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Bioremediation potential of diesel-contaminated Libyan soil

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ABSTRACT

Bioremediation is a broadly applied environmentally friendly and economical treatment for the clean-up of sites contaminated by petroleum hydrocarbons. However, the application of this technology to contaminated soil in Libya has not been fully exploited. In this study, the efficacy of different bioremediation processes (necrophytoremediation using pea straw, bioaugmentation and a combination of both treatments) together with natural attenuation were assessed in diesel contaminated Libyan soils. The addition of pea straw was found to be the best bioremediation treatment for cleaning up diesel contaminated Libyan soil after 12 weeks. The greatest TPH degradation, 96.1% (18,239.6 mg kg^{-1}) and 95% (17,991.14 mg kg^{-1}) were obtained when the soil was amended with pea straw alone and in combination with a hydrocarbonoclastic consortium respectively. In contrast, natural attenuation resulted in a significantly lower TPH reduction of 76% (14,444.5 mg kg^{-1}). The presence of pea straw also led to a significant increased recovery of hydrocarbon degraders; 5.7 log CFU g^{-1} dry soil, compared to 4.4 log CFU g^{-1} dry soil for the untreated (natural attenuation) soil. DGGE and Illumina 16S metagenomic analyses confirm shifts in bacterial communities compared with original soil after 12 weeks incubation. In addition, metagenomic analysis showed that original soil contained hydrocarbon degraders (e.g. *Pseudoxanthomonas* spp. and *Alcanivorax* spp.). However, they require a biostimulant (in this case pea straw) to become active. This study is the first to report successful oil bioremediation with pea straw in Libya. It demonstrates the effectiveness of pea straw in enhancing bioremediation of the diesel-contaminated Libyan soil.

1. Introduction

Soil pollution as a result of contamination with petroleum hydrocarbons represents a serious global issue. The extent of hydrocarbon contamination in the environment is not surprising given the amount of oil used and transported around the world. Eighty four million barrels of crude oil are consumed around the world per year, almost 50% of which is transported by sea which leads to the increased chance of oil tanker accidents and in turn large-scale water and soil pollution (Hasan et al., 2010; McKew et al., 2007; Rhodes, 2010).

Diesel is one of the most commonly found hydrocarbons in the environment, consisting of alkanes and aromatic compounds which can be released during storage and transportation (Gallego et al., 2001). According to the Oil and Gas Journal and the Organization of the Petroleum Exporting Countries (OPEC), Libya has the largest proven oil reserves in Africa, with production running at 1.65 million barrels per day of high quality crude oil in 2010 and gross proven oil reserves of 47.1 billion barrels in 2012. Such a large production has environmental consequences too since Libya has already faced one of the largest oil spills in world history when 59 million litres of oil were released in an area southeast of the capital city Tripoli, with the spill covering about 800 km^2 of Libyan soil (O'Rourke and Connolly, 2003).

Traditional physico-chemical methods such as soil washing, soil vapour extraction, incineration, the use of oil booms and solidification have been used for the clean-up of oil contaminated sites; however they are disruptive, labour intensive and relatively expensive processes (Huang et al., 2004; Tang et al., 2010). Over the past twenty years, there has been an increasing global interest in the field of bioremediation due to the limitation of landfills and the growing remediation costs. Among strategies for hydrocarbon contamination management, bioremediation has received considerable attention. One strategy to improve the efficiency of bioremediation processes is the introduction of highly specialized

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microorganisms into the polluted environment. Inoculating the soil with contaminant degrading microbes is generally known as bioaugmentation (Barathi and Vasudevan, 2003; Wu et al., 2008; Wu et al., 2011). Bioaugmentation has been successfully employed in the field of environmental pollutant mineralization through inoculation of the affected soil with exogenous hydrocarbon utilizing microbial strains (bacteria or fungi) to enhance the uptake of contaminants. This method is mainly beneficial when the abundance of relevant catabolic genes (such as the *alkB* alkane hydroxylase gene) among the native microbial community is insufficient (Vomberg and Klinner, 2000).

Some of the hydrocarbonoclastic microorganisms reported include members of the *Pseudomonas*, *Acinetobacter*, *Alcaligenes, Brevibacillus* and *Bacillus* genera; all have been listed as among the most important hydrocarbon degraders in marine and soil environments (Leahy and Colwell, 1990). Among the *Bacillus* genera, several *Bacillus* strains have been reported to degrade diesel oil (Bento et al., 2005; Ghazali et al., 2004), crude oil (Das and Mukherjee, 2007), phenanthrene (Doddamani and Ninnekar, 2000), naphthalene (Tuleva et al., 2005) and benzene (Aburto-Medina and Ball, 2015; Dou et al., 2010) among other hydrocarbons (Cooper and Goldenberg, 1987; Menezes Bento et al., 2005, Morán et al., 2000).

