Ecotoxicology and Environmental Safety 125 (2016) 153–160

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Evaluation of microbial population and functional genes during the bioremediation of petroleum-contaminated soil as an effective monitoring approach

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ARTICLE INFO

Article history:
Received 8 June 2015
Received in revised form
18 November 2015
Accepted 22 November 2015
Available online 10 December 2015

Keywords:
Bioremediation
Biostimulation
Hydrocarbon-degrading bacteria
Soil
Horizontal gene transfer

ABSTRACT

This study investigated the abundance and diversity of soil n-alkane and polycyclic aromatic hydrocarbon (PAH)-degrading bacterial communities. It also investigated the quantity of the functional genes, the occurrence of horizontal gene transfer (HGT) in the identified bacterial communities and the effect that such HGT can have on biostimulation process. Illumina sequencing was used to detect the microbial diversity of petroleum-polluted soil prior to the biostimulation process, and quantitative real-time PCR was used to determine changes in the bacterial community and functional genes (alkB, phnAc and nah) expressions throughout the biostimulation of petroleum-contaminated soil. The illumine results revealed that γ-proteobacteria, Chloroflexi, Firmicutes, and δ-proteobacteria were the most dominant bacterial phyla in the contaminated site, and that most of the strains were Gram-negative. The results of the gene expression results revealed that gram-negative bacteria and alkB are critical to successful bioremediation. Failure to maintain the stability of hydrocarbon-degrading bacteria and functional gene will reduce the extend to which alkanes and PAHs are degraded. According to the results of the study, the application of a C:N:P ratio of was 100:15:1 in the biodegradation experiment resulted in the highest rate at which petroleum hydrocarbons were biodegraded. The diversity of pollutant-degrading bacteria and the effective transfer of degrading genes among resident microorganisms are essential factors for the successful biostimulation of petroleum hydrocarbons. As such, screening these factors throughout the biostimulation process represents an effective monitoring approach by which the success of the biostimulation can be assessed.

1. Introduction

Today, petroleum and its derivatives are the main source of energy for industry and domestic consumption (Rahman et al., 2003). Petroleum hydrocarbons are based on multiple carbon bonds that develop intense and raised complex structures when they bound with other characteristic molecules, hence the latest hydrocarbons have variety of forms and consist of short, medium and long aliphatics (i.e. alkanes, alkenes), aromatics and polycyclic aromatic hydrocarbons (PAHs) of different proportions (Zhang et al., 2011; Steliga et al., 2012). The accidental release of these hydrocarbons into the environment represents a major source of water and soil contamination (LeFevre et al., 2012; Gao et al., 2014). The hydrocarbons that are released through soil and water pollution can have a toxic effect on any multicellular tissues that are exposed to such chemicals (Steliga et al., 2012). Petroleum accumulation in the tissues of an organism can lead to mutation and eventually cancer (Steliga et al., 2012). Burying, combusting, extracting soil vapor, soil washing, and dispersion are among the physical and chemical technologies that are commonly used to treat petroleum-contaminated soil (Reddy et al., 2011; Xu et al., 2013). However, these methods are not economically practical and may not be capable of completely decomposing contaminants. In some cases, they may even lead to the development of compounds that are more environmentally toxic than they were before treatment (Megharaj et al., 2011; Wang et al., 2012; Xu et al., 2013).

Unlike the above-mentioned methods, bioremediation consists of emerging technologies that are feasible and cost-effective (Wang et al., 2012; Lu et al., 2014). During this process, the microorganisms may convert the affected chemicals to nontoxic compounds or entirely degrade the toxic hydrocarbon compounds to CO_2 and H_2O as opposed to simply transferring them form one

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http://dx.doi.org/10.1016/j.ecoenv.2015.11.029
0147-6513/

phase to another (Megharaj et al., 2011; Rahman et al., 2003). Bioaugmentation, which involves adding microbial strains to the natural existing population to improve the microbial community's ability to degrade petroleum and other hydrocarbons, has its own challenges (Gao et al., 2014). Determining whether the introduced microorganisms or the naturally existing ones play the main role in destruction process is difficult. Furthermore, concerns about the use of genetically engineered microorganisms and their potential impact on the environment are commonly raised (Megharaj et al., 2011). It is widely accepted that biostimulation represents a more effective method of achieving sustainable soil bioremediation than bioaugmentation (Megharaj et al., 2011).

Microbial activity in the intrinsic bioremediation process is generally restricted by a number of factors such as low nutrient supplies, low level of electron donors and low bioavailability of contaminants (Ron and Rosenberg, 2014). These limitations can be overcome by introducing essential growth-limiting nutrients, electron acceptors/donors, and surfactants to the polluted site to improve the biodegradation potential of the indigenous microorganisms (Masakorala et al., 2013). Nitrogen and phosphorus are the limiting nutrients that are most commonly employed in biostimulation practice (Chang et al., 2010; Steliga et al., 2012). Theoretically, 150 mg of Nitrogen and 30 mg of phosphorous are required to degrade 1 g of hydrocarbons. Therefore, a stoichiometric ratio of C:N:P of 100:5:1 has been accepted as a common formula for biostimulation practice (Zhu et al., 2001). However, different microbial concentrations have been reported as a result of the application of different N:P ratios. Since every petroleum source has different properties and every contaminated site has different treatment factors, the appropriate N:P ratios need to be identified for each contaminated site. For instance, Turgay et al. (2009) reported a successful bioremediation of crude-oil contaminated soil through the regulation of C:N:P ratio to 100:15:1. Qin et al. (2013), achieved a biodegradation of 60% by using a C:N:P ratio of 100:10:1 and Yerushalmi et al. (2003) applied a ratio of 50:10:1 to biodegrade a contaminated site.

