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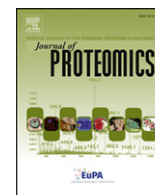

The ecological and physiological responses of the microbial community from a semiarid soil to hydrocarbon contamination and its bioremediation using compost amendment

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ABSTRACT

The linkage between phylogenetic and functional processes may provide profound insights into the effects of hydrocarbon contamination and biodegradation processes in high-diversity environments. Here, the impacts of petroleum contamination and the bioremediation potential of compost amendment, as enhancer of the microbial activity in semiarid soils, were evaluated in a model experiment. The analysis of phospholipid fatty-acids (PLFAs) and metaproteomics allowed the study of biomass, phylogenetic and physiological responses of the microbial community in polluted semiarid soils. Petroleum pollution induced an increase of proteobacterial proteins during the contamination, while the relative abundance of *Rhizobiales* lowered in comparison to the non-contaminated soil. Despite only 0.55% of the metaproteome of the compost-treated soil was involved in biodegradation processes, the addition of compost promoted the removal of polycyclic aromatic hydrocarbons (PAHs) and alkanes up to 88% after 50 days. However, natural biodegradation of hydrocarbons was not significant in soils without compost. Compost-assisted bioremediation was mainly driven by *Sphingomonadales* and uncultured bacteria that showed an increased abundance of catabolic enzymes such as catechol 2,3-dioxygenases, *cis*-dihydrodiol dehydrogenase and 2-hydroxymuconic semialdehyde. For the first time, metaproteomics revealed the functional and phylogenetic relationships of petroleum contamination in soil and the microbial key players involved in the compost-assisted bioremediation.

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1. Introduction

Crude oil and related products are the major source of global energy and the risk for accidental spills during exploration, refining, industrial processes, and transport is an important concern of production companies and environmental authorities [1]. Linear and branched alkanes, cycloalkanes, and other mono- and poly-aromatic compounds comprise major fractions of hydrocarbon-containing products and have unintentionally been released to the environment causing the contamination of soil ecosystems and water bodies [2] with subsequent risks for humans and the environment. For instance, polycyclic aromatic hydrocarbons (PAHs) have potential toxic, mutagenic, and carcinogenic effects and therefore successful removal of these compounds from the environment is a goal of spill remediation and cleanup.

The scarce nutrient content and organic matter, as a consequence of the dry climate conditions in semiarid areas, makes these soils particularly problematic for microbial development [3,4]. For this reason, the impact of hydrocarbon contamination may cause severe damage in semiarid environments. Under these circumstances, the addition of exogenous sources of nutrients (i.e. compost, sludges) has been applied to improve soil quality [5,6] and to foster the activity and growth of microbial communities which are able to degrade hydrocarbons. These communities are often nutrient limited [7] and the addition of organic amendments palliates these deficiencies and support biodegradation of hydrocarbons [1,7,8].

However, due to the tremendous level of microbial diversity and the complex biotic and abiotic processes governing microbial activities in soil, the microbial responses to hydrocarbon contamination and

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stimulated biodegradation by organic amendments are still obscure in soil habitats. Multidisciplinary and novel approaches are needed to decipher these complex relationships. To date, the impact of hydrocarbon contamination on soil microbiota has been evaluated by the use of soil quality indicators such as enzyme activity [9], phospholipid-fatty acid analyses [10] and genomic approaches [8]. However, these approaches cannot easily link phylogenetic and functionalities in the microbial community. Nowadays, a definitive linkage between functional and taxonomic relationships of the microbial populations inhabiting hydrocarbon-polluted soils is feasible due to the recent advance in the field of environmental [11,12] and, particularly in soil [13,14] metaproteomics.

To the best of our knowledge, despite proteomic technologies have been applied to understand the physiology of cultures degrading hydrocarbons in the recent past [15–18], metaproteomics have not yet been applied to understanding the effects of hydrocarbon contamination and bioremediation strategies in a real soil containing a complex matrix including organic, inorganic and mineral constituents along with a high level of microbial diversity.

