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Bioaugmentation of butane-utilizing microorganisms to promote cometabolism of 1,1,1-trichloroethane in groundwater microcosms

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Abstract

The transformation of 1,1,1-trichloroethane (1,1,1-TCA) in bioaugmented and non-augmented microcosms was evaluated. The microcosms contained groundwater and aquifer materials from a test site at Moffett Field, Sunnyvale, CA. The initial inoculum for bioaugmentation was a butane-utilizing enrichment from the subsurface of the Hanford DOE site. The non-augmented microcosm required 80 days of incubation before butane-utilization was observed while the augmented microcosms required 3 days. Initially the augmented microcosms were effective in transforming 1,1,1-TCA, but their transformation ability decreased after prolonged incubation. The non-augmented microcosms initially showed limited 1,1,1-TCA transformation but improved with time. After 440 days, both the non-augmented and augmented microcosms had similar transformation yields (0.04 mg 1,1,1-TCA/mg butane) and had similar microbial composition (DNA fingerprints). Subsequent microcosms, when bioaugmented with a Hanford enrichment that was repeatedly grown in 100% mineral media, did not effectively grow or transform 1,1,1-TCA under groundwater nutrient conditions. Microcosm tests to study the effect of mineral media on transformation ability were performed with the Hanford enrichment. Microcosms with 50% mineral media in groundwater most effectively utilized butane and transformed 1,1,1-TCA, while microcosms with groundwater only and microcosms with 5% mineral media in groundwater lost their 1,1,1-TCA transformation ability. DNA fingerprinting indicated shifts in the microbial composition with the different mineral media combinations. Successful bioaugmentation was achieved by enriching butane-utilizers from Moffett Field microcosms that were effective in groundwater with no mineral media added. The results suggest that successful in-situ bioaugmentation might be achieved through the addition of enriched cultures that perform well under subsurface nutrient conditions.

Abbreviations: 1,1,1-TCA – 1,1,1-trichloroethane; 1,1-DCA – 1,1-dichloroethane; 1,1-DCE – 1,1-dichloroethylene; CAH – chlorinated aliphatic hydrocarbon; GC – gas chromatograph; MCL – maximum concentration limit; MF – Moffett Field; p-MMO – particulate methane monooxygenase ; s-MMO – soluble methane monooxygenase; TCE – trichloroethylene; TSS – total suspended solids.

Introduction

A contaminant frequently found in groundwater in the United States is the chlorinated aliphatic hydrocarbon (CAH) 1,1,1-trichloroethane (1,1,1-TCA). Its ubiquity in the environment is due to its wide use as a solvent in industry and in household products (NRC 1994; Pankow and Cherry 1996). Subsurface contamination with 1,1,1-TCA is exacerbated by its relatively high solubility (1350 g/m^3 at 20 °C, Mercer and Cohen 1990) and limited sorption, resulting in rapid transport in the subsurface. The water maximum concentration limit (MCL) in drinking water of 1,1,1-TCA of 0.2 g/m^3 is five orders of magnitude lower than its solubility in water.

Transformation of 1,1,1-TCA is possible through various processes. 1,1,1-TCA is abiotically transformed in water to form 1,1-dicloroethylene (1,1-DCE; MCL of 0.005 g/m^3) via a dehydrohalogenation reaction (Vogel and McCarty 1987). 1,1,1-TCA is also biologically transformed by anaerobic or aerobic processes (McCarty and Semprini 1994). The anaerobic transformation of 1,1,1-TCA leads to the formation of 1,1-dichloroethane (1,1-DCA), a product that can be more resistant to further transformation (Vogel and McCarty 1987). Aerobic transformation of 1,1,1-TCA results in products less resistant to further transformation into innocuous byproducts. The transformation of 1,1,1-TCA by aerobic conditions is via cometabolism (McCarty and Semprini 1994). Several cometabolic substrates have been used to grow microorganisms capable of transforming 1,1,1-TCA and include methane (Broholm et al. 1990; Strand et al. 1990), butane (Kim et al. 1997a; Kim et al. 1997b) and propane (Keenan et al. 1993; Tovanabootr and Semprini 1998).

In-situ aerobic cometabolism of CAHs has been tested in a shallow aquifer at the Moffett Field (MF) test site where 1,1,1-TCA is present as a contaminant (Roberts et al. 1990). MF was a Naval Air Base in Sunnyvale (CA) that was closed in 1994 and is now under the custody of the NASA Ames Research Center. Substrates used in in-situ tests or in microcosm studies with groundwater and aquifer solids from MF include: phenol (Hopkins et al. 1993a; Hopkins et al. 1993b; Hopkins and McCarty 1995), toluene (Hopkins et al. 1993b; Hopkins and McCarty 1995), methane (Lanzarone and McCarty 1990; Semprini et al. 1990; Semprini and McCarty 1991; Hopkins et al. 1993b) and ammonia (Hopkins et al. 1993b). However, none of these substrates were effective towards inducing 1,1,1-TCA cometabolism. 1,1-DCE was also found to be a strong inhibitor for tricloroethylene (TCE) cometabolism (Hopkins and McCarty 1995). Thus the study of systems that might be effective towards transforming both 1,1,1-TCA and 1,1-DCE is of interest.

The transformation of CAHs by butane-utilizers is thought to occur through oxygenase enzyme system(s). The transformation ability among different butane-utilizers differs; e.g. two butane-utilizing cultures, isolate CF8 (Hamamura et al. 1997, Hamamura and Arp 2000) and *Pseudomonas butanovora*, were able to degrade chloroform, trichloroethylene (TCE) and vinyl chloride, but only isolate CF8 was able to degrade 1,1,2-trichloroethane (Hamamura et al. 1997). Kim et al. (1997a) found that microcosms with stimulated butane-utilizers effectively transformed 1,1,1-TCA at aqueous concentrations as high as 2400 μg/L but butane-utilizers stimulated in microcosms with groundwater and aquifer solids from the McClellan Air Force Base (CA) showed effective butane utilization but no ability to transform 1,1,1-TCA (Tovanabootr and Semprini 1998). These results suggested that indigenous butane-utilizers at different sites have different potentials for 1,1,1-TCA cometabolism. It may, however, be possible to bioaugment into the subsurface butane-utilizers enriched in the laboratory with the ability to cometabolize 1,1,1-TCA. Bioaugmentation is an effective way to transform the target contaminant, improving transformation efficiency and reducing long lag times to establish a viable population (Frosyth et al. 1995; Mayotte et al. 1996; Munakata-Marr et al. 1996; Vogel 1996; Munakata-Marr et al. 1997a; Munakata-Marr et al. 1997b; Dybas et al. 1997).

