Aerobic biodegradation of benzene, toluene and ethylbenzene in liquid medium by a bacterial consortium, isolated from non-history clay soil, and their interrelation effect

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Abstract
The present study was carried out in order to investigate the ability of isolated subsurface bacteria, from a non-history clay soil, to biodegrade the non-aqueous phase-liquids (NAPLs), monoaromatic hydrocarbons: benzene, toluene and ethylbenzene. First stage of the study was focussed on stand-alone biodegradation of each contaminant under described conditions. Benzene (100, 260 and 500 mg/l) exposed to isolated soil bacteria for 14 days, was biodegraded 100, 70 and 50%, respectively, ethylbenzene (100, 260 and 500 mg/l) at 85, 87 and 90%, respectively and toluene (100, 260 and 500 mg/l) revealed the lowest rate of 45, 50 and 52%. Toluene and ethylbenzene showed a direct increase in biodegradation associated with increase in their concentration. The second stage was the biodegradation of benzene, toluene and ethylbenzene admixture (all three compounds at the very same concentrations, w/v) in glucose absence and supplemented with Tween 80 (10 and 15 mg/l). The overall biodegradation improved when contaminants were mixed together.

Keywords Benzene, toluene and ethylbenzene (BTE); biodegradation; aerobic; liquid medium; clayey soil; bacteria; non-history soil

Introduction
Benzene, toluene and ethylbenzene (BTE) are among the most frequent aromatic substances of gasoline contaminated areas. BTE group is ranked as the second pollutant following trichloroethylene (TCE), among contaminants occurring in USA groundwater (Olsen et al., 1994). Microorganisms able to degrade these compounds are abundant in nature and have the potential for both in-situ and ex-situ bioremediation (Mikesell et al., 1991). Biological processes have been used extensively to reduce organic compound concentration in the environment such as the activated sludge process for treatment of municipal waste. Indigenous bacteria have the advantage of adapting to the physico-chemical characteristics of various sites, yet they may not be able to biodegrade specific hydrocarbons (Nishino et al., 1994). Some BTE compounds have been reported to persist in the environment at levels that are beyond the threshold, even after addition of nutrients and electron acceptor (Atlas, 1981). The rate and extent of microbial adaptation to biodegrade hydrocarbons is influenced by several factors such as contaminant concentration, temperature, pH, availability of inorganic nutrients and electron acceptors (Alvarez and Vogel, 1991). Bacterial biodegradation under aerobic condition has been studied for more than 10 years. For example toluene aerobic biodegradation has five pathways as demonstrated by several authors, though not all of the monoaromatic hydrocarbons (such as benzene, ethylbenzene and xylene) are metabolized through these aerobic pathways (Mikesell et al., 1993). In spite of the numerous studies that have been carried out to investigate the biodegradation of BTE compounds, few have focused on the antagonistic or synergistic effects of combined pollutants and mixed bacterial culture. Alvarez and Vogel (1991) reported that the presence of p-xylene increased the lag period of bacteria involved in biodegradation of toluene and benzene. These authors also reported that the acclimation period of benzene degraders

increased when both toluene and p-xylene were present. The primary conclusion of this study was that albeit BTX have similar chemical structure, different microorganisms could metabolize certain BTX compounds but not others (e.g. hexachlorobenzene). Dyreberg et al. (1996) studying the influence of creosote compounds on degradation of toluene and benzene, observed an inhibitory effect of creosote on benzene and toluene degradation, albeit toluene is well known to be easily degraded under aerobic conditions. In this study two major inhibition phenomena were identified: (a) extension of the lag phase in presence of more than one compound, and (b) a decrease in degradation rates.

The present study assessed the capability of subsurface bacteria mixed culture to biodegrade monoaromatic hydrocarbons (BTE) in liquid medium and the interrelations between the various contaminants admixture on their biodegradation.