Different studies have attempted to determine the microbial community that is responsible for the degradation of petroleum hydrocarbons. Diverse bacterial groups can degrade petroleum hydrocarbons. Some bacteria, such as Acinetobacter spp., Pseudomonas spp., Streptomyces spp., Arthrobacter spp., and Bacillus spp., are able to effectively degrade groups of alkanes (Arvanitis et al 2008; Fuentes et al 2014). Whereas genera, such as Pseudomonas spp., Flavobacterium spp. Alcaligenes spp., Sphingomonas spp. and so on, are capable of degrading aromatic hydrocarbons (Das and Chandran, 2011). From the Gram stain assay point of view, both Gram-negative and Gram-positive bacteria play an important role in the bioremediation of petroleum-polluted sites. While the work of some researchers indicates that Gram-negative bacteria play the main role in the biodegradation of petroleum hydrocarbons, others have demonstrated the importance of Gram-positive bacteria, specifically those with a high C+G content (Vandecasteele 2008; Cébron et al., 2008). Although research in this area and the subsequent isolation and determination of the related bacterial strains is promising, the dynamics of the microbial populations and functional genes present in the petroleum-contaminated soil is an important aspect of the monitoring process required to optimize the success of bioremediation approach that remains unclear (Megharaj et al., 2011).

Hence, developing an understanding of the bioremediation process lies in understanding the microbial diversity (Yadav et al., 2015). This involves conducting a molecular analysis of the contaminant degradation genes and microbial populations found in contaminated sites that are undergoing bioremediation, and has become a fundamental aspect of biodegradation monitoring and subsequent decision-making (LeFevre et al., 2012). Moreover, research studies report that horizontal gene transfer supports the biodegradation process by accelerating the development of an efficiently degrading microbial community upon contamination. Therefore, the determination of given degradation genes on mobile genetic elements or the determination of genes that are able to promote HGT can be subsequently perceived as evidence that a bioremediation process has been successful (Wilson et al., 2003; Nie et al., 2014). A real-time PCR approach can be used to quantify amplified targeted DNA and to identify the copy numbers of a given gene that are present in the samples obtained from the site as a result of the bioremediation process (Cyplik et al., 2011). The value of this technique as an automated, fast, sensitive and reliable monitoring tool has also been confirmed in bioremediation studies. The alkB coding for alkane monooxygenase enzyme, phnAc and naphthalene dioxygenase (nah) genes are ideal markers for the purposes of studying the microbial community's potential to degrade petroleum hydrocarbons in the bioremediation site (Laurie and Lloyd-Jones, 2000; Yang et al., 2015). The use of real-time PCR to target these genes and quantify n-alkane and PAH-degrading bacterial communities has been proven to represent an effective method of characterizing the abundance and diversity of a degrading bacterial community (Cébron et al., 2008; Juhanson et al., 2009; Aydin et al., 2015a,b).

The aim of this research was to understand the functional diversity of hydrocarbon degrading bacteria as well as alkane and PAHs degradation genes (*alkB*, *phnAc* and *nah*) in association with a total and active bacterial community. In addition, the study aimed to assess the horizontal transfer potential of functional genes as a means of monitoring the efficiency of a bioremediation process.

2. Methods

2.1. Sampling and soil characteristics

Petroleum polluted soil samples were collected at four different points from coastal site of an old petroleum sludge storage pit in Turkey. Samples were sealed in polyethylene materials, transported to the laboratory and stored at 4 °C. Collected samples were homogenized and mixed thoroughly. Forest soil samples were obtained from five different point of Emirgan Forest (Istanbul, Turkey). Obtained samples were mixed completely and stored at 4 °C. Table S1 summarizes some physicochemical characteristics of collected samples.

2.2. Microcosms set ups and biostimulation experimentations

Petroleum contaminated soil and forest soil were mixed in different ratios to gain four different mixture experimental groups based on their final amount of total organic carbon (TOC). Four different experimental setups of T_1, T_2, T_3, and T_4 with TOC of %, 10%, 15%, and 25% respectively were prepared.

Biodegradation experiments were carried out in 16 microcosm setups. 300 g of obtained mixtures were weighed into microcosms and each experimental group was amended by four different nutrient ratios of 100:5:1, 100:10:1, 100:15:1, and 100:25:1. The nitrogen and phosphorus correction was performed using Urea ($(NH_2)_2CO$) and KH_2PO_4 solutions, respectively. Urea was selected as a nitrogen source based on its effectiveness in microbial respiratory activity that reported by Ghaly et al. (2013) and Dave et al. (2011). Furthermore, Kauppi et al. (2011) have declared utilization of urea as nitrogen source causes a significant rise in pH. Due to this and in light of the fact that nutrient overdose is a potential problem in in-situ bioremediation of contaminated sites, the probable effect of a high amount of urea using the nutrient