Dual augmentation for aerobic bioremediation of MTBE and TCE pollution in heavy metal-contaminated soil

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Abstract

In this work we isolated from soil and characterized several bacterial strains capable of either resisting high concentrations of heavy metals (Cd^{2+} or Hg^{2+} or Pb^{2+}) or degrading the common soil and groundwater pollutants MTBE (methyl-*tert*-butyl ether) or TCE (trichloroethylene). We then used soil microcosms exposed to MTBE (50 mg/l) or TCE (50 mg/l) in the presence of one heavy metal (Cd 10 ppm or Hg 5 ppm or Pb 50 or 100 ppm) and two bacterial isolates at a time, a degrader plus a metal-resistant strain. Some of these two-membered consortia showed degradation efficiencies well higher (49–182% higher) than those expected under the conditions employed, demonstrating the occurrence of a synergetic relationship between the strains used. Our results show the efficacy of the dual augmentation strategy for MTBE and TCE bioremediation in the presence of heavy metals.

Keywords Bioremediation · Soil · Heavy metal · TCE · MTBE · Cadmium · Lead · Mercury

Introduction

Remediation of sites co-contaminated with organic and metal pollutants is a complex problem, as the two components often must be treated differently. A high proportion of hazardous waste sites are co-contaminated with organic and metal pollutants (Sandrin and Maier 2003).

MTBE (methyl-*tert*-butyl ether) is the most widely used fuel ether and is very soluble in water (40 g/l) and thus able to migrate easily to groundwater producing large contamination plumes in impacted aquifers (USEPA 1997). It is known that MTBE can be degraded by co-metabolism as was observed during growth of various microorganisms on different carbon substrates (Brusseau et al. 1990; Bowman et al. 1993; Fayolle et al. 2003).

TCE (trichloroethylene) is used mainly as a solvent to remove grease from metal parts, namely for cleaning aircrafts at air-force bases, but it is also an ingredient in adhesives, paint removers, typewriter correction fluids and spot removers. As a pollutant, it has been found in underground aquifers, many surface waters and soils as a result of its use and improper disposal (ATSDR 2003). In aerobic conditions TCE disappearance was related to the co-metabolic activity of local microflora: methanotrophs using either type of methane monooxygenase (Di-Spirito et al. 1991) or aromatic-degraders expressing enzymes such as toluene monooxygenase (Shields et al. 1995).

For clean up of groundwater and soils contaminated by chlorinated solvents natural attenuation may be inadequate and bioaugmentation may be the only practical option (Aulenta et al. 2005; Olaniran et al. 2006; Adebusoye et al. 2007).

Co-contaminated soils are considered difficult to remediate because of the mixed nature of the contaminants. Many microorganisms are known to degrade a variety of organics, and likewise a number of metal-resistant microorganisms are known to detoxify metals such as lead, mercury or cadmium. Some works have analyzed the possibility of remediation of organic pollution in heavy metal-contaminated soils (Roane et al. 2001; Lee et al. 2006).

To date, several studies have shown the acute effects of heavy metals on microbial communities. Results from these studies indicated that heavy metal amendments have a negative impact, resulting in severe reduction of metabolic activity, microbial biomass and bacterial abundance (Sandrin and Maier 2003). The inhibition of the degradation of organic pollutants is directly related to heavy metal concentrations. Subsequent shifts in the microbial community toward a more metal-tolerant or metal-resistant population has also been observed with increasing inputs of heavy metals (Sandrin and Maier 2003). Metals may inhibit remediation through interaction with the enzymes directly involved in biodegradation or through interaction with general microbial metabolism (Sandrin and Maier 2003).

Metal inhibition has also been observed in metal-contaminated soils systems. For example, cadmium added at levels of 60 mg total cadmium/kg was found to inhibit biodegradation of 2,4-dichlorophenoxyacetic acid (2,4-D) in a soil system inoculated with the 2,4-D-degrader *Ralstonia eutropha* strain JMP134. For rapid degradation of 2,4-D to be achieved, it was necessary to co-inoculate str. JMP134 and a cadmium-resistant isolate, *Pseudomonas* H1, which accumulates cadmium intracellularly (Roane et al. 2001). These results suggest that in the presence of a toxic metal, inoculation with metal-resistant microorganisms that reduce bioavailable metal concentrations via sequestration will foster the increase of biodegradation. In another study, in order to reduce the cadmium potentially available to plants, soil bioaugmentation was performed by using *Bacillus* sp. (Je´ze´quel et al. 2005). A more recent example (Lee et al. 2006) involved recombinant rhizobacteria (*Pseudomonas* and *Rhizobium*) expressing a cystein-rich peptide at the cell surface and thus improving their TCE degradation in the presence of Cd.