Materials and methods

**Materials** – Benzene was purchased from Bio-Lab (Jerusalem, Israel), toluene from Frutarom (Haifa, Israel) and ethylbenzene from Aldrich (Milwaukee, WI, USA). All solutions were prepared in tri-distilled water including phosphate buffer saline (PBS). Minimal media contained (g/l): K2HPO4 7, KH2PO4 2, (NH4)2SO4 1, MgSO4 0.1 and glucose 0.1 (pH–7.0). Heterotrophic viable count was performed on R2A agar plates (Difco, Detroit, MI, USA). Oxygen demand was measured by the azide modification method (APHA, 1995).

**Bacteria isolation and identification** – Clayey soil (5 g) was introduced in a sterile 100 ml milk dilution bottle and resuspended in 95 ml sterile phosphate buffer saline (pH 7.0). The bottle was placed horizontally over an orbital shaker and rotated at 200 rpm at room temperature (24±2ºC) for 24 hours. Following the extraction procedure and allowing the soil to settle, 0.5 ml of liquid phase was inoculated to experimental serum bottles. Another fraction of 0.5 ml was plated on R2A agar plates for bacterial growth and further identification. Colonies obtained on the agar plates were further isolated on the same agar in order to obtain pure cultures. Each pure culture colony was grown in diluted nutrient broth (1:2). Log phase bacteria were washed five to six times with PBS (pH 7.0) as requested by the Biolog system (Biolog Co., CA, USA). The Biolog system has two microwell plates types: Gram (+) and (–). The plates were selected according to previous Gram staining result. Bacteria resuspended in PBS at 0.2–0.3 O.D. 660nm were inoculated to Biolog microwell plates (150 µl/well) and incubated aerobically for 24 hours at 36ºC. Positive wells (violet colour) were recorded and analyzed by MicroLog software.

**Experimental system** – All experiments were conducted in 100 ml serum bottles, filled with minimal media, bacteria, and contaminant and sealed with rubber caps and aluminum septa. A 25 ml volume of minimal media was introduced in each bottle and sterilized (15 minutes at 121ºC). Following addition of 0.5 ml bacterial suspension and defined concentrations of the contaminant, bottles were sealed and agitated on an orbital shaker (Labotron HT, Austria) at 100 rpm at room temperature (24±2ºC) for 14 days.

Periodically, 0.5 ml of suspension was removed by a sterile syringe, diluted and plated for bacterial count. The same experimental bottle was subjected to sampling for residual contaminant at different time intervals. Following warm up of the bottle to 60ºC for 30 minutes in a waterbath, headspace gas was sampled with a gastight syringe (Dynateck, LO, USA) compressed and injected to the gas chromatograph. Bottles sampled for bacteria and residual contaminant were sacrificed and excluded from the system. Control bottles containing contaminants at various combinations and sterile medium without bacteria were sampled to determine abiotic losses.
Gas chromatography – Gas chromatography analysis was performed on a Unicam GC 610 (UK) equipped with flame ionization detector (FID). Separation column was DB-VRX (J and W Scientific, CA, USA) (L=75 m x I.D.=0.45 mm and F=2.55 µm). Injection temperature was 220ºC. Column temperature was maintained at 88ºC for 1 minute and gradually increased by 4ºC/minute until it reached 92ºC and was held for 15 minutes at this temperature. A second increase by 50ºC/minute was performed to reach 220ºC and held for an additional 3 minutes. Analysis was performed by direct injection of 1 ml gas sample. Under these conditions, the retention time of benzene was 5.3 minutes, toluene 6.8 minutes and ethylbenzene 8.1 minutes.

