Soil Biology & Biochemistry 99 (2016) 66–74

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

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

Hydrocarbon biostimulation and bioaugmentation in organic carbon and clay-rich soils

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ARTICLE INFO

Article history:
Received 31 December 2015
Received in revised form
20 April 2016
Accepted 23 April 2016
Available online 8 May 2016

Keywords:
Bioremediation
Rhodococcus erythropolis
Hydrocarbons (HC)
Oxitop
qPCR
16S-rRNA gene sequencing

ABSTRACT

Hydrocarbon-contaminated organic carbon-rich clayey soils are challenging for bioremediation stakeholders since the pollutant is heterogeneously distributed and poorly bioavailable due to its strong adsorption on clay and organic particles. In addition, biodegradation rates are restricted by limited diffusion of oxygen and nutrients to hydrocarbon-degrading aerobes. This study assessed the benefits of bioaugmentation with the strain *Rhodococcus erythropolis* T902.1 versus those from biostimulation and anaerobic natural attenuation in terms of hydrocarbon (HC) degradation efficiency and changes in the bacterial community structure in a diesel-polluted clay-rich soil. Three soil samples with a similar total organic content but with a different HC concentration (0.2, 1.0 and 6.5 g/kg) were compared in a microcosm experiment. Despite a limitation in oxygen transfer, *R. erythropolis* T902.1 enhanced a greater HC degradation compared to the biostimulation treatment. However, this advantage decreased with time as the proportion of *Rhodococci* declined from 25% initially to 1% of the global community after 80 days of treatment. Similarly, the *alkB* gene proportion in bioaugmented soils decreased to levels close to those of biostimulated soils. Consequently, further engineering was suggested to improve the resilience of the inoculum to ensure its long-term presence and activity in such polluted environments.

1. Introduction

Hydrocarbons (HC, including mineral oil, BTEX and PAH) are widely spread in nature as a result of both long-term use and frequent release. In Europe, they occur on average in 45% of identified contaminated sites and this frequency reaches 85% in Belgium (Van Liedekerke et al., 2014). Furthermore, they are particularly difficult to remediate in carbon-rich clayey soils because of their lower bioavailability and the difficulty to provide oxygen for fast HC degrading aerobes. Several limiting factors can be considered: heterogenic distribution of organic matter and associated HC, restricted oxygen and nutrients transfer, competition between carbon sources and between microorganisms. Consequently, even linear HC degrade slowly or not at all in such soils while they are relatively rapidly biodegraded in other soils. Treatment technologies that rely on stimulating indigenous microorganisms (biostimulation) or adding specific degraders (bioaugmentation) might be stymied by these rate-limiting conditions. Bioaugmentation is considered to have the same dependence on physicochemical conditions as biostimulation even if the active microbial population should be higher. Although bioaugmentation is an ecologically- and cost-effective technique, its efficiency and usefulness is thus debatable and there is a need for improved understanding of the causes that can lead to its failure, such as diverse environmental constraints and poor adaptation ability of laboratory-cultivated microorganisms (Vogel, 1996; El Fantroussi and Agathos, 2005; Boon and Verstraete, 2010; Tyagi et al., 2011).

Rhodococcus sp. appears to be a good candidate for use in bioaugmentation, since this genus is ubiquitous (Bell et al., 1998) and

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http://dx.doi.org/10.1016/j.soilbio.2016.04.016
0038-0717/

can degrade a broad range of aliphatic, branched, cyclic, (polycyclic) aromatic, sulfur-containing and chlorinated HC (Martinkova et al., 2009; Larkin et al., 2010a, 2010b). *Rhodococcus* sp. can also withstand environmental stresses, such as low temperature, low water content, high pH, high salinity, lack of nutrient, presence of toxic solvents or pollutants (Whyte et al., 1999; de Carvalho, 2010, 2012; de Carvalho et al., 2014). *Rhodococci* synthesize mycolic acids and trehalolipids to modify their membrane hydrophobicity in order to protect themselves from these adverse environments and to solubilize and assimilate hydrophobic substrates that are poorly bioavailable in soil (Lang and Philp, 1998; Kuyukina et al., 2005; Kuyukina and Ivshina, 2010). In this study, the desiccation-tolerant strain *Rhodococcus erythropolis* T902.1 was tested. This strain is suitable for large production in bioreactors and conservation in powdered form before its use on the field (Weekers et al., 1999).

The objective of this work was to assess the potential benefits of bioaugmentation with *R. erythropolis* T902.1 versus those for aerobic biostimulation and anaerobic natural attenuation for the effective degradation of poorly available hydrocarbons in carbon-rich soils. Three levels of pollution ($[HC_{C10-C40}] = 0.2$, 1.0 and 6.5 g/kg dry matter) were tested in a microcosm study. HC degradation and oxygen uptake were monitored to evaluate performance differences between the treatments. In addition, microbiological parameters thought to confirm the degradation processes were measured. These include the concentrations of the alkane monooxygenase (*alkB*) gene and the different microbial populations such as *R. erythropolis*. The alkB gene was widely studied to assay the biodegradation potential of n-alkanes in soils (e.g. Kloos et al., 2006; Powell et al., 2006). It encodes an alkane monooxygenase, *i.e.* a membrane-bound hydroxylase involved in the assimilation and oxidation of linear and branched HC, which catalyzes this critical reaction step enabling HCs to be used in the general microbial metabolism. This gene has been observed in contaminated soil (Kloos et al., 2006) and was expressed during active bioremediation of diesel-contaminated soils (Yergeau et al., 2009, 2012). However, *alkB* gene expression (transcripts) was not taken into account since it is not necessarily correlated to HC level and degrading activity, according to a previous study on chronically polluted sediments (Paisse et al., 2011). Finally, changes in the microbial community structure as a function of the HC level, time and the type of treatment were evaluated using 16S-rRNA (*rrs*) gene analysis.