One of the major goals in bioaugmentation is to create and maintain the appropriate environment for the inoculated strain to grow and survive (Vogel 1996). In this study we tested butane as a substrate for growing microorganisms capable of cometabolizing 1,1,1-TCA. The studies were conducted in batch-fed microcosms constructed with groundwater and aquifer soils from the MF test site. Non-augmented microcosms from the MF test site subsurface were stimulated with butane, and their 1,1,1-TCA transformation abilities were compared with bioaugmented microcosms. Since inoculated strains were grown in mineral media in the laboratory, the effects of mineral media addition on bioaugmentation was also evaluated. DNA fingerprinting was used to determine whether different microbial populations were stimulated under the different mineral media conditions. The development of an enrichment that performs well under groundwater conditions with no nutrient amendments is also discussed.

Materials and methods

Chemicals

Butane (10.2% in nitrogen), 1,1,1-TCA and TCE (99.9% {GC} grade) were obtained from Aldrich Chemical Co. (Milwaukee, WI). GeneAmp®PCR core reagents (Ampli Taq DNA Polymerase, dNTPs, 10X PCR buffer II and $MgCl_2$) were purchased from Perkin Elmer (Foster City, CA). All reagents used to prepare

mineral growth media were reagent grade from Fisher Scientific (Pittsburgh, PA) or from Sigma Chemical Co. (St. Louis, MO).

Microcosm preparation and operation

Batch-fed microcosms were constructed using 125 ml amber serum bottles (Wheaton Glass Co., Millville, NJ). The bottles were filled with 15 ml of aquifer core material and 50 ml groundwater. Both groundwater (Table 1) and aquifer core material were collected from the Stanford Test Facility at Moffett Field (MF). The aquifer solids were aseptically collected from the inner portion of 5-cm cores to avoid microbial contamination. The solids were wet-sieved through a No. 8 (2.38 mm opening) sieve under a laminar flow hood to remove large pebbles. Details of the microcosm construction are provided by Kim et al. (1997a). The bottles were crimp-sealed with a Teflon^{TM}/butyl rubber septum to accommodate frequent sampling. Butane was added to each microcosm by the gaseous addition of 18.6 ml of 10% butane in nitrogen (4.5 mg). The required volume of saturated aqueous solution of 1,1,1-TCA was added to the microcosm to achieve the desired initial mass addition. Microcosms with 1,1,1-TCA added, but with no butane, served as controls.

The microcosms were incubated at room temperature (22 °C) on a rotary shaker at 100 rpm and the butane and 1,1,1-TCA concentrations in the headspace monitored. The gas pressure was equilibrated to atmospheric pressure by adding oxygen to the headspace. After each batch incubation with butane and 1,1,1-TCA, the microcosm groundwater was exchanged to replenish mineral nutrients and to prevent by-product accumulation. Prior to exchanging the groundwater, the microcosms were centrifuged at 2,000 rpm (RCF 510) for 20 min to settle aquifer materials and cells. The rubber septa were replaced under a laminar flow hood and 60% of the groundwater was exchanged. The mass of 1,1,1-TCA added was gradually increased as the batch exchanges proceeded, while the amount of butane fed was held constant. This permitted estimates of a transformation yield (mass of 1,1,1-TCA transformed/mass of butane degraded). The total mass of butane utilized and 1,1,1-TCA transformed was estimated using the headspace concentration by applying the appropriate Henry's Law constants (Mackay & Shiu 1981) and determining the masses in the headspace and aqueous phases.

Table 1. Basic media (Kim et al. 1997a) and groundwater (Roberts et al. 1989) chemical composition

Major ions	Concentration in basic media	Concentration in groundwater
Cations		
Na^+	23.7 mM	2.3 mM
K^+	None added	0.07 mM
$Ca2^+$	0.1 mM	5.0 mM
$NH4^+$	0.5 mM	<0.01 mM
$Mg2^+$	None added	4.17 mM
Anions		
Cl^-	0.2 mM	1.2 mM
Br^-	None added	0.01 mM
HCO_3^-	None added	5.51 mM
NO_3^-	23.5 mM	0.14 mM
PO_4^{3-}	15.0 mM	0.001 mM
SO_4^{2-}	0.5 mM	9.38 mM

Trace chemicals		Dissolved	Total
Fe	22.60 μM	Not detected	9.64 μM
Mn	1.50 μM	5.45 μM	5.64 μM
B	1.00 μM	13.64 μM	18.18 μM
Zn	0.50 μM	0.15 μM	0.46 μM
Sr	None added	0.77 μM	0.87 μM
Ba	None added	0.15 μM	0.15 μM
Co	0.10 μM	Not detected	Not detected
Mo	0.45 μM	Not detected	Not detected
Ni	0.10 μM	Not detected	Not detected

Growth of bioaugmentation enrichments

The bioaugmentation enrichments were grown in 275 ml Wheaton media bottles (Wheaton Glass Co., Millville, NJ) filled with 175 ml of mineral salt growth media. Butane-utilizers used in the initial bioaugmentation study and the study of mineral-nutrient effects on bioaugmentation were from enrichments obtained from the Hanford (WA) DOE site (Kim et al. 1997a). For the final bioaugmentation studies microorganisms were obtained from MF soil microcosms after 440 days of stimulation.

The mineral salt medium composition is described in Table I and elsewhere (Kim et al. 1997a). Five milliliters of suspended liquid taken from a given enrichment were used to inoculate the growth bottles. Butane was added to the bottles (20% vol/vol in the headspace). The growth bottles were placed on a rotary shaker at 100 rpm at 30 °C and grown to optical densities (OD_{600}) of 1.3 to 1.9 (30 days). The

cells were washed with groundwater to remove growth media before adding them to the microcosms. Pressures were equilibrated daily to atmospheric pressure by adding oxygen.