Results and discussion
The clayey soil subjected to buffer extraction yielded a series of Gram (–) and (+) bacteria. Identification of pure cultures performed with the Biolog system showed two species (>70% identification): Rhizobium loti B and Corynebacterium jeikeium A. Figure 1a shows benzene biodegradation after 14 days in capped bottles. Bacteria exposed to benzene alone showed the highest biodegradation at 100 mg/L (100%) and decreased with increase in contaminant concentration (260 and 500 mg/L, 70% and 50%, respectively). Exposure of the bacterial consortium to increased concentration of the contaminant may reduce its biodegradability due to several factors: influence on adaptation rate of bacteria to environmental conditions, toxicity of the contaminant itself and toxicity of by-products such as catechol or phenol (Alvarez and Vogel, 1991).

Supplementation of toluene to benzene (1:1 v/v) increased the degradation of benzene at 260 mg/L and decreased it at 500 mg/L. Ethylbenzene as supplement (1:1 v/v) did not alter benzene biodegradation at 100 and 260 mg/L but decreased it at 500 mg/L to 25%. Combination of the three contaminants (BTE) showed no alteration in biodegradation percentage at 100 mg/L, but was significantly reduced at 260 mg/L (35%) and equalled the biodegradation of 500 mg/L obtained with benzene alone. The biodegradability of benzene alone was concentration dependent. The variability in biodegradation of benzene in combination with toluene and ethylbenzene seems to be dependent on the solubility of each component and its influence on the bacterial consortium’s biological activity. Toluene biodegradation is shown in Figure 1b. Toluene solely showed low biodegradability at all three concentrations from 45% to 52%. It should be mentioned that toluene solubility is 515 mg/L, while we tested 500 mg/L, that is, still below it. Addition of benzene (1:1 v/v) increased the biodegradability of toluene at all tested concentrations. Ethylbenzene supplementation also increased toluene biodegradation but to a lower extent (58 to 75%). Combining all three contaminants improved overall biodegradability at all concentrations.

Ethylbenzene biodegradation is shown in Figure 1c. Ethylbenzene solely revealed a middle to high grade of biodegradability that is located between benzene and toluene (regarding all three concentrations). Supplementation of benzene significantly reduced ethylbenzene degradation compared to ethylbenzene alone.

Figure 2(a,b,c) represents biodegradation of benzene, toluene and ethylbenzene where each contaminant was measured separately in combination with the other two contaminants in the absence of glucose in the liquid media, or by addition of Tween 80 at two concentrations (10 and 15 mg/L). Tween 80, a nonionic surfactant, has a CMC (critical micellar concentration) of 13 mg/L, therefore the rationale of the two tested concentrations was to observe biodegradation efficiency below and above CMC. Glucose was selected to act as an available and simple carbon source to stimulate hydrocarbon biodegradation. As already pointed out by other authors (Gonzales and Hu, 1991), addition of co-substrate increases the biodegradation rates. Panneton et al. (1995) investigated the influence of glucose supplementation on biodegradation of pentachlorophenol (PCP). Their results showed that
PCP as the sole carbon source has higher rates of biodegradation, compared to PCP supplemented with glucose. Edgehill (1995) found that addition of a carbon source has a positive impact on cell biomass but not on the removal rates of PCP.

Poor solubility of the hydrocarbons can be a possible cause for the low biodegradation. Addition of surfactants to environments contaminated with hydrophobic hydrocarbons may increase the bioavailability of these compounds to hydrocarbon degrading microorganisms, however, results from different studies are somewhat contradictory. Results shown by Nelson et al. (1996) revealed that addition of surfactants to a mixed culture of indigenous bacteria enhanced the degradation rates. Use of chemical surfactants did not show an increase in mineralization as confirmed by Atlas and Barta (1973). Benzene biodegradation decreased when glucose was omitted from the medium at 100 mg/L (Figure 2a), while the other two concentrations were not affected. No effects were observed with toluene and ethylbenzene at all tested concentrations (Figures 2b and 2c). The present results do not support the premise that addition of glucose as a co-metabolite can enhance biodegradation, being in agreement with other studies. Addition of Tween 80 at 10 mg/L (below the CMC) did not induce benzene biodegradation at all concentrations (Figure 2), though 15 mg/L increased the biodegradation at concentrations of 260 mg/L and 500 mg/L. Tween 80, at both concentrations had a minor effect on toluene biodegradation (Figure 2b).

