Applied Soil Ecology 105 (2016) 76–85

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Monitoring the changes in a bacterial community in petroleum-polluted soil bioaugmented with hydrocarbon-degrading strains

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ARTICLE INFO

Article history:
Received 16 September 2015
Received in revised form 12 February 2016
Accepted 11 April 2016
Available online 19 April 2016

Keywords:
Petroleum hydrocarbons
Bioaugmentation
qPCR
DGGE
PLFA
CLPP

ABSTRACT

Bioaugmentation is a strategy used to enhance degradation of petroleum compounds in contaminated soils, however little is known about the interactions between introduced bacteria and autochthonous microflora. Therefore the objective of the study was to assess any changes in the structure and metabolic activity of the soil bacterial communities as a result of the introduction of *Bacillus subtilis* T′-1 or *Pseudomonas* sp. P-1, as well their consortium, into petroleum-contaminated soil. The bioaugmentation experiment was carried out under laboratory conditions using soil taken from an industrial area located around a refinery in Czechowice-Dziedzice. After the bioaugmentation process, a significant ($P < 0.05$) decrease in the TPH content was reported in all inoculated soils. Inoculation of the soil with the bacterial consortium resulted in a three times greater removal of TPH compared to soils inoculated with single strains. It has been reported that all of the strains had an ability to survive in the environment during the experimental period. The introduction of bacterial strains led to increase in the number of 16S rRNA gene copies in soil on 1 and 7 days of the experiment as well as *alkB* gene copies during 91 days of the study compared to the non-treated soil. Analysis of the 16S rRNA and *alkB* genes-based DGGE fingerprints showed that introduced bacteria changed the genetic diversity of the total bacterial communities as well as the communities that have the genes involved in the degradation of hydrocarbons. Analysis of the PLFA profiles showed that the bacterial strains caused short-term changes in the amounts of fatty acids characteristic for Gram-positive and Gram-negative bacteria. The CLPPs indicated differences in soil metabolic activity between the inoculated and non-inoculated soils after the bioaugmentation process.

1. Introduction

The contamination of soil by petroleum hydrocarbons, which are known to belong to the family of carcinogens and neurotoxic organic pollutants (Abioye, 2011), is a serious problem prevalent across the globe. Currently, a variety of technologies can be applied for the remediation of environments that have been polluted with such compounds. Among them, bioaugmentation, which is the addition of specific strains or the microbial consortium that has the desired catabolic properties into the contaminated soil, is recommended as an efficient, economic, versatile and environmentally sound solution (Liu et al., 2011). The bacteria used in the bioaugmentation of petroleum-polluted soils very often have the ability to produce surface active compounds. Inoculation with bacteria that are able to degrade hydrocarbons and produce biosurfactants increase the bioavailability of hydrophobic compounds and thus accelerate hydrocarbon biodegradation (Pacwa-Płociniczak et al., 2011). However, it has been observed that bioaugmentation does not always bring the desired effects. One of the reasons for the failure may be the interactions between the indigenous populations of microorganisms and the introduced strains (Mao et al., 2012). Whilst the design and implementation of remediation technologies are relatively well established, analysis of the changes in the microbial activity and community structure

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http://dx.doi.org/10.1016/j.apsoil.2016.04.005
0929-1393/

in bioaugmented hydrocarbon-polluted ecosystems has still been a challenge for scientists. These shortfalls in understanding the dynamics and shifts in soil during the degradation of hydrocarbons are constantly compared to the ecological "black box" of biological remediation systems (Whiteley and Bailey, 2000). The presence of hydrocarbons in soil selectively promotes the growth of the microorganisms capable of utilising them. Thus, in long-term contaminated soils, changes towards the dominance of hydrocarbon degrading microorganisms can be observed (Vázquez et al., 2009). On the other hand, in soils subjected to the bioremediation process, shifts in the structure of the microbial communities might occur once again, especially if a bioaugmentation strategy was applied. Studies on the diversity of soil microbial communities during bioremediation are necessary in order to evaluate the impact of introduced bacterial strains may have on the structure of that community (Vázquez et al., 2009). Additionally, the ability to monitor the diversity and structural composition of the soil microbial communities may provide good information about the health and condition of the soil during the degradation of contaminants (Whiteley and Bailey, 2000). The lack of knowledge about changes in the structure of microbial communities in soil subjected to bioremediation comes from the fact that many of the microbes may not have been isolated in the laboratory or may have specific community associations that prevent the isolation of pure cultures for analysis. Nonetheless, the application of culture-independent molecular and biochemical techniques can facilitate complex analyses of environmental samples (Whiteley and Bailey, 2000).

The aim of the study was to assess any shifts in the genetic, structural and functional diversity of the soil autochthonous microbial communities as a result of the introduction of *Bacillus subtilis* T′-1 or *Pseudomonas* sp. P-1, as well their consortium, into petroleum-hydrocarbon contaminated soil.

