Bioremediation of humic soils contaminated with benzene*

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ABSTRACT

Bioremediation uses degradation capacity of indigenous or inoculated microorganisms to biodegrade organic constituents adsorbed to soils. It is a technology that generally requires long periods of treatment to reach the desired clean-up goals. The objectives of the reported work were to evaluate the capability of bioremediation to achieve legal clean-up goals in a humic soil with an organic matter content of 14% and contaminated with benzene. The benzene quantification was performed by gas chromatography. The results of the experiments were used to calculate process efficiency and remediation time. It was concluded that: a) the bioremediation is an effective process; b) bioremediation efficiencies were near 100%; and c) the remediation times were 92, 139 and 354 hours for soils contaminated with benzene levels of 70, 90 and 120mg·kg^{-1}, respectively.

INTRODUCTION

Improper utilization, handling and disposal of industrial chemicals have led to numerous cases of soil contamination. One of these contaminants is benzene that is one of the most common petroleum hydrocarbon components. Benzene is commonly used as a constituent in motor fuels, as a solvent, in photogravure printing and in the manufacture of detergents, explosives and pharmaceuticals.

In the last 25 years, the group of contaminants constituted by the benzene, toluene, ethylbenzene and xylene (BTEX) has been among the most addressed contaminants in soil remediation projects. These contaminants were found in 238 remediation projects that correspond of 24% of the total number of ongoing or completed projects in the United States of America (USEPA 2007). Due to its toxicity properties, benzene has become one of the most intensely regulated substances in the world. It has been recognized as a class I carcinogenic agent from the International Agency for Research on Cancer (IARC), as long-term exposure to high benzene concentrations is known to cause bone marrow damage, leukemia and aplastic anemia (Fredriksson et al. 1989).

Several remediation technologies can be applied to soils contaminated with volatile contaminants such as benzene (Khan et al. 2004). Bioremediation (BR) was the second most used remediation technology in the United States (13% of all in-situ remediation projects) (USEPA 2007). BR uses the degradation capacity of indigenous or inoculated microorganisms to biodegrade organic constituents occurring within soils. The technology generally needs long periods of treatment to reach desired clean-up goals. The process requires optimization of certain parameters, such as temperature and the presence of nutrients and soil moisture; in some cases these requirements are present but in others it is necessary to add or adjust some. The ideal range for soil moisture should be between 40 and 85% of the water-holding capacity (field capacity) of the soil, or about 12% to 30% by weight (Boopathy 2000). In general, the soil should be moist but not wet. Excessive soil moisture also restricts the movement of air through the subsurface thereby reducing the availability of oxygen which is necessary for aerobic bacterial metabolic processes (Boopathy 2000; USEPA 1994).

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Microorganisms require inorganic nutrients such as nitrogen and phosphorus to ensure cell growth and sustain bioremediation processes. However, excessive amounts can repress microbial metabolism. The typical carbon : nitrogen : phosphorus ratio necessary for biodegradation falls within the range of from 100.0 : 10.0 : 1.0 to 100.0 : 1.0 : 0.5, depending upon the specific constituents and microorganisms involved in the biodegradation process (Boopathy 2000; USEPA 1994).

Regarding temperature, bioremediation tests of diesel fuel performed in gravel and sand from Alaska under different temperatures (6 and 20°C) concluded that in cold soil, the diesel fuel showed minimal degradation, i.e. less than 5% total mineralization of the hydrocarbons in the fuel (Horel and Schiewer 2009). This study reports the results obtained in BR experiments performed in a laboratory environment using a humic soil with 14% of organic matter content and contaminated with benzene. These experiments sought to evaluate the capability of bioremediation to achieve a level that meets the legal clean-up goals in a humic soil with an organic matter content of 14% and contaminated with benzene. The rest results were used to calculate: i) process efficiency, and ii) remediation time.

MATERIAL AND METHODS

Enrichment and growth of strains

The growth of all strains were performed in minimal medium (MinE) buffered with 10mM phosphate (pH 6.8) and supplemented with 0.1% yeast extract at 26°C. All bacteria strains were tested for growth on solid medium with a C1 compound (methanol), organic acid (lactate) or benzoate in MinE plus $16g \cdot l^{-1}$ agar as described by Kelly et al. (1994).

Microbial inocula were obtained from spoonfuls of soil samples taken from polluted soils collected in the vicinity of the Estarreja Channel of Ria de Aveiro, northern Portugal, as described in Fernandes et al. (2009). Those strains showed, in previews works, capacity to degrade several compounds. Resistance to the presence of benzene was assayed for all the isolates used in this study by testing growth on solidified MinE medium at increasing concentrations (10ppm and 20ppm). Data for all strains tested in this study are shown in Table 1.

Table 1. Classification of bacterial strains studied in the contaminated soil.

