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Assessment of the efficiency of *in situ* bioremediation techniques in a creosote polluted soil: Change in bacterial community

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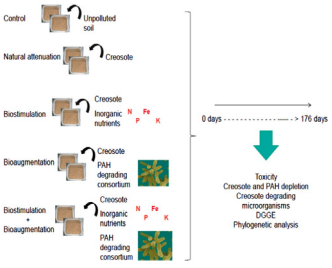
HIGHLIGHTS

- *In situ* bioremediation of a soil polluted with creosote.
- Biostimulation and bioaugmentation are effective in low temperatures.
- Degrading capacity of a bacterial consortium from a soil not previously exposed to creosote.
- Bacterial population distribution changed along all bioremediation treatments.
- Influence of autochthonous population on allochthonous community in bioaugmentation experiments.

GRAPHICAL ABSTRACT



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ABSTRACT

This work aimed to assess the effectiveness of different *in situ* bioremediation treatments (bioaugmentation, biostimulation, bioaugmentation and biostimulation, and natural attenuation) on creosote polluted soil. Toxicity, microbial respiration, creosote degradation and the evolution of bacterial communities were analyzed. Results showed that creosote decreased significantly in all treatments, and no significant differences were found between treatments. However, some specific polycyclic aromatic hydrocarbons (PAH) were degraded to a greater extent by biostimulation. The dominance of low temperatures (8.9 °C average) slowed down microbial creosote and PAH uptake and, despite significantly creosote degradation (>60%) at the end of the experiment, toxicity remained constant and high throughout the biodegradation process. DGGE results revealed that biostimulation showed the highest microbial biodiversity, although at the end of the biodegradation process, community composition in all treatments was different from that of the control assay (unpolluted soil). The active uncultured bacteria belonged to the genera *Pseudomonas*, *Sphingomonas*, *Flexibacter*, *Pantoea* and *Balneimonas*, the latter two of which have not been previously described as PAH degraders. The majority of the species identified during the creosote biodegradation belonged to *Pseudomonas* genus, which has been widely studied in bioremediation processes. Results confirmed that some bacteria have an intrinsic capacity to degrade the creosote without previous exposure.

1. Introduction

Creosote is a complex mixture of persistent organic compounds derived from coal pyrolysis and the further distillation of the oily product obtained and is widely used as a wood preservative. It is composed of approximately 85% polycyclic aromatic hydrocarbons (PAH), 10% phenolic compounds, and 5% nitrogen and sulfur. As

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PAH are toxic, mutagenic, bioaccumulative and persistent in environmental compounds, they are considered priority pollutants by the US-EPA. Compared to physicochemical methods, bioremediation is a more effective, versatile and economical technique for removing PAH from the environment. Microbial degradation is the main process in natural decontamination and the biological removal of pollutants in chronically contaminated soils [1] where degrading bacteria are abundant [2]. However, recent studies have reported the potential ability of microorganisms to degrade PAH in soils which have not been previously exposed to these toxic compounds [3–5]. The technique based on the degradation capacity of indigenous bacteria is called natural attenuation. This method avoids damaging the habitat [6], allowing the ecosystem to revert back to original conditions and converting toxic compounds into harmless ones [7,8]. However, it takes a long time to remove toxic components, because degrading microorganisms in soils can represent about only 10% of the total population [9]. Thus, many bioremediation studies focus on bioaugmentation, which consists of adding allochthonous degrading microorganisms [10] which can be a pre-adapted pure bacteria strain or consortium, genetically engineering bacteria or the addition of relevant genes in a vector to be transferred by conjugation [11]. However, bioaugmentation is a complex technique, because a negative or positive effect depends on the interaction between the inocula and the indigenous population, due to resource competition, mainly for nutrients [12]. Biostimulation is another bioremediation technique which consists of increasing the degrading capacity of the indigenous community by adding nutrients to avoid metabolic limitations [13].

However, inconsistent results have been obtained with these techniques. Previous studies have shown that biostimulation can increase biodegradation rates [14] without negatively effecting degradation rates [9,15]. Similarly, when bioaugmentation was applied, biodegradation rates were enhanced [10], but not significantly [12,13].

It should be noted that each contaminated site can respond differently [13]. Therefore, laboratory-scale assays should be designed before carrying out an *in situ* bioremediation process to determine the most efficient technique and evaluate its effect on microbial diversity. Furthermore, previous works [16] have shown that although PAH were depleted, toxicity was still significant. Although most reported works did not perform toxicity assays, they should be carried out to assess the effectiveness of biodegradation on the final result. The main goal of the present study is to determine the most effective bioremediation technique in the decontamination of a creosote contaminated soil through microcosm assays, evaluating changes in the bacterial community and toxicity.