2. Materials and methods

2.1. Study area and soil collection

The soil, which was historically contaminated, was obtained from an industrial area located around an oil refinery in Czechowice-Dziedzice, Upper Silesia, Poland. Nearly a century of continued use of a sulfuric acid-based oil refining technology by the Czechowice Oil Refinery has produced an estimated 120 thousand tons of acidic, highly weathered petroleum sludge that has been deposited into a waste lagoon. The soil that was used for the bioremediation experiment was collected from a site adjacent to lagoon. The soil (prepared from eight different sub-samples taken from an area of 25 m^2) was collected from the surface to a depth of about 0.2 m. Prior to experiment, the soil was passed through 1.2 mm sieve and stored at 4 °C. The soil used in the study was classified as silty clay loam (Orthic Luvisols, according to FAO system) (FAO, 2014). Its detailed chemical and physical parameters are listed in Table 1.

Table 1
Selected physicochemical properties of the soil used in the experiment.

Parameter	Value
Sand (%)	31 ± 3.1
Silt (%)	45 ± 4.5
Clay (%)	24 ± 2.4
Textural classification	silty clay loam
Density (g cm^{-3})	1.145 ± 0.002
pH_{H2O}	4.02 ± 0.01
Organic matter (%)	6.81 ± 0.03
N_{tot} (%)	0.079 ± 0.001
C_{org} (%)	1.58 ± 0.12
P (mg kg^{-1})	505.40 ± 29.32
Fe (mg kg^{-1})	20740.00 ± 782.56

±Stand. Dev. of three independent experiments.

2.2. Isolation and selection of rifampicin-resistant mutants and preparation of the inoculum

Hydrocarbon-degrading and biosurfactant-producing *Bacillus subtilis* T′-1 and *Pseudomonas* sp. P-1 strains were isolated from the soil used in this study (Płaza et al., 2011; Pacwa-Płociniczak et al., 2014). In order to monitor the survival of the strains after their introduction into the contaminated soil, spontaneous rifampicin-resistant mutants of the strains were selected according to Płociniczak et al. (2013). To prepare the inoculum for bioaugmentation, the rifampicin-resistant mutants of T′-1 and P-1 strains were grown on a molasses medium on an orbital shaker at 120 rpm (28 °C) for 48 h. The number of bacteria in the inoculum was evaluated based on the turbidimetry and plating techniques. The proper volume of bacterial suspensions was centrifuged (12,000 rpm, 4 °C, 20 min) and the harvested bacteria were washed twice with sterile saline and resuspended in 40 mL of sterile saline.

2.3. Experimental design

The bioremediation study was carried out under laboratory conditions. The experiment had a completely randomised block design with three replications that had four treatments: (1) soil inoculated with strain T′-1, (2) soil inoculated with strain P-1, (3) soil inoculated with mixture of T′-1 and P-1 strains and (4) the control soil treated with sterile saline instead of a bacterial suspension. Four hundred grams of contaminated soil was placed into pots and then 40 mL of the bacterial solutions of the T′-1, P-1 and T′-1 + P-1 strains were added into the soil up to 10^7 bacterial cells g^{-1} dry weight (dw) soil. Afterwards soils (inoculated with bacteria and control) were gently mixed for equal distribution of bacteria in soil. The soil pots were incubated for 91 d at room temperature On days 1, 3, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84 and 91 the soil samples were collected with diameter auger and immediately analysed for the survival of the T′-1 and P-1 strains. For other analyses, soil samples of 15 g were taken on days 1, 7, 42 and 91, and immediately stored at −20 °C.

2.4. Total petroleum hydrocarbons (TPH)

The total petroleum hydrocarbon concentration in the soil before and after the bioremediation study was quantified as hydrocarbons with a carbon number between 10 and 40 ($TPHc_{10-40}$) following the ISO 16703:2011 protocol. Five grams of soil was briefly shaken with 10 mL of acetone and after the addition of 5 mL of *n*-heptane containing a C_{10} and C_{40} standard solution, and then the mixture was shaken for 1 h. After the solid material settled, the supernatant was transferred into glass tubes with Teflon seals and then, the organic phase was washed twice by shaking thoroughly for 5 min with 20 mL of water in order to remove the acetone. The organic layer was cleaned up using dual-layer Florisil/Na_2SO_4 columns (Supelco, USA). TPH was analysed using a gas chromatograph (Hewlett-Packard 6890, USA) equipped with a flame ionisation detector (FID) with an Rxi—5 ms capillary column (25 m × 0.2 mm ID × 0.33 μm); the injection volume was 2 μL and hydrogen was used as the carrier gas (2.1 mL min^{-1}). The operation program was started with injector and detector temperatures of 300 °C. Oven temperature was initially programmed at 60 °C, held for 10 min, increased to 320 °C at 30 °C min^{-1} and then held for 10 min.