Successful application of bioaugmentation is reliant on the subsequent survival and activity of the degrading strains once introduced into the target habitat. An alternative approach, which has also increased the chances of successful bioremediation by maintaining high rates of microbial adaption, persistence and activity, has been the use of plant biomass (Shahsavari et al., 2015, 2013b). Plant residues such as hay and straw are among the cheapest and most plentiful agricultural waste products in the world, with an estimated annual production of more than 2900 million tonnes (Sun et al., 2004) and they have been successful used in the degradation of petroleum hydrocarbons (Barathi and Vasudevan, 2003; Rojas-Avelizapa et al., 2007; Shahsavari et al., 2013a; Zhang et al., 2008).

Often in contaminated soils, nutrients, aside from C are depleted. Therefore in order to increase the efficiency of bioremediation, the addition of nutrients such as nitrates and phosphates to enhance the growth of hydrocarbonoclastic microbes is crucial (Mohan et al., 2008) and this is termed biostimulation (Molina-Barahona et al., 2004). In recent decades, biostimulation together with bioaugmentation, necrophytoremediation and phytoremediation technologies have become valuable alternatives to physical and chemical treatments. The advantages of these biological treatments include low cost, ease of implementation, environmentally friendliness, applicability over large areas, and often results in the complete mineralization of the contaminant (Guo et al., 2014).

The microbial communities in the soil are the main driver for the degradation of contaminants; therefore, assessment of the microbial communities during a bioremediation study is highly desirable. Various methods such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), ribosomal intergenic spacer analysis (RISA) or automated RISA (ARISA) have been developed and used for assessments of microbial communities in soils. Recently next generation sequencing (NGS) has become a popular technique since it provides the largest amount of data (up to one billion short reads per run) with a relatively low cost (Metzker, 2010; Schuster 2007). Therefore, this technique is ideal for the identification of the microbial community.

While several studies have assessed a combination of bioaugmentation and biostimulation worldwide (Calvo et al., 2009; Coulon et al., 2010; Grace Liu et al., 2011; Kauppi et al., 2011, Łebkowska et al., 2011; Sheppard et al., 2011; Zhao et al., 2011), studies in regard to bioremediation technology in Libyan soils are limited and there exists a lack of information (Mansur et al., 2014; Shaieb et al., 2015). In addition, our knowledge about the microbial communities in these types of soils remains low. High-throughput sequencing or NGS not only gives us valuable information about microbial communities in Libyan soils but also underpins any bioremediation process by providing essential information on the diversity and activity of the soil microbial community during the degradation of petroleum hydrocarbons.

Therefore, the aim of this study was to provide an assessment of the potential for cheap and readily available bioremediation technologies for the remediation of petroleum contaminated Libyan soil. Four different approaches including natural attenuation, necrophytoremediation (addition of pea straw), bioaugmentation (addition of a hydrocarbonoclastic bacterial consortium including several *Bacillus*) and a combination of necrophytoremediation and bioaugmentation were evaluated on the degradation of diesel contaminated soil. Moreover the microbial community was elucidated by metagenomic analysis.

2. Materials and methods

2.1. Soil sample collection

The petroleum hydrocarbon contaminated soil used in this study was collected from the top layer (0–15 cm; 20 kg) of a diesel contaminated site in Libya. The original soil contamination resulted from an oil pipeline leak from the main oil reservoirs in Tripoli, Libya. The initial level of contamination, in terms of Total Petroleum Hydrocarbon (TPH) was 18,966 mg kg^{-1} dry soil.

The soil was imported to RMIT University, Melbourne, Australia, coded and stored in a quarantine facility at the university. Prior to use, stones were manually removed from the soil, and the samples were passed through a sieve (4 mm). Plant residue (pea straw) was kindly donated by Johnson's Stockfeed and Horticultural Products Co. (Australia). The straw was chopped using a blender to small pieces (2 mm) prior to use.

2.2. Physico-chemical analysis

The soil was analysed for soil texture, moisture content, pH and water-holding capacity (WHC) using methods previously described (Rayment and Higginson, 1992) (Table 1). The percentage of organic carbon (C), nitrogen (N) and hydrogen (H) was analysed (Chemical Analysis Facility, Macquarie University) using a model LECO TruMac CNS analyser following the manufacturer's instructions. The concentration of different elements including Ca, K, Mg Fe, P, S and Zn in the soil samples were determined using, x-ray fluorescence spectrometry following the method previously described (Norrish and Hutton, 1969) (Table 1).

The concentrations of heavy metals in the tested soil were analysed using inductively coupled plasma mass spectrometry (ICPMS) (Varian Model Spectra AA 220) as per the manufacturer's protocol. Briefly, using a hot block, soil samples were digested with HNO_3 (5 ml, 65–70%) and hydrogen peroxide (5 ml, 30% v/v) at 60 °C for 1 h. Soil samples were further heated at 120 °C for 5 h. The tubes were then cooled at room temperature; the solutions were filtered through No. 1 filter paper.

2.3. Preparation of microbial consortia

A microbial community with previously assessed hydrocarbonoclastic activity was selected for use in this study. The hydrocarbonoclastic microorganisms used in this study were *Bacillus lentus* A5019 ST, *Bacillus megaterium* B 5013, *Bacillus pumilus* C

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