Studies of nutrient effects on bioaugmentation

Three sets of augmented microcosms with varying ratios of groundwater and mineral salt growth media were prepared. The mineral salt growth media used was the same as in the growth bottles. Each set contained 2 microcosms, one with butane and 1,1,1-TCA added, the other with only butane added. The microcosms contained 5 mg cell mass, as measured by total suspended solids (TSS), 5 ml of aquifer material, and 50 ml of liquid with the various ratios of groundwater and mineral media. The compositions of the liquid in the microcosms by percent were: 100% groundwater; 95% groundwater with 5% mineral media; and 50% groundwater with 50% mineral media. Butane and 1,1,1-TCA were added in the same fashion as in the bioaugmentation study.

Analytical methods

TCE and 1,1,1-TCA were analyzed by withdrawing 100 μl of headspace gas from the microcosms and injecting the sample into a Hewlett Packard 5890 gas chromatograph (GC) equipped with a capillary column (HP-624 19091 v-433; 30 m $\times$ 0.25 mm $\times$ 1.4 μm, Hewlett Packard, Wilmington, DE) and electron capture detector. The oven temperature was set to 80 °C. Butane was also analyzed by withdrawing 100 μl of headspace gas from the microcosms. The sample was then injected into the GC but now equipped with a 1.0-m Hayesep Q stainless steel micropacked column (Restek Corporation, Bellefonte, PA) and a flame ionization detector. The oven temperature was 120 °C. The concentrations were estimated by comparison to external standards with known concentrations.

DNA fingerprinting

Liquid/solid samples (500 μL) were obtained from well-shaken microcosms for DNA fingerprinting using the polymerase chain reaction (PCR). Total DNA was extracted as described (Wilson, 1994). The DNA was dissolved in water and gel-purified. Gel plugs containing the DNA were cut and immersed in water to diffuse the DNA into solution. An aliquot from the diffused DNA was used for the PCR. The PCR was carried out as described by the manufacturer (Perkin Elmer Foster City, CA) using the arbitrary primer GCA12 (CGT GCC GAG CTG) (Caetano-Anolles and Bassam 1993). In this technique the primer anneals randomly to genomic DNA, but because a limited number of annealing sites with opposite orientation within a range for PCR amplification are available, the result is a limited number of amplified DNA fragments. The amplified DNA fragments are a "fingerprint" characteristic of the DNA source. The PCR amplification was carried out in a thermocycler (Ericomp Power Block^{TM} Easy Cycler^{TM} series). Cycling conditions were 95 °C for 45 sec, 47 °C for 45 sec and 72 °C for 60 sec, 10 times, then 92 °C for 45 sec, 45 °C for 45 sec and 72 °C for 90 sec, 20 times. After this process, DNA fragments were resolved by electrophoresis. Electrophoresis was carried out in a 7 $\times$ 9 $\times$ 0.75 cm 7% polyacrylamide/bisacrylamide gel prepared in TBE (0.089 M Tris-borate, 0.002 M EDTA). 20 μl of sample containing 0.02% bromophenol blue as tracking dye were loaded into the gel. The gel was run in TBE at 70 V in a Hoefer (Piscataway, NJ) SE200 apparatus. The DNA was visualized by staining the gel at the end of the run with ethidium bromide.

Results and discussion

Indigenous versus augmented butane-utilizers

The transformation of 1,1,1-TCA was determined in microcosms stimulated by the addition of butane, with and without the augmentation of butane-utilizing microorganisms. Two microcosms (B1 and B2) were initially fed butane to stimulate indigenous butane-utilizers and were then challenged with 1,1,1-TCA. Butane consumption and 1,1,1-TCA transformation in the two microcosms were monitored during the first 94 days of stimulation (Figure 1). By day 57, no substrate utilization was observed in either microcosm. To test for bioaugmentation potential, B2 was inoculated with cells from the Hanford butane-utilizing enrichment. After three days of further incubation (day 60), the concentration of butane in B2 started to decrease implying the establishment of butane-utilizing microorganisms. Concomitantly, the concentration of the 1,1,1-TCA decreased in B2, and by the time all the butane was completely utilized, 90% of 1,1,1-TCA was transformed. Minimal loss of 1,1,1-TCA was observed in the control microcosm that lacked substrate addition. The non-augmented microcosm B1

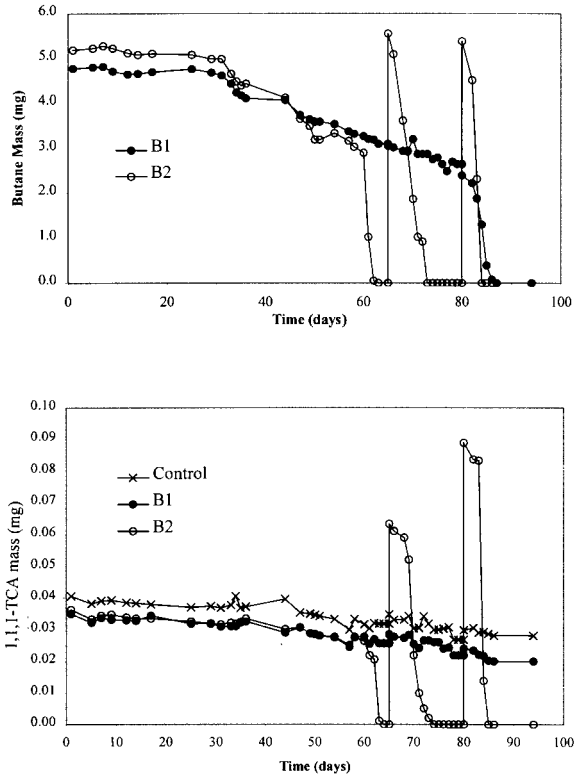

Figure 1. Butane degradation and 1,1,1-TCA transformation in microcosms B1 and B2 (bioaugmented) during the first 94 days of stimulation.

began to utilize butane after 80 days of incubation. Although the butane was completely consumed in B1, no 1,1,1-TCA transformation was observed. The different 1,1,1-TCA transformation abilities of B1 and B2 was probably due to the establishment of different microbial populations (indigenous and augmented). The 80-day lag observed in B1 can be attributed to a very low initial microorganism number coupled with a slow rate of growth. However, we can not rule out that butane utilization in B1 by day 80 was possibly due to microbial contamination introduced by the repeated sampling. Replicates that are not sampled as frequently are recommended in future studies to reduce the possibility of microbial contamination.