2. Materials and methods

2.1. Soil sampling and characterization

Soil was sampled from a site polluted with diesel and heating fuel. The site is an operating truck-fill station where several potential leaks were assumed. A transect of eight 3 m-long wells (piezometers 501 to 508) was realized at one of the site border to delimit the contaminant plume (details are provided in Appendix A).

From core drilling, three soil samples (3 kg) were selected and characterized (Table 1). They originate from three different horizons (20–100, 100–200 and 200–300 cm, cf. Appendix A), possess a similar total organic carbon content (2.6%) but are differently contaminated ($[HC_{C10-C40}] = 0.2$, 1.0 and 6.5 g/kg dry matter). The monoaromatic HC content is quite low: 3.39 ± 0.20 mg/kg of dry matter for the most polluted soil and concentrations are under limit of detection (LOD) for lesser polluted soil samples. The soil structure mainly consists of a low permeable and clay-rich loam that limits O_2 diffusion (no NO_3^-, 1 ± 0.6 mg/L NH_4^+, pO_2 of 0.9 ± 0.4 mg/L and negative redox potential in the groundwater from the piezometers developed). In addition, peat is also present and heterogeneously mixed within the loam in the least and moderately polluted soils, which contributes to their high carbon content.

2.2. Microcosms: experimental conditions and oxygen consumption monitoring

Each soil underwent three treatments: an anaerobic natural attenuation (An), a biostimulation with air (Ox) and a bioaugmentation with air and *R. erythropolis* T902.1 (Re). A sample (150 g) of soil was placed in either a 250 mL bottle that had been degassed with N_2 and sealed with a rubber cap (An) or in a pre-calibrated Oxitop® bottle that measured O_2 consumption once every day (Ox and Re treatments). One digital unit measured by the Oxitop system corresponded approximately to 1.9 mg of O_2 consumed in the bottle *i.e.* 20 mg O_2/kg dry soil. Inoculation consisted of spraying soil with 2 mL of a concentrated resuspension (0.85% NaCl) of a pure liquid culture of *R. erythropolis* T902.1. This corresponded to a concentration of approximately 8×10^7 CFU per gram of soil. Two mL of sterile 0.85% NaCl solution were sprayed on non-inoculated soils. Each treatment was run in triplicate and the 27 bottles were maintained at 22 °C without agitation. Bottles were opened every 5 days, or sooner if ¾ of the maximum oxygen uptake (30 digits measured by the manometer) was reached, in order to prevent oxygen limitation. During each bottle opening, bottles were agitated to ensure complete reoxygenation of the gas phase and dissolved NaOH pellets in the CO_2 trap were replaced.

2.3. Hydrocarbon monitoring

A C_{10}-C_{40} HC analysis by GC-FID was performed on 20 g of soil sampled from the microcosms after 0, 40, 80 and 100 days (ISO 16703, Wessling, Saint-Quentin-Fallavier, France).

2.4. DNA extraction

DNA was extracted from 0.30 g of wet soil after 0, 20, 40 and 80 days of the experiment, with the NucleoSpin® Soil kit (Macherey Nagel, Düren, Germany) following the manufacturer's instructions but with a double lysis step (SL1+SX then SL2). DNA was finally resuspended in 100 μL of elution buffer (5 mM Tris–HCl, pH 8.5) and gDNA concentration was measured with the Qubit® dsDNA HS Assay kit and a Qubit® fluorometer (ThermoFisher Scientific, Waltham, USA). Then, DNA was stored at −20 °C and kept on ice in further manipulations.

2.5. qPCR analyses

Quantitative PCR were performed on the *alkB* gene coding for alkane monooxygenase and on the *16S-rRNA* gene with either specific primers for the species *R. erythropolis* or wide-range primers covering Eubacteria (Table 2) (Bell et al., 1999; Fierer et al., 2005; Powell et al., 2006). The amplification performance was first assessed by a PCR with a gradient temperature (T gradient thermocyler, Biometra, Göttingen, Germany) on DNA isolated from the pure strain T902.1 and from some soil samples to establish the optimal annealing temperature (Table 2). Amplicon specificity was checked on a 2% agarose gel and with a GeneRuler Low range DNA ladder (ThermoFisher Scientific, Waltham, USA). For all primer pairs, the crude extracts of soil gDNA did not exhibit PCR or qPCR inhibition.

Two μL of crude-extracted gDNA, DNA-free water or of standard DNA were added to a premix containing 0.8 μL of each primer at 10 μM, 6.4 μL of DNA-free water and 10 μL of 2× SensiFast™ SYBR No-Rox mix (Bioline, London, UK). All the qPCR reactions were