Abbreviation	Strain
PfST	*Pseudomonas fluorescens* ST
PpKT	*Pseudomonas putida KT2440*
OX1	*Pseudomonas stutzeri*
F11	*Labrys portucalensis*
EP1	*Xanthobacter*
EHg5	*Methyloversatilis*
Mi1	*Methylobacterium*
EHg7	*Methylophilus*

Bacterial inocula were grown aerobically at 26°C in MinE buffered with 10mM phosphate (pH 6.8) and supplemented with 0.1% yeast extract. The carbon source used for the study was lactate. Each strain was cultured in 50ml broth until an optical density (600nm) of 1.5–2.0 was attained; following this they were harvested by centrifugation and resuspended in 15ml of the same medium.

Apparatus and chromatography

A Shimadzu GC-2010 chromatograph equipped with a flame ionisation detector was used for the quantification of benzene. The column used was a Teknokroma TRB-35 ($30m \cdot 0.53mm \cdot 0.52\mu m$). The injector and the detector were set at 250°C and the oven worked isothermically at 200°C. Detector gases were air, at $400cm^3 \cdot min^{-1}$, and hydrogen at $40cm^3 \cdot min^{-1}$. The carrier gas was nitrogen at $13cm \cdot min^{-1}$. Chromatographic data were recorded using GCsolution Analysis software. The direct calibration method was used. Under the above described experimental conditions, the benzene showed a retention time of 0.6 minutes.

Soil preparation and characterization

The soil used in this study was prepared by mixing differing proportions of a sandy soil collected at 3m depth in a beach (41°14'23"N; 8°43'32"W), and a superficial humic soil collected in a forest (41°16'49"N; 8°35'00"W), both from the Porto region in Portugal. Both soils were stored in hermetic plastic boxes. The sandy soil was prepared in three steps: a) washing with deionized water until visually clean water was obtained; b) drying, first at room temperature over 5 days and then at 110°C for 24 hours; and c) sieving in a 2mm mesh sieve.

The humic soil was dried at 50°C for 48 hours and sieved through a 2mm mesh sieve. Both soils were subsequently characterized using international standard methods, including

apparent density, particle density, porosity, pH, and the contents of water and natural organic matter (Albergaria 2003).

The experimental soil was prepared by mixing the exact amount of both sand and humic soil in order to create the desired composition.

Bioremediation experiments

Microbial resistance tests

Microbial resistance to the presence of benzene was assayed for all isolates used in this study by testing strain growth on solidified medium at 26°C. The strains were sealed in a plastic box after the creation of a contaminated atmosphere (50mg·l^{-1} and 100mg·l^{-1} of benzene). An adequate volume of benzene was placed in a small tube taking into account the total box volume subtracted by the volume of the Petri dishes. These experiments were compared to control tests performed in similar boxes with a contaminant-free atmosphere. These studies were performed in triplicate.

Flask experiments

Each microcosm contained 30g of soil inoculated with 15ml of MinE obtained from a single CFU and 1.0 of turbidance at 600nm. Inoculation was followed by vigorous mixing of the soil. Incubation was performed in 500ml Erlenmeyer flasks closed with Teflon valves (Mininert^{TM}, VICI ®, Valco instruments) to prevent losses due to volatilization. The headspace in each flask was approximately 450ml of air. In parallel, soil controls (sterile and non-sterile) inoculated with sterile MinE were monitored. A small tube was inserted in each flask with an adequate volume of benzene to obtain the right concentration in flask headspace and incubated at 26°C.

The headspaces of the microcosms were sampled daily over 9 days and analyzed by GC-FID to determine the concentrations of benzene in the headspace and consequently calculate the rates of degradation of benzene. Headspace samples (in duplicate) were extracted using a gas-tight syringe through the septum.

Column experiments

Tests were performed in the stainless steel columns of 37cm height and of 10cm internal diameter and involved two main stages: column preparation and bioremediation monitoring. The column preparation consisted of 250g of soil followed by 90ml of deionized water and the benzene creating contaminations of 70, 90 and 120mg·kg^{-1}. This procedure was repeated until a total of 2000g of soil were introduced into the column. The column was then closed and left isothermically at 23°C to reach equilibrium. After this point, the concentration of benzene in the gas phase of the soil was extracted from the four sampling ports of the column (Figure 1) and monitored by gas chromatography. The average of the four sampling ports was calculated.

Another column was prepared with sterile mixed soil contaminated with the legal limit of benzene (10mg·kg^{-1}). The concentration of benzene in the gas phase of the soil was determined after the establishment of the equilibrium and the obtained value (0.40mg·l^{-1}) was used to indicate the end of the bioremediation. The time needed to reach this concentration was considered the remediation time.

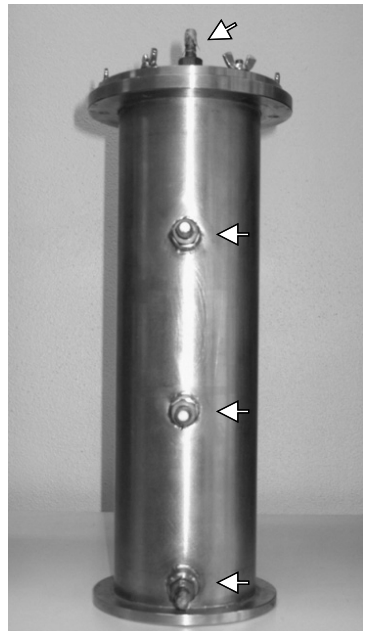

Figure 1. Experimental column (arrows point sampling ports).