Shorter lag times for the establishment of microbial populations have been observed in other systems. For example, lag times of about 10 to 15 days are common for methane-utilizers in MF aquifer solids/groundwater microcosms (Lanzarone and McCarty 1990), and lag times of approximately 25 days were observed in microcosms from both the Hanford DOE Site, WA (Kim et al. 1997a), and the McClellan AFB, CA (Tovanabootr and Semprini 1998). It is interesting to note that Lanzarone and McCarty (1990) did not stimulate propane-utilizers in their MF column studies. Thus microbes that grow on propane or butane are either absent or are at very low numbers at the MF test site.

Long term performance of augmented and non-augmented microcosms

The responses of microcosms B1 and B2 between 100 and 425 days of stimulation are shown in Figures 2 and 3. Whenever the butane in the B1 and B2 microcosms was depleted, the groundwater was exchanged, the butane was replenished, and the 1,1,1-TCA concentration was increased. Microcosm B1 started to show some limited 1,1,1-TCA transformation after 130 days of stimulation, and by 190 days, 90% of the 1,1,1-TCA added was being transformed (Figure 2). Transformation yields in the non-augmented and augmented microcosms for three different time periods are presented in Figure 4. The transformation yield represents the amount of 1,1,1-TCA transformed to the amount of butane consumed in each incubation batch (Tovanabootr and Semprini 1998). The yields are conservative estimates since, in many of the incubations, all of the 1,1,1-TCA present was transformed. With repeated incubations, the transformation yields of microcosm B1 gradually improved while those of the bioaugmented microcosm (B2) decreased. After 340 days, the transformation yields in B1 and B2 were nearly identical. It is possible that the population shifted with the repeated stimulation by butane and by the increasing exposure to 1,1,1-TCA. The results indicate that short-term microcosm studies may not accurately predict long-term performance.

The maximum 1,1,1-TCA transformed in microcosm B2 was 0.71 mg at an aqueous concentration of 8.3 mg/L. This resulted in a transformation yield of 0.16 mg 1,1,1 TCA/mg butane. The highest 1,1,1-TCA transformation efficiency was obtained during the first 190 days of stimulation when butane was rapidly consumed. Only 20% of the 1,1,1-TCA was transformed when 1 mg of 1,1,1-TCA at an aqueous concentration of 11.7 mg/L was added to microcosm B2 on day 220. The limited 1,1,1-TCA transformation was associated with a reduction in the rate of butane consumption. After 340 days, the 1,1,1-TCA mass was reduced to 0.27 mg (3.16 mg/L), resulting in an increase of 1,1,1-TCA transformation efficiency. The

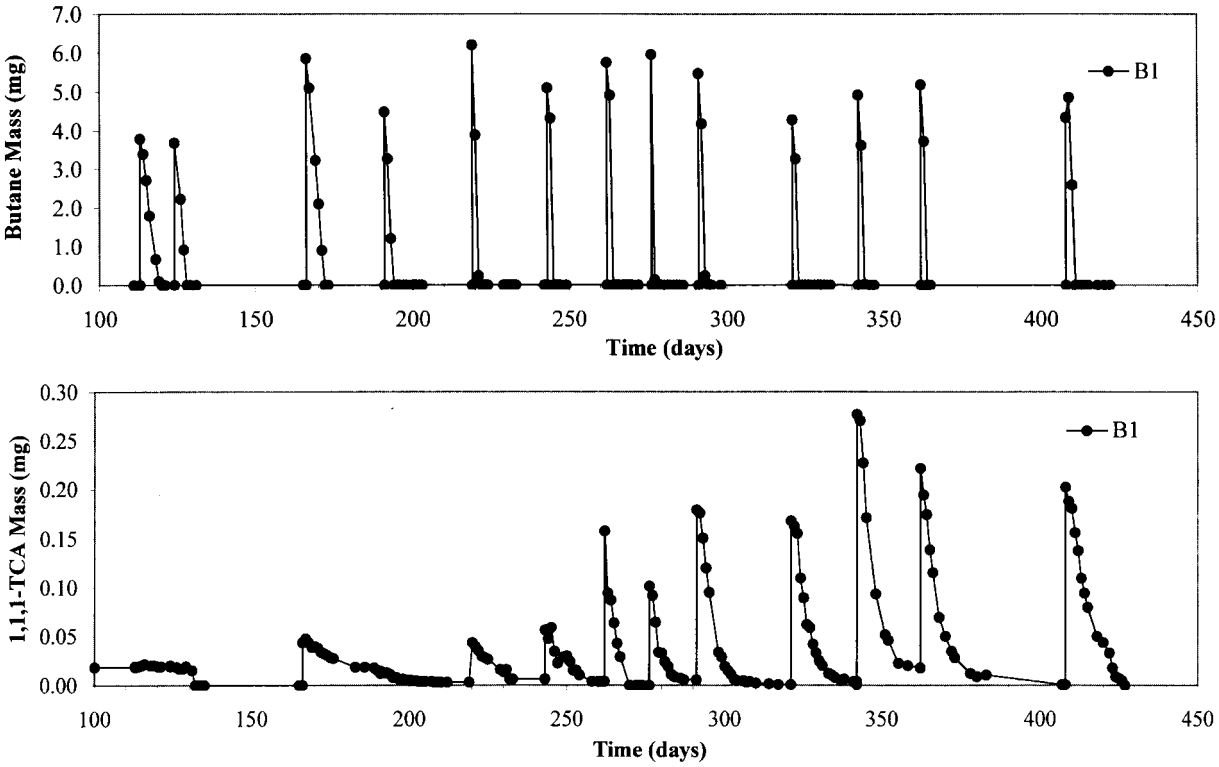

Figure 2. Butane degradation and 1,1,1-TCA transformation in microcosms B1 from 100 to 420 days of stimulation.