In this study, the effect of soil bioaugmentation with natural bacterial strains on the biodegradation of TCE or MTBE was investigated in order to assess the possibility to improve aerobic remediation of these compounds in contaminated soils. The approach used in this study was to coinoculate a metal-detoxifying population with an organic-degrading population that cooperatively functioned to remediate organic pollution in such co-contaminated systems.

Material and methods

Enrichment and growth of strains

All enrichments were performed in tryptic soy broth (TSB) at 26°C with shaking and by sub-culturing in TSB with growing concentrations of metals (5–1,500 mg/l Cd^{2+} or 5–30 mg/l Hg^{2+} or 5–1,000 mg/l Pb^{2+}). All strains were tested for growth on solid medium with a C_1 compound (methanol), organic acid (lactate) or glycerol in minimal medium MinE plus 16 g/l agar as described in Kelly et al. (1994), buffered with 10 mM phosphate (pH 6.8). When metals were used, minimal medium MinE was buffered with 100 mM MOPS pH 7 to avoid metal precipitation with phosphate. The original inocula were spoonfuls of soil samples obtained from polluted soils collected in the vicinity of the Estarreja Channel of Ria de Aveiro, northern Portugal. This channel has received mercury-rich effluent of a chloralkali industrial plant since the 1950s and the soil samples were contaminated with levels of Hg, Zn, Cd and Pb above the EU limits for agricultural soils (Commission of the European Communities 1986).

Five of the bacterial strains used in the bioaugmentation experiments had been isolated previously using enrichment techniques from pristine or contaminated soil samples, as described in De Marco et al. (2004). The remaining 12, the VF series, were obtained in this study. Resistance to the presence of heavy metals was assayed for all the isolates used in this study by testing growth on solidified MinE medium at increasing concentrations of $CdCl_2$, or

HgCl$_2$ or PbCl$_2$ (Sigma–Aldrich). Data on all the strains are shown in Table 1.

Microcosm inocula were grown aerobically at 26°C in minimal medium MinE buffered with 10 mM phosphate (pH 6.8) and supplemented with 0.1% yeast extract. The carbon sources used for the study were lactate or glycerol. Cultures were harvested by centrifugation and resuspended in the same medium to an optical density (600 nm) of 1.5–2.

The strains tested in this study are listed in Table 1.

Classification of the isolates

DNA was extracted from ca. 300 mg of wet cell pellet of each isolate by resuspending in 50 μl s.d.w. and immersing in boiling water for 10 min. After centrifuging, 1–2 μl of the supernatant were used as template in PCR reactions using universal 16S rDNA primers f27 and r1492 (Lane 1991). The amplicons obtained were cloned into plasmid vector pGEM®T-EASY (Promega) and partially sequenced using vector-based primer M13rev which yielded reads of 500–700 bps. The sequences obtained were used in BLAST searches of the non-redundant NCBI database (www.ncbi.nlm.nih.gov/Blast.cgi) in order to obtain an approximate classification of each strain.

Composition of the soil

Microcosms were set up using sieved (10 mesh) commercial garden substrate. The soil thus obtained was analyzed and the results were: pH = 5.75, 55% organic matter, 61% humidity, 9.86% porosity and particle density was 0.352 g/ml.

Soil microcosms

Each microcosm contained 25 g of soil inoculated with 15 ml of the singular bacterial cultures at OD$_{600}$ of 1.5–2. When combinations of two strains were used, 7.5 ml of each culture were employed. When heavy metals were used, each salt (CdCl$_2$ or HgCl$_2$ or Pb(NO$_3$)$_2$—Sigma–Aldrich) was dissolved in a maximum volume of 150 μl sterile distilled water and

Table 1 Metal resistance levels and pollutant degradation efficiency of the strains used in this study