Ethylbenzene biodegradation was only enhanced by addition of 15 mg/L of Tween 80 at 260 mg/L (Figure 2c). Among the substantial limiting factors involved in aerobic biodegradation of BTE, oxygen is of the utmost importance as an electron acceptor. Biodegradation rates of BTE have been shown, in several studies, to be greater under aerobic conditions (Mikesell et al., 1991). Oxygen measurements were performed in experimental systems containing benzene, toluene and ethylbenzene concurrently at the three tested concentrations (100, 260 and 500 mg/L).
The initial concentration of dissolved oxygen, in all the systems, was 5 mg/L, and during the 14 days of the experiment did not go below 2.5 mg/L (data not shown), showing biodegradation under aerobic conditions.

Gibson et al. (1970) reported on the higher toxicity of direct addition of benzene opposite vapor phase substrate. In our experimental study, benzene, toluene and ethylbenzene were partially in the vapor phase but the main difference in the tolerance for aromatic compounds, was mainly because microorganisms were previously isolated from a history soil. The cumulative toxicity was not proved, and in some cases such as benzene and toluene but not ethylbenzene, addition of other aromatic hydrocarbons enhanced the biodegradation of the initial contaminant. Omission of glucose from the minimal medium had only a small impact on benzene biodegradation but not on toluene and ethylbenzene. Tween 80, a non-ionic surfactant at two tested concentrations (10 and 15 mg/L), increased the biodegradation of benzene at 15 mg/L especially at higher concentrations (260 and 500 mg/L)(Figure 2). Nevertheless, Tween 80 did not increase the biodegradation of toluene and ethylbenzene (Figures 2b and 2c). The effect of various surfactants on bacterial membrane permeability was extensively described. According to our results, surfactants did not reduce the biodegradability, which may be related to a protective effect against the toxicity of the tested aromatic compounds, but this is pure speculation and was not within the scope of this study.

Conclusions

The present study was performed in order to answer questions on the capability of a non-history soil microbial consortium to biodegrade BTE. In spite of previous unexposure of these bacteria to BTE, the results showed that biodegradation took place at all concentrations.
tested (100, 260 and 500 mg/L) with variations in concentration dependent on efficiency. The following conclusions were drawn.

1. Bacterial consortia from a clayey non-history soil were capable of biodegrading benzene, toluene and ethylbenzene (BTE) and their admixture.

2. Benzene as a stand-alone contaminant subjected to biodegradation, revealed a decreased efficiency rate reverse to concentration increase (100>260>500 mg/L). Toluene and ethylbenzene did not show a significant decrease in biodegradation rates at all three concentrations tested. This characteristic can be attributed to the higher toxicity of benzene.

3. The biodegradation rates of benzene, toluene and ethylbenzene alone and in combination, showed different patterns of enhancement or reduction. At the moment it is difficult to explain the differences due to lack of sufficient information and explanations are based on speculation alone.

4. Benzene biodegradation without a co-metabolite such as glucose showed decreased rates, yet toluene and ethylbenzene were independent of glucose absence.

5. Addition of Tween 80 as a non-ionic surfactant did not significantly improve the biodegradation of the three contaminants, in spite of previous reports in the literature. It may be postulated that surfactants are more efficient in a soil system than in a liquid one (used in the present study), involving contaminant desorption from soil particles.

In summary BTE biodegradation by a bacterial consortium from a non-history soil shows that many bacterial species previously not known to biodegrade contaminants, can accomplish this task. This feature promotes new hopes that oil spills and other environmental hazards can be treated by biological means without previous acclimatization. The present investigation raised several additional questions among them a most important one on the interaction of one contaminant with other contaminants and their interrelation on the efficiency of the biodegradation process.

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References