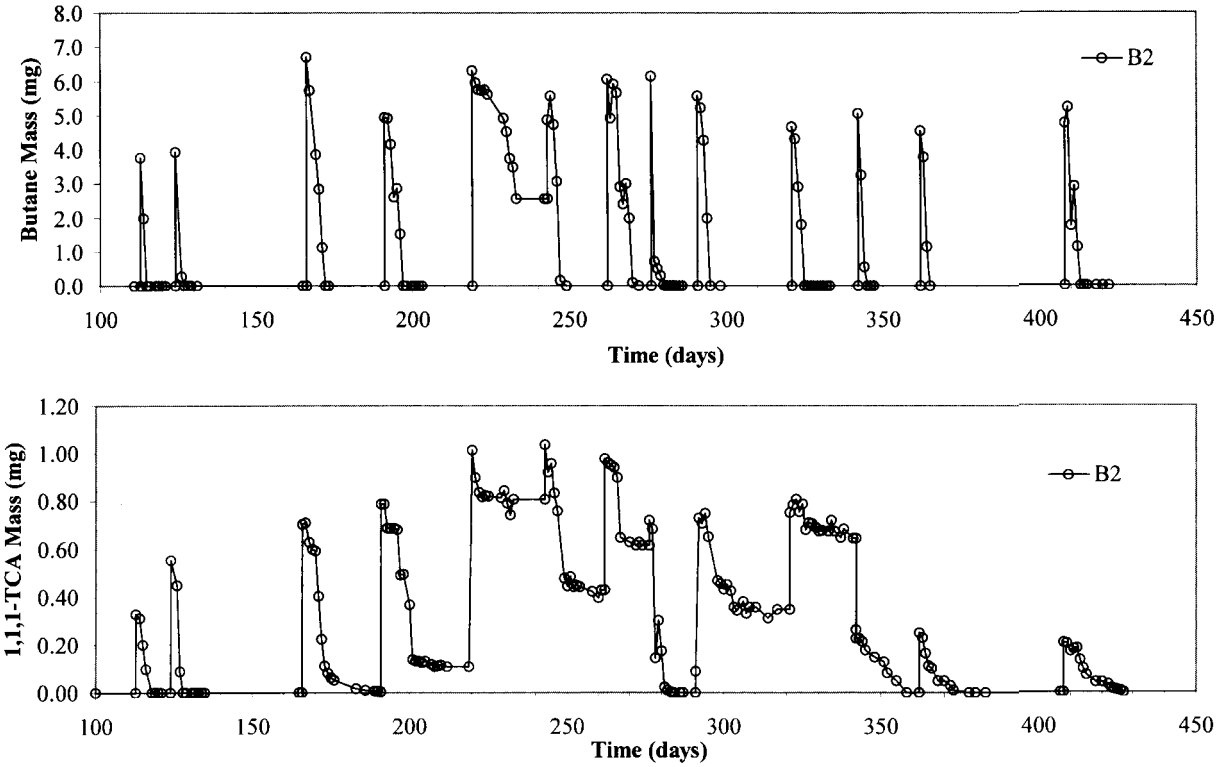

Figure 3. Butane degradation and 1,1,1-TCA transformation in microcosms B2 from 100 to 430 days of stimulation.

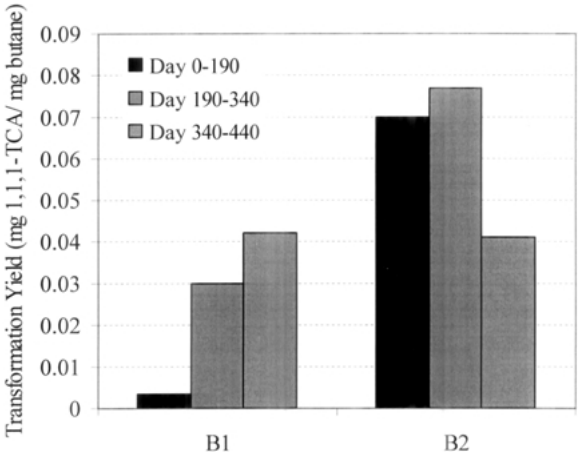

Figure 4. Transformation yields achieved by microcosms B1 and B2 at three different time periods.

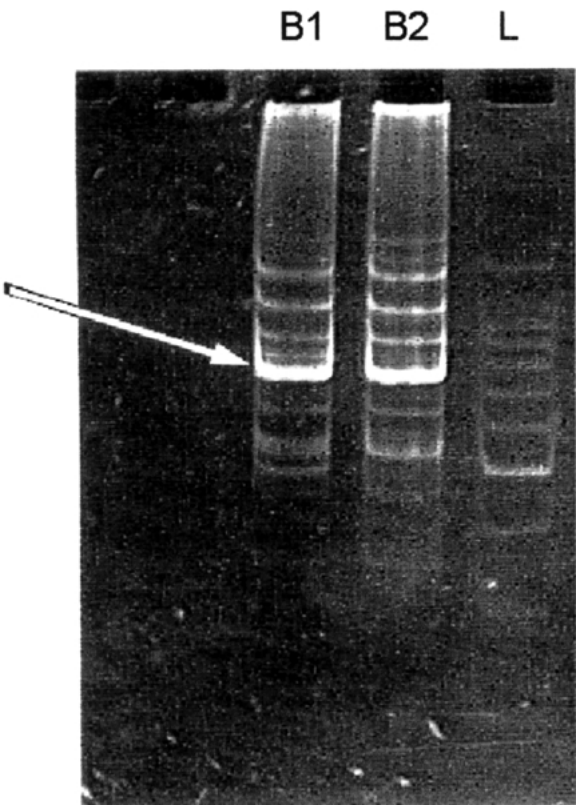

Figure 5. DNA fingerprints of butane-utilizers obtained from microcosms B1 and B2 on day 440 (L = Ladder).

control bottle showed little loss during the 425 days of the study.

In both microcosms, 1,1,1-TCA transformation continued after the butane was consumed indicating that the enzymes promoting the transformation remained active in the absence of substrate utilization. For example, on day 320, the butane was totally consumed after 3 days, while 1,1,1-TCA transformation continued for almost 20 days (Figure 2). Similar long-term activity was observed by Kim et al. (1997a) in butane-fed Hanford microcosms transforming 1,1,1-TCA.

The DNA fingerprints (PCR analysis) after 440 days of stimulation showed similar DNA fragment sizes in both microcosm samples, suggesting that the microbial populations in both microcosms were similar (Figure 5). A possibility is that an indigenous population was gradually enriched and predominated in both microcosms at the time tested. Unfortunately, microbial samples for PCR analysis from the early stages of this study were not available to show if initially different mixed populations existed in the microcosms. These DNA fingerprints do not specify the different microorganisms or their numbers in the microcosms. More detailed studies are required to establish the identity of the microorganisms in the microcosms by phylogenetic analysis of their 16S ribosomal genes (Li 1997), an approach that will be used in future studies.