Trait strain	Approximate classification (DNA seq. identity)	Cadmium (Cd)/ppm[d]	Mercury (Hg)/ppm[d]	Lead (Pb)/ppm[d]	Degradation of TCE[e] (%)	Degradation of MTBE[e] (%)
PM1	*Methylobacterium*[a]	500	0	250	16.15	23.9
Mi1	*Methylobacterium*[a]	500	1	400	29.46	40.14
F5.4	*Methylobacterium*[a]	800	15	300	33.67	6.93
EHg5	*Methyloversatilis*[a,b]	40	5	500	25.92	21.15
EHg7	*Methylophilus*[a]	40	0	200	31.80	0
VF1	*Alcaligenes faecalis* (99%)	700	1	300	0	0
VF2	*Alcaligenes faecalis* (99%)	700	5	300	15.78	22.84
VF3	*Methylobacterium*[c]	1,500	5	100	5.48	39.93
VF4	*Methylobacterium*[c]	1,000	5	100	3.90	0
VF5	*Ralstonia gilardii* (99%)	20	0	50	0	24.95
VF6	*Micrococcus luteus* (99%)	50	0	100	4.90	7.55
VF7	*Bacillus cereus* (99%)	100	1	100	2.61	25.96
VF8	*Bacillus cereus* (100%)	100	1	100	0	3.58
VF9	*Ralstonia taiwanensis* (99%)	100	0	100	11.13	0
VF10	*Brevundimonas diminuta* (99%)	20	0	100	0	0
VF11	*Methylobacterium*[c]	700	5	100	5.47	0
VF12	*Methylobacterium*[c]	700	10	100	2.57	6.03

VF strains are new isolates obtained in this study. [a]Strains obtained earlier as described in De Marco et al. (2004) or [b]Kalyuzhnaya et al. (2006); [c]phenotypical classification (see ''Material and methods''); [d]maximum concentration of metal withstood by the strain in solid medium; [e]these percentages of degradation were measured in microcosms in all identical to those described in the ''Material and methods''. The results were obtained after two weeks of incubation

added to the microcosm together with the bacterial inoculum. Final metal concentrations were 1–20 ppm for Cd, 1–10 ppm for Hg and 50–100 ppm for Pb. Inoculation was followed by vigorous soil mixing. Incubation was performed in 500 ml flasks stoppered with Teflon valves (Mininert™, VICI®, Valco instruments) to prevent losses due to volatilization. The headspace in each flask was made up of approximately 450 ml of air. In parallel, soil controls inoculated with sterile minimal medium MinE were monitored. The stoppered soil microcosms were injected through the septa with 22.5 mg of the organic compounds (TCE or MTBE; corresponding to a final headspace concentration of 50 mg/l) and incubated at 20°C. The kinetic seen in control microcosms without bacterial inoculation showed a clear initial drop in the concentrations of the volatile organics for the first half to 1 h likely due to equilibration between the gas and solid phases, followed by a stabilization that lasted for the 15 days of the span of these tests (data not shown). Although the soil used was not sterile nor was any of the flasks autoclaved, the uninoculated microcosms showed a remarkable absence of degradation activity with either TCE or MTBE. Thus, the base-line

concentrations of the organics were determined 30 min after injection. This initial adjustment corresponded to a drop of 20–40% of the initial headspace concentration. The headspaces of the microcosms were then regularly sampled and analyzed by GC-FID to determine the rates of degradation of the pollutant. Concentrations of headspace TCE and MTBE were measured three times each week, typically at days 0, 3, 5, 7, 10, 12 and 15, for a total of 15 days. Headspace samples (in duplicate) were extracted using a gas-tight syringe through the septum. Each soil experiment was performed in triplicate. The results shown in this study (Figs. 1, 2) are already net results at day 15 of incubation, where the background disappearance of pollutant measured in negative controls has been subtracted.

