BH4.4 (Fig. 1B) and BH1.1 subcultures. Interestingly, the BH1.1 (data not shown) and BH4.4 cultures differed from the BH4.3 culture by forming a distinctly different spectrum of transformation products, which was characterized by transient formation of 2,4,5-TCP and the accumulation of 3,4-DCP and to a less extent 3-CP and 2,5-DCP (Fig. 1B). This suggests that 2,4,5-T was transformed initially via ether cleavage followed by dechlorinations at *ortho*- and *para*-positions (Fig. 2). This transformation pathway has also been reported for sewage sludge by Mikesell and Boyd (1985). Our results suggest that 2,4,5-T transformation via 2,4,5-TCP is a common property of the microbial communities in the bioremediation cells in Bienhoa.

In contrast, in the BH4.3 cultures only low concentrations of 2,4,5-TCP and 3,4-DCP were measured detected but considerable amounts of 2,5-DCP were transiently formed and finally converted to 3-CP after 5 days and disappeared after 10 days (Fig. 1A) . The formation of 2,5-DCP requires a combination of *para*-dechlorination and ether cleavage. Although, the order of these reactions has still to be elucidated (Fig. 2), the fact that 2,5-DCP is not formed during the dehalogenation of 2,4,5-TCP (see the results of the primary enrichment cultures) strongly supports that 2,5-D was the first transient intermediate. 2,5-DCP was further transformed to 3-CP. This was in accordance with results of Gibson and Suflita (1990), Bryant (1992) and Chang *et al*. (1998).

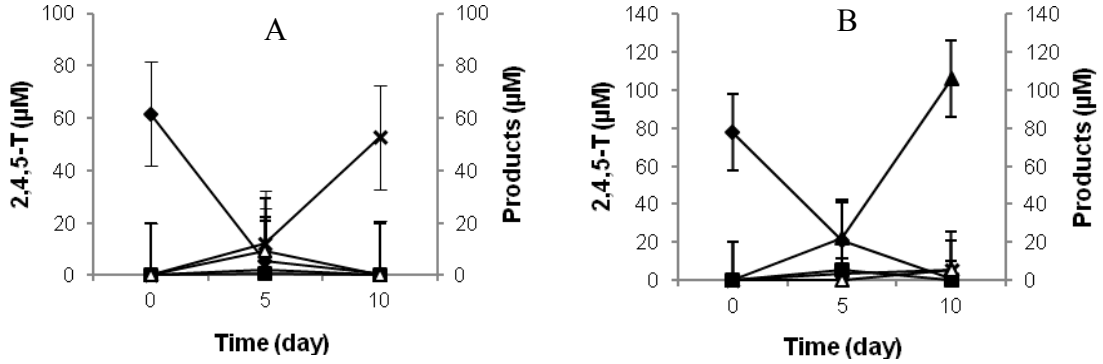

Fig 1. Dynamics of 2,4,5-T transformation in cultures from BH4.3 (A) and BH4.4 (B) under anaerobic conditions. Symbols: solid diamond, 2,4,5-T; solid square, 2,4,5-TCP; solid triangle, 3,4 DCP; multiplication, 3-CP; empty triangle, 2,5-DCP

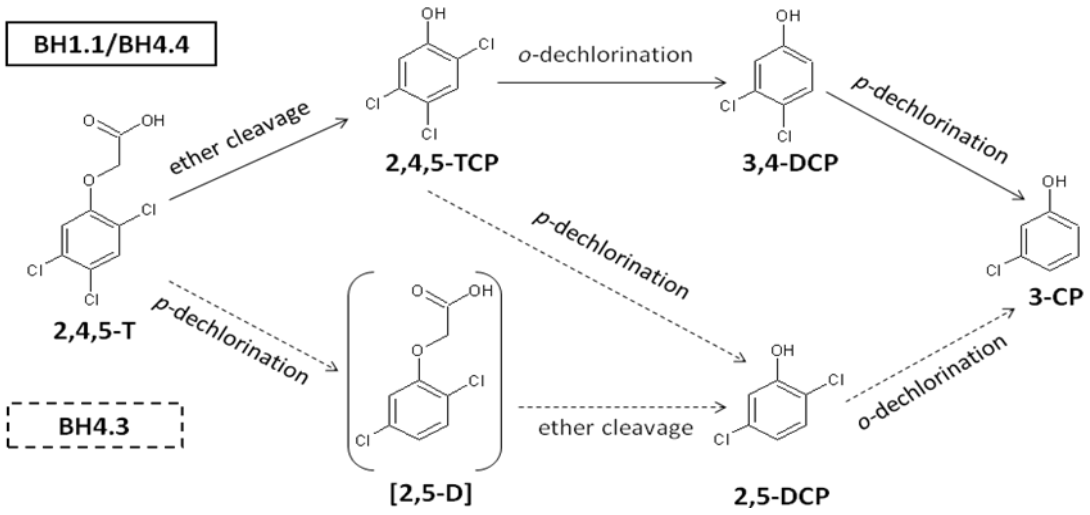

Fig 2. Proposed anaerobic transformation of 2,4,5-T in the Bien Hoa biotreatment cells as inferred from products observed in enrichment cultures. The main transformation routes were via 2,4,5-TCP and 3,4-DCP in BH1.1 and BH4.4 (solid lines) and via 2,5-DCP in BH4.3 (broken lines) cultures.

Our results demonstrate that a highly active and diverse herbicide transforming and dechlorinating anaerobic community exists in the bioremediation cells. The occurrence of two different pathways of 2,4,5-T transformation suggests that different organisms are present at different sites in the cells of the active landfill. The primary formation of 2,5-D might be more advantageous with respect to desired bioremediation of 2,4,5-T because it results in formation of a less chlorinated product, 3-CP, which might be finally degraded by aerobic or facultative anaerobic bacteria.

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