Mineral media effect on 1,1,1-TCA transformation

Bioaugmentation tests were repeated with the Hanford enrichment that had been repeatedly enriched in mineral media for over 150 days. The bioaugmentation of aquifer solids/groundwater microcosms proved unsuccessful. This likely resulted from the selection of butane-utilizing strains that perform well only in a nutrient-rich environment. To test the effect of the addition of mineral media, three new sets of microcosms with different ratios of groundwater and mineral media were prepared and augmented with the Hanford enrichment that was grown in mineral media (Figure 6). Butane and 1,1,1-TCA were added to microcosms BT3, BT4 and BT5, while only butane was added to microcosms B3, B4, and B5. The microcosms were treated in the same manner as the B1 and B2 microcosms.

Microcosms B3 and BT3 (groundwater only) had the slowest rate of butane utilization and 1,1,1-TCA transformation, while microcosms B5 and BT5 (50% mineral media) had the fastest rates (Figure 6). The butane utilization rates between each pair of microcosms (B3 and BT3, or B4 and BT4, or B5 and BT5) were similar, indicating that 1,1,1-TCA aqueous concentrations of up to 3.51 mg/L did not inhibit butane utilization. The amount of mineral media added was

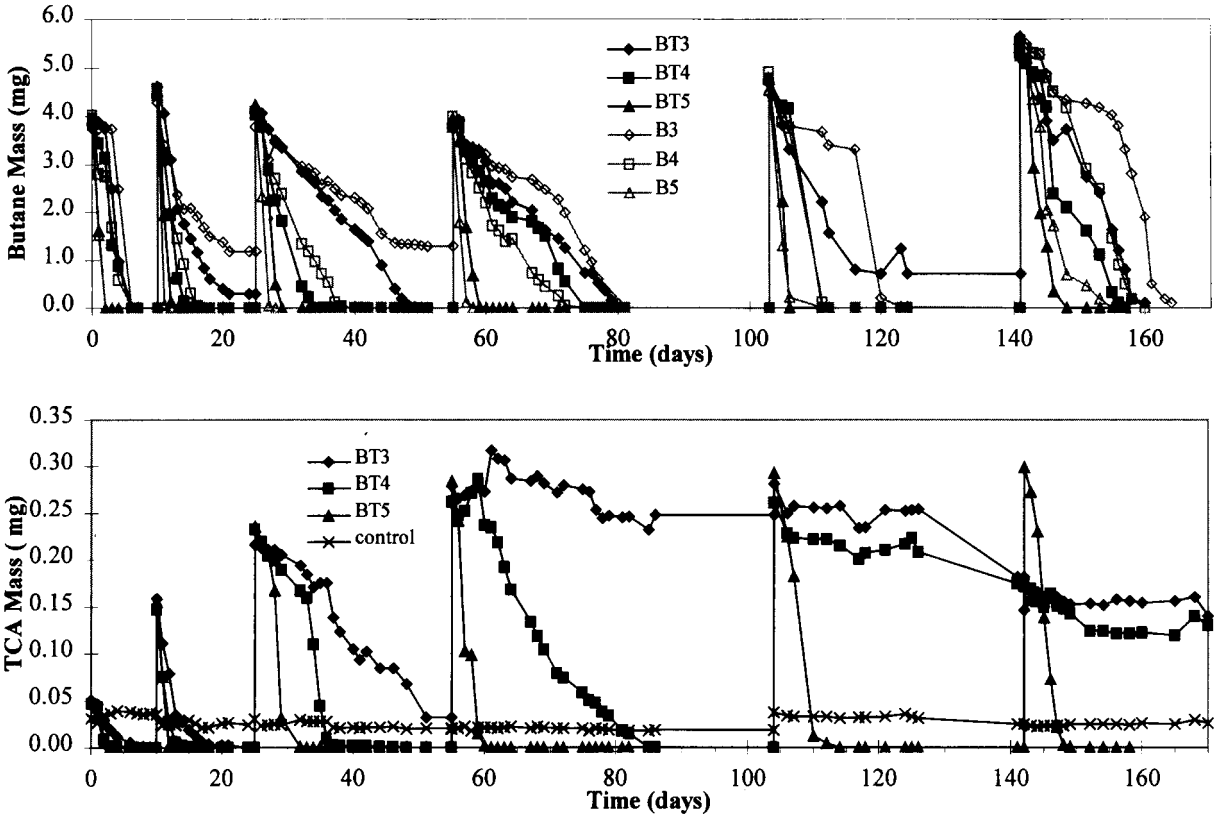

Figure 6. Butane-utilization and 1,1,1-TCA transformation in microcosms bioaugmented with the Hanford Enrichment. The microcosms had the following amounts of nutrient addition: B3 and BT3 (groundwater only); B4 and BT4 (5% mineral media and 95% groundwater); B5 and BT5 (50% mineral media and 50% groundwater). The BT series had butane and 1,1,1-TCA added while the B series had only butane added.

the main factor for the differences in butane utilization rates among the microcosm pairs.

In microcosms B3 and BT3 (groundwater only) the rates of butane utilization and 1,1,1-TCA transformation decreased with repeated additions of butane, and 1,1,1-TCA transformation eventually ceased (Figure 6). Microcosms B4 and BT4 (5% mineral media in groundwater) showed similar trend but with faster rates of butane consumption and 1,1,1-TCA transformation. After 105 days of incubation, less than 10% of the 1,1,1-TCA added was transformed. The results indicated that bioaugmented enrichments needed mineral media to maintain 1,1,1-TCA transformation efficiencies, and that 1,1,1-TCA transformation was associated with effective rates of butane utilization. 5% mineral media in groundwater was inadequate for the prolonged treatments. Microcosms B5 and BT5 displayed high activities throughout the study, indicating that the 50% mineral media supplement helped maintain the enrichments' transformation efficiencies.