Analytical method

TCE and MTBE degradation in the different micro-cosms was monitored by headspace samples analysis in a gas chromatograph (GC) (Achten and Püttmann 2001). Using a gastight syringe, 1 ml headspace samples were injected into a gas chromatograph equipped with a flame ionization detector (FID) (Chrompack CP
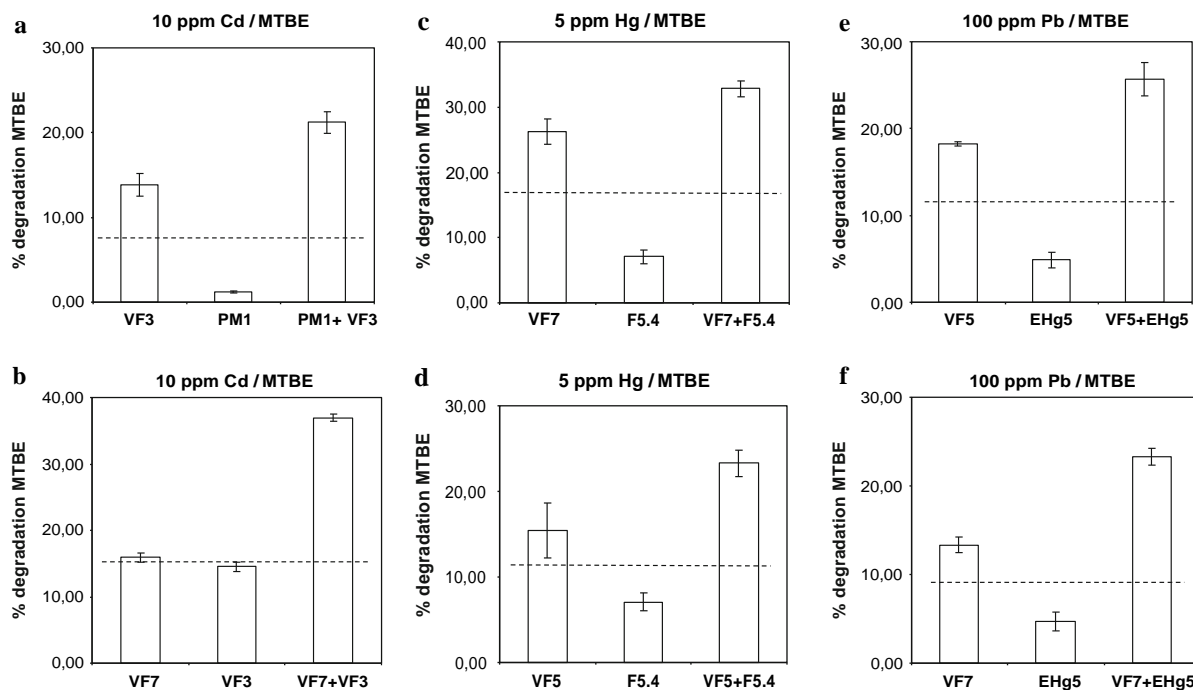

Fig. 1 Degradation of MTBE in the presence of the heavy metals (Cd, Hg, or Pb) by the doubly inoculated microcosms, as indicated. The *dashed line* shows the expected degradation level (mean of the two single strains)
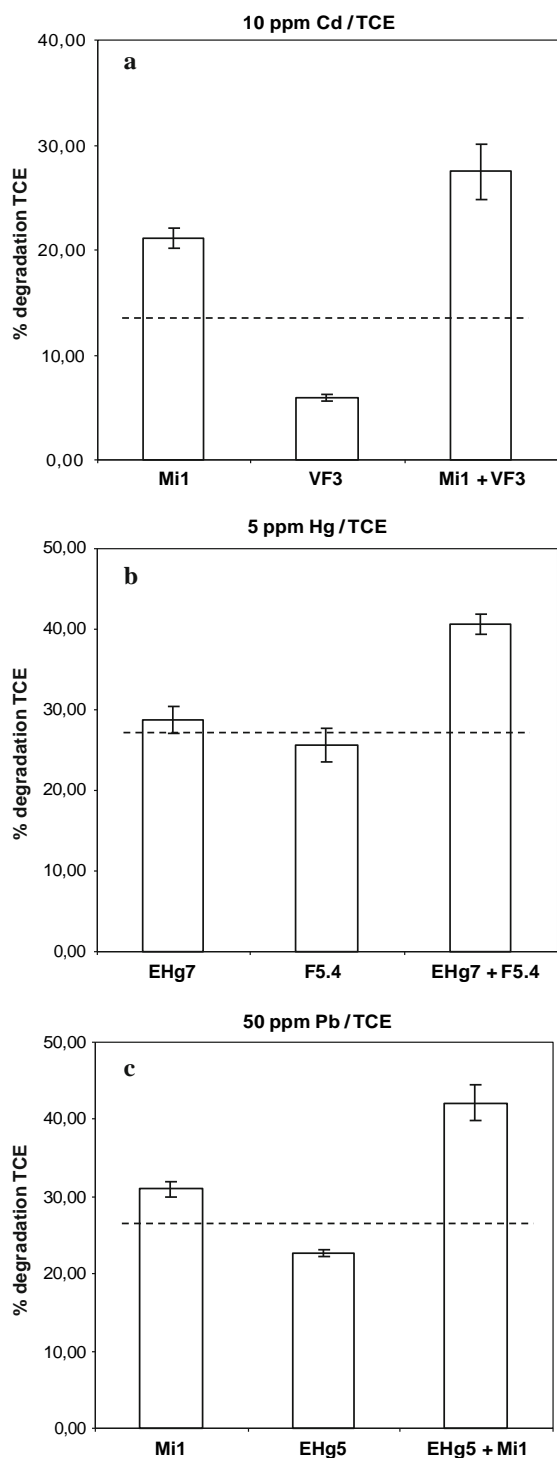
Fig. 2 Degradation of TCE in the presence of the heavy metals (Cd, Hg, or Pb) by the doubly inoculated microcosms, as indicated. The *dashed line* shows the expected degradation level (mean of the two single strains)