RESULTS AND DISCUSSION

Soil characterization

The characteristics of the sand, humic soil and the prepared mixed soil are shown in Table 2. The presence of shell debris was responsible for the relatively high values of pH observed.

Bioremediation experiments

Microbial performance

The microbial resistance studies allow choosing *P. fluorescens* (PfST), *P. putida* (PpKT), *P. stutzeri* (OX1) and *Labrys portucalensis* (F11). These four strains showed CFU growth with benzene at 20ppm.

Flask experiments

The objective of the tests performed with non-sterile soil (NSS) mixed with the bacterial strains (PfST, PpKT, OX1 and F11) was the identification of the most efficient strain to degrade the benzene present in the soil matrix and to be used in the column tests. The test performed with the sterile soil (SS) and its comparison with the non-sterile soil (NSS) aimed to evaluate the degradative capacity of the indigenous bacteria of the soil. The results obtained in these experiments are presented in Figure 2.

Table 2. Characteristics of the study soils (particle size < 2mm).

Soil	Apparent density ($g \cdot ml^{-1}$)	Particle density ($g \cdot ml^{-1}$)	Porosity (%)	pH	Water content (%)	Natural organic matter content (%)
Sandy	1.50	2.5	42	8.8	0.0	< 0.02
Mixed	1.00	2.8	64	7.2	4.3	14.00
Humic	0.55	3.1	82	6.1	7.6	7.60

For all tests performed with non-sterile soil, the concentration of benzene in the gas phase of the soil decreased to near-zero levels, indicating that the soil was successfully remediated. This indicates that the indigenous bacteria of the soil have a strong capacity to degrade benzene as well all the selected bacteria. *Pseudomonas fluorescens* (PfST) was chosen to perform the column experiments based on growth rate of the bacteria with 1.0 of turbidance within 48h at 600nm.

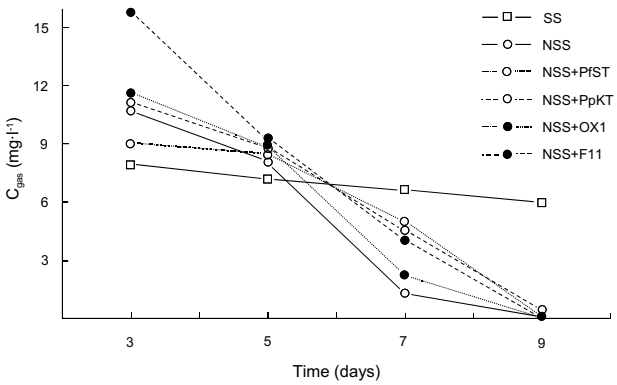

Figure 2. Results of the flask experiments.

Column tests

The maximum level of benzene in soil that is allowed by Spanish law is 10mg·kg^{-1} (Real Decreto 9/2005 2004). The bioremediation tests should guarantee that in the end of the remediation, the level of benzene in soil is below that limit. The concentration obtained in the equilibrium was 0.40mg·l^{-1}. This indicated that the bioremediation process can be stopped when the concentration of benzene in the gas phase of the soil reaches that level. The monitoring of the bioremediation experiments is presented in Figure 3. The horizontal line represents the legal limit that indicated the end of the bioremediation. Bioremediation times for the three soils were 92, 139 and 354 hours, respectively, for 70, 90 and 120mg·kg^{-1} levels of contamination.

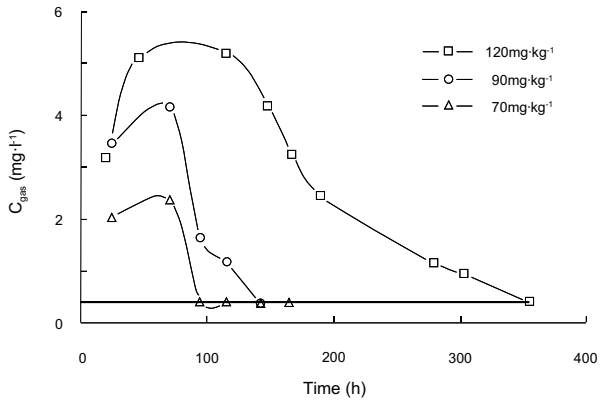

Figure 3. Benzene concentrations of soils in column studies.

CONCLUSIONS

The results obtained in this work allowed concluding that:

- bioremediation is an efficient technology to remediate soils similar to those experimentally contaminated with benzene,
- the bacteria showed good resistance to and degradation capacity of benzene,
- the experimental soil had indigenous bacteria capable of efficient degradation of benzene,
- the remediation times for the treatment of the soils with levels of contamination of 70, 90 and 120mg·kg^{-1} were respectively 92, 139 and 354 hours.

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