To evaluate the microbial composition of the microcosms, DNA fingerprints were carried out. DNA fingerprints of samples taken on day 80 are shown in Figure 7. Of the fragments amplified, fragment A is present in all cultures, and fragment B appears in cultures grown only in groundwater. The other minor fragments may be correlated to the relative concentration of the different microorganisms in the mixed culture. These minor fragments appear at various positions in the different treatments. Comparison of the DNA fingerprints among microcosms shows greater differences resulting from the mineral media amendments than from the exposure to 1,1,1-TCA. The transformation of 1,1,1-TCA appears to have caused less of an effect on the mixed population than the variations in the amount of mineral nutrients.

Another set of DNA fingerprints was obtained on day 160 after the 1,1,1-TCA transformation activity ceased in microcosms BT4 and BT5 (not shown). The major bands (A and B) observed in day 80 were present. However, the minor bands were slightly dif-

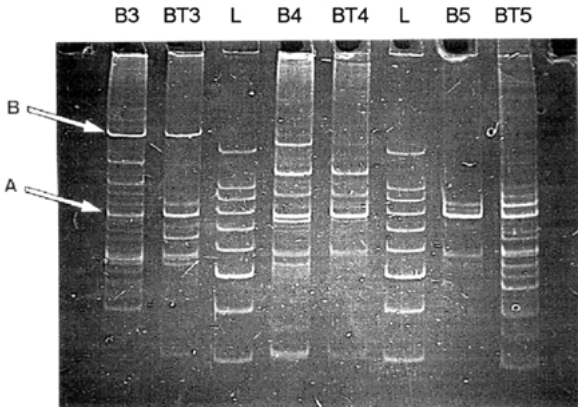

Figure 7. DNA fingerprints of microbial populations obtained from microcosms B3, B4, B5, BT3, BT4 and BT5 on day 80 (L = Ladder).

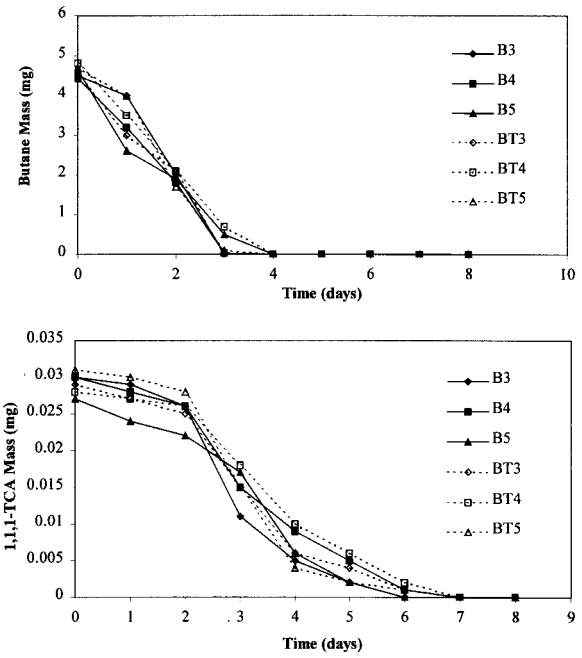

Figure 8. Butane degradation and 1,1,1-TCA transformation in batch bottles with mineral media that were inoculated with enrichments from B3, B4, B5, BT3, BT4 and BT5.

ferent. This suggested that the microbial population had likely changed between days 80 and 160 of stimulation. Again, changes in the mineral salt media caused more variations in the DNA profiles than the exposure to 1,1,1-TCA.

Recovery of transformation activity

Tests were performed to determine if rapid butane uptake and effective TCA transformation could be recovered in cultures that lost their 1,1,1-TCA transformation ability in the nutrient study (Figure 6). At the end of the nutrient study, butane-utilizers from six microcosms were each inoculated in separate batch-growth bottles. A 5-ml soil groundwater slurry sample from a microcosm was added to a growth bottle containing mineral media and butane, and headspace air. The enrichments were incubated for 30 days in the absence of 1,1,1-TCA and were then tested for 1,1,1-TCA transformation ability (Figure 8). Enrichments from BT3 and BT4, which stopped transforming 1,1,1-TCA in soil microcosms, transformed 1,1,1-TCA after regrowth in mineral media. All the microcosms transformed 1,1,1-TCA at similar rates, including enrichments from B3, B4 and B5 that had not been exposed to 1,1,1-TCA in the microcosm tests. The results indicated that butane degradation and 1,1,1-TCA transformation efficiencies could be recovered after growth in mineral media. Previous exposure to 1,1,1-TCA did not alter the transformation efficiencies. The replenishment of the endogenous energy cell reserves combined with new cell growth through effective butane utilization likely caused the recovery of 1,1,1-TCA transformation ability.

Bioaugmentation of the butane-utilizers from microcosms B1 and B2

Butane-utilizers in the augmented (B2), and eventually in the non- augmented (B1), microcosms transformed 1,1,1-TCA efficiently under the groundwater conditions with no mineral media addition (Figure 2 and 3). Tests were conducted to determine whether bioaugmentation would be more successful if these strains were enriched and augmented into aquifer-solids/groundwater microcosms that lacked mineral media additions. On day 440 of the microcosm study, 5-ml soil groundwater slurry from microcosms B1 and B2 were inoculated in growth bottles and incubated in mineral media with 10% butane in the headspace. After growth to an optical density (OD_{600}) between 1.3 to 1.9, 5 mg of groundwater-washed cells were reinoculated into new aquifer solids/groundwater microcosms that lacked nutrient amendments.

Rapid butane utilization and 1,1,1-TCA transformation were achieved in the augmented microcosms B1A and B2A (Figure 9). This is in contrast to the long lags in the original microcosms B1 and B2 (Figure 1) and those in a repeated microcosm stimulation with aquifer solids/groundwater from the MF site (data not shown). The mass of 1,1,1-TCA added was increased