9000). The GC column (CP-Wax 52 CB, 25 m by 0.53 mm, Chrompack) was run at isothermic temperature (200°C). The detector and injector temperatures were 325 and 275°C, respectively. N_2 served as carrier gas at a flow rate of 7 ml/min.

To obtain concentration standards, five 200 ml flasks were used. Each of these flasks was sealed with a gastight Mininert™ valve. Different amounts of MTBE (Fluka, assay C 99.5%) or TCE (Fluka, assay C 99.5%) between 1 and 20 ll were injected into the flasks using a gastight syringe, resulting in five different calibration standards for each compound in the appropriate range of 5–50 mg/l. Sampling was performed after 15 min, when all the liquid compound added was vaporized.

For the calibration curves, 1 ml samples were taken from each flask and injected directly into the GC-FID. All these samples were taken in triplicate. The correlation coefficient of the calibration curves for both TCE and MTBE was 0.996. The limits of detection and quantification were 3.95 and 13.16 mg/l for MTBE and 4.10 and 13.68 mg/l for TCE.

Results

Selection of strains

Strains PM1, Mi1, F5.4, EHg5 and EHg7 were selected from the culture collection obtained during a previous study on robust soil methylotrophs (De Marco et al. 2004). The 12 strains of the VF series were obtained in new enrichments as described in the ''Material and methods''. VF3, VF4, VF11 and VF12 had all the typical characteristics of the genus *Methylobacterium* (pink colonies, facultative growth on methanol and/or methylamine, Gram negative rods). For this reason, they were empirically classified as *Methylobacterium* strains. The other VF strains were preliminarily classified based on their 16S rDNA sequences and the results are summarized in Table 1. It is clear that the enrichment conditions favored Gram-negatives, which are the most abundant group in the newly obtained collection. However, two *Bacillus* strains and a *Micrococcus* were also present.

All the strains listed in Table 1 were tested for their resistance to the three chosen heavy metals. The highest concentrations tolerated were 1,500 ppm

(mg/l) of Cd (by str. VF3), 500 ppm of Pb (by str. EHg5) and 15 ppm of Hg (by str. F5.4).

A second round of selection was performed based on the strains' ability to degrade MTBE or TCE in microcosm conditions. As can be seen in Table 1, the best degraders of MTBE were, in descending order, strains Mi1, VF3, VF7 and VF5. As for TCE, strains F5.4, EHg7, Mi1 and EHg5 were the best performers. Strains VF1, VF2, VF10, VF11 and VF12 displayed erratic growth in liquid medium and due to this reason were eliminated from this study. All strains tested showed a decreasing capacity of MTBE or TCE removal with increasing concentrations of heavy metals, which clearly proves that the action of the bacterial biomass on the organic pollutants was due to biological activity.

Preliminary microcosm experiments (results not shown) were performed with the best degraders and increasing concentrations of metals (Cd 1, 5, 10, 20 ppm; Hg 1, 5, 10 ppm; Pb 50 and 100 ppm). The degradation rates of the organic pollutants dropped dramatically (80–90%) at 20 ppm Cd, 10 ppm Hg or 100 ppm Pb, so the next lower concentration for each metal was chosen.