2. Materials and methods

2.1. Chemical, media and inoculated consortium

Creosote contained 87 wt% of PAH and derived compounds thereof. For the purpose of the present work, 6 key PAH, representing a wide range of molecular weight and structures, were used to monitor the bioremediation process. The compositions of these key compounds in creosote were: 0.5 wt% naphthalene, 5.1 wt% phenanthrene, 12.2 wt% anthracene, 3.1 wt% pyrene, 1.3 wt% dibenzofurane and 4.7 wt% acenaphthene. Creosote was diluted in acetonitrile (Sigma–Aldrich, Steinheim, Germany) in a stock solution (0.439 g/ml final concentration) containing0.117 gPAH/ml. Luria–Bertani (LB) and Bushnell-Haas Broth (BHB) media were purchased from Panreac (Barcelona, Spain). Biostimulation treatments were amended with BHB as a source of inorganic nutrients, whose composition was previously optimized [17] for a PAH-degrading consortium (C2PL05). Tween-80 (300 μl/ml) was used as optimum surfactant for PAH biodegradation using C2PL05 consortium [18]. Bioaugmentation treatments were inoculated with PAH-degrading consortium C2PL05, extracted from PAH-contaminated soil at a petrochemical plant and described elsewhere [19].

2.2. Experimental design

The efficiency of five different types of treatments for creosote removal was compared: control or untreated (C), natural attenuation (NA), biostimulation (BS), bioaugmentation (BA) and biostimulation and bioaugmentation (BS + BA). Experiments were carried out in duplicate microcosms for five sampling times: 0, 6, 40, 145 and 176 days from December 2009 to May 2010. Thus, a total of 40 microcosms were prepared. Microcosms consisted of plastic trays containing 550 g of soil samples, collected from unpolluted soil in the area of Rey Juan Carlos University. Soil samples were obtained from the top 20 cm of soil and sieved to obtain the fraction with a particle size < 2 mm. Mesocosms were randomly arranged outdoors in a terrace and protected with plastic film to avoid direct rain and snow. Except for the control treatment, each tray was spiked with 5.6 ml of a creosote solution (0.439 g/ml) in *n*-hexane to a final amount of 2.5 g of creosote per tray. All microcosms were maintained at 40% water holding capacity (WHC) [13]. For BS, microcosms were hydrated with the required amount of optimum BHB, while deionized and sterilized water was used in treatments without BS. BA microcosms were inoculated with 5 ml of C2PL05 consortium containing $2.0 \times 10^7 \pm 4.3 \times 10^6$ cells/g soil of heterotrophic microorganisms and $1.8 \times 10^5 \pm 1.0 \times 10^5$ cells/g soil of creosote-degrading microorganisms. Air temperature was continuously recorded during the whole experiment using temperature data loggers (MicroLogEC650, Fourier Systems Ltd., Barrington, RI, USA).

2.3. Characterization of soil and environmental conditions

Soil NO_3^- concentration was estimated using a SKALAR San++ Analyzer (Skalar, Breda, The Netherlands) after shaking the soil sample with distilled water (1:5 ratio) for one hour. Water holding capacity (WHC) was measured following the method described by Wilke [20], and water content was calculated as the difference between wet soil weight and soil weight after drying at 60 °C for 24 h. One gram of dried soil was suspended in deionized water (1:10) and incubated in an orbital shaker at 150 rpm and 25 °C for 1 h. Then, the pH of the suspension was measured using a GLP 21 micro pH meter (Crison, Barcelona, Spain). Ambient temperature was continuously recorded with temperature loggers (Tidbit Loggers, Onset Computer, Pocasset, MA, USA) placed on site.

Total heterotrophic microorganisms (HM) and creosote-degrading microorganisms (CDM) of the microbial population in the natural soil were counted using a miniaturized most probable number (MPN) technique and 96-well microtiter plates with eight replicates per dilution [21]. The number of cells was calculated with Most Probable Number Calculator software version 4.04 [22]. To extract microorganisms from the soil, 1 g of soil was resuspended in 10 ml of phosphate buffer saline (PBS) and shook at 150 rpm and 25 °C for 24 h. HM were determined in 180 μL of LB medium with glucose (15 g/L), and CDM were counted in 180 μl of BHB medium with 10 μL of creosote stock solution as a carbon source.

2.4. MPN, respiration and toxicity assays

CDM in microcosms were estimated by MPN at 6, 40, 145 and 176 days. For respiration assays, 10 g of soil (moistened with deionized water to 40% of WHC) were incubated in duplicate in closed desiccators at 25 °C for 14 days. Each replicate contained a vial with 14 ml 0.2 M NaOH to absorb and neutralize the CO_2 produced by