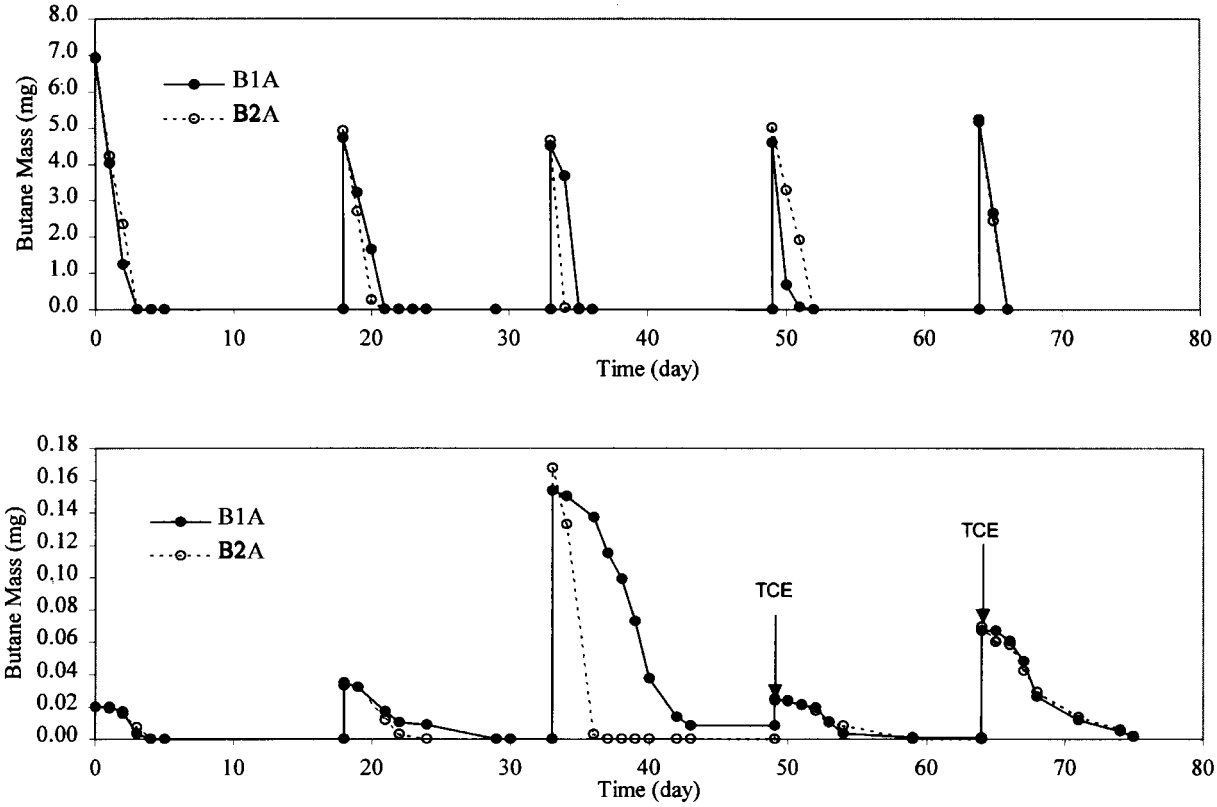

Figure 9. Butane degradation and 1,1,1-TCA transformation of groundwater microcosms B1A and B2A inoculated with enrichments obtained from microcosms B1 and B2.

to 0.17 mg on the third addition, and transformation yields of 0.037-0.04 mg 1,1,1-TCA per mg butane were achieved. This was close to the average transformation yields observed in the original microcosms between days 340 and 440 (Figure 4). After three incubations with butane and 1,1,1-TCA, the microcosms were tested for their TCE transformation abilities (Figure 9). TCE transformation yields of 0.015 mg TCE per mg butane were achieved (Figure 9).

These results indicate that augmented butane-utilizers were able to effectively grow under groundwater conditions after only a single enrichment in mineral media prior to inoculation. After three additions of butane and 1,1,1-TCA, butane was more rapidly consumed and 1,1,1-TCA was more rapidly transformed compared with butane- utilizers grown under groundwater conditions (BT3 in Figure 6). These microcosms, however, were not operated for as long a period at high concentrations of 1,1,1-TCA as those presented in Figure 6. Thus, the long term effectiveness that can be achieved requires further study.

Conclusions

The results demonstrate that butane-utilizers are capable of transforming 1,1,1-TCA in microcosms containing aquifer solids and groundwater from the MF test site. Previous microcosm studies with groundwater and aquifer solids from the test site showed no, or limited, ability to transform 1,1,1-TCA when methane, phenol, toluene, or ammonia were used as cometabolic substrates. Concentrations of 1,1,1-TCA as high as 8 mg/L were completely transformed in the microcosm tests.

The DNA fingerprints confirmed that the presence or absence of mineral media supplements altered the community. Mineral media had to be added into the soil microcosms to assure effective butane utilization and 1,1,1-TCA transformation with the bioaugmented enrichments from the Hanford Site. However, it would be more practical to bioaugment with strains that were able to transform 1,1,1-TCA under groundwater nutrient conditions.

This work indicates that bioaugmentation has the potential for reducing lag periods and providing effective cometabolic cultures when such cultures are absent in the subsurface. Enrichments that perform well in groundwater nutrient conditions are needed for this approach. The butane-utilizers enriched from microcosms B1 and B2 were able to transform 1,1,1-TCA under groundwater nutrient conditions. These enriched cultures were first grown in mineral media and then inoculated into the microcosms. More studies are needed to determine how enrichments are best grown for bioaugmentation, what mineral media formulations work best, and whether concentrated minerals from the site groundwater can be used to grow the bioaugmented cultures.

Butane-utilizers may also have advantages over other alkane-utilizers for the in-situ transformation of 1,1,1-TCA. For example, at the MF site where mineral nutrients are present in the subsurface, butane-utilizers would likely perform better than methane-utilizers. During in-situ tests at the MF test site, methane-utilizers showed limited transformation of TCE and 1,1,1-TCA (Semprini et al. 1990). Methane-utilizers transform TCE and 1,1,1-TCA when the soluble methane monooxygenase (sMMO) is expressed, but transformation is limited when particulate methane monooxygenase (pMMO) is expressed (Oldenhuis et al. 1990). Particulate methane monooxygenase is expressed when copper is not limiting, as was likely the case at the MF site (Semprini, 1997). However, not all butane oxidizers effectively transform CAHs. For example, butane-utilizers stimulated in microcosms fabricated with groundwater and aquifer solids from the subsurface of the McClellan Air Force Base showed no ability to transform 1,1,1-TCA (Tovanabootr & Semprini, 1998). The composition of butane-utilizers and their transformation abilities likely differs among sites. While the bioaugmentation of effective butane-utilizing cultures requires future investigation, these results demonstrate the potential of this technique to achieve successful bioremediation.

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