Best degraders were then coupled to most resistant strains for each metal and the analyses repeated in the same manner. Some of the couples (e.g. Mi1 ? VF12 with TCE ? Hg 5 ppm or with MTBE and Hg 5 ppm) showed combined degradation levels approximate to the mean of the values of the two single strains, as expected. Other pairs showed values below the mean (e.g. EHg7 ? VF3 with TCE and Cd, −28% or VF3 ? Mi1 with MTBE and Cd, −25%). A few other combinations showed higher performances when taken together than singularly: of these cases we describe below the best examples.

MTBE degradation tests

In the experiments with MTBE and in the presence of metals, strains VF3, VF7 and VF5 were chosen as degraders while strains PM1, VF3, F5.4 and EHg5 were used as resistant members of the consortia (PM1 or VF3 for Cd; F5.4 for Hg; EHg5 for Pb). Strain VF3 was actually used in both roles due to its good performance in degrading MTBE and its resistance to Cd.

In all the double inoculation experiments only half of the biomass of each strain was added to the microcosm, so degradation levels corresponding to the arithmetic mean of the two singular strains were expected. However, in the examples shown in this work, when the microcosms received both strains, the final levels of organic removal in the presence of a heavy metal were well above the ones anticipated. When Cd was used, the expected degradation values of MTBE for pairs VF3 ? PM1 and VF7 ? VF3 were exceeded by 182 and 142%, respectively (Fig. 1a, b). In the case of Hg, pairs VF7 ? F5.4 and VF5 ? F5.4 exceeded the expected values by 97 and 107%, respectively (Fig. 1c, d). When Pb was present, the strain pairs chosen were VF5 ? EHg5 or VF7 ? EHg5. In these cases the degradation levels were 122 and 158% higher than the predicted values (Fig. 1e, f).

TCE degradation tests

In the case of TCE, strains Mi1 and EHg7 were selected as degraders, while strains VF3, F5.4 and EHg5 were chosen as resistant members of the pairs (VF3 for Cd; F5.4 for Hg; EHg5 for Pb). In the instances shown, when the microcosms were inoculated with two strains, clearly a synergistic effect was observed since the double inoculation yielded values of organic removal well higher than the expected levels: strains Mi1 ? VF3 (?Cd) caused the disappearance of 103% more TCE than the expected mean between the two strains taken singularly; EHg7 ? F5.4 (?Hg) exceeded the predicted value by 49%; Mi1 ? EHg5 (?Pb) together outdid the anticipated level by 57%.

Discussion

Remediation of sites co-contaminated with organics and heavy metals can be very difficult due to the inhibiting effect that toxic metals exert on the microflora (Sandrin and Maier 2003). A few works have demonstrated that in principle it is possible to alleviate some of the inhibition caused by heavy metals by employing metal-resistant microorganisms that, each in its own way, somehow lessen the toxic burden of the metal ions (Roane et al. 2001; Je´ze´quel et al. 2005). In most cases these microorganisms will lower bioavailability of the metals by sequestering the ions on extracellular anionic polysaccharides

(EPS) or on thiol-rich proteins (either intracellular or anchored to the cell surface). In some cases, such type of trait was deliberately engineered in microbial cells (Kuroda et al. 2001; Lee et al. 2006).

In this work we isolated and selected several bacterial strains capable of withstanding considerable concentrations of $Cd^{2?}$ or $Hg^{2?}$ or $Pb^{2?}$ and coupled them with strains that showed good performance at degrading the common soil and water pollutants MTBE or TCE. Some of the combinations of strains we examined showed microcosm degradation efficiencies frankly higher than those expected on the grounds of a purely additive interaction: between 49 and 103% higher for TCE and between 97 and 182% for MTBE. This demonstrates that synergy between the members of these consortia is indeed occurring, resulting in a cooperative effect. Since many sites are polluted with both organic and inorganic contaminants, several works have explored the possibility of obtaining metal-resistant organic-degrading microorganisms. However, such microbes are difficult to recover by enrichment techniques and attempts have been made to introduce metal resistance traits in natural degrading strains by genetic engineering. The idea of dual bioaugmentation (Roane et al. 2001) resides in the usage of two separate microbial species, each with its separate task of detoxifying the heavy metal on the one hand and degrading the organic pollutant on the other. We applied this design to soil microcosms and showed that dual bioaugmentation, even without making use of genetically modified microrganisms, are a viable option for the remediation of soils polluted with MTBE or TCE and heavy metals.

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