In this study, we aim to correlate the changes in biomass, composition and functionalities of the microbial community of a semiarid soil contaminated with crude-oil and the bio-stimulating capacity of compost amendment. Moreover, we aim to reveal the microbial key-players involved in the biodegradation of hydrocarbons. For these purposes, a model experiment with the integration of methodologies based on hydrocarbon quantification, microbial activity proxies, phospholipid-fatty acid analysis and metaproteomics was performed. We hypothesized that compost will increase biomass but also promote a change in the microbial composition that may be related to the expression of proteins involved in the biodegradation of various hydrocarbon compounds.

2. Material and methods

2.1. Experimental design

A semiarid soil was taken from a 100 m^2 abandoned agricultural area located in Santomera, in the Province of Murcia, South-east Spain. The soil is classified as Haplic calcisol [19] and represents a low-degraded soil [4]. Plant cover was around 15% and was dominated by xerophytic shrubs. The soil particle distribution was 18.8% clay, 9.5% silt, and 71.7% sand. Santomera soil has a total N content of 0.5 g kg^{-1} and total organic C was 5.3 g kg^{-1}. Within this area three plots ($n = 3$) of 20 m^2 each were selected. Six subsamples were taken from the upper 15 cm of each plot and pooled to obtain one composite sample of 5 kg per plot for further incubation assays. Soil samples were sieved to a size of <2 mm.

In order to study the effects of hydrocarbon pollution and compost bio-stimulation in controlled conditions, a microcosm study was established by incubating 200 g of soil in containers containing 2% w/w crude oil. Soils were incubated at 28 °C for 50 days and the water-holding capacity was gravimetrically adjusted to 60%. Two incubation times were independently established (an initial time of 2 days of exposure to hydrocarbons, and a short/medium term of 50 days) in order to study the impact of petroleum contamination in the soil microbial community. Containers with soil but without crude oil were prepared in the same conditions as the controls. In order to evaluate the capacity for hydrocarbon bioremediation, an additional treatment was prepared by the addition of compost to the petroleum-contaminated soil. The results were compared at 50 days of incubation with the corresponding unamended contaminated sample. The compost was produced from garden and crop prunings, the composting process lasting three months. The highest temperature reached (65 °C) and was maintained for a minimum of 48 h. The compost dose consisted of 5% (w/w) to raise the total organic C of the soil to 3-times of the autochthonous content. The contents of total organic C, total N and total P of compost were 184.8, 14.6 and 5.9 g kg^{-1}, respectively. Each treatment was prepared in identical replicates ($n = 3$) for each incubation time.

2.2. Nutrients, basal respiration and enzyme activities

Total organic carbon (TOC) and total nitrogen were determined using an elemental analyzer (C/N Flash EA 112 Series Leco Truspec). Water-soluble carbon (WSC) was determined via soil extraction (2 h shaking with a soil:distilled water ratio of 1:5), followed by centrifugation, filtration, and analysis of the extract solution on a C analyzer for liquid samples (Multi N/C 3100, AnalytikJena). Microbial respiration (CO_2 emission) was measured in 10 ml capped tubes containing 1 g of soil. Vials were hermetically closed and incubated in the dark at 28 °C for 11 days. The concentration of CO_2 was periodically analyzed with a gas chromatograph (Trace Ultra Thermo Scientific, Milan, Italy) using a packed column (Trace PLOT TG-BOND Q GC, Thermo Scientific, Milan, Italy). The urease, phosphatase and β-glucosidase enzyme activities were determined using the methods described by Kandeler and Gerber (1988) [20], Tabatabai and Bremmer (1969) [21], and Eivazi and Tabatabai (1987) [22], respectively.

2.3. Hydrocarbon degradation

Soil (5 g) was extracted with a mixture hexane-acetone (3:1, v/v) by sonication followed by centrifugation at 3500 rpm for 10 min. The extract from 3 consecutive extractions were combined and dried. Triphenylamine was used as an internal standard. Samples were processed by GC–MS in full scan mode. Calibration curves were linear from 2–7 to 240 ng ($R^2 > 0.99$).

2.4. Phospholipid fatty-acid analysis

Phospholipids were extracted from 6 g of fresh soil using the chloroform-methanol extraction as described by Bligh & Dyer[23], and fractionated and quantified using the procedure described by Frostegard et al.[24]. Phospholipids were transformed into fatty acid methyl esters (FAMEs) by alkaline methanolysis (Guckert et al.) [25], and designated as described by Frostegard et al.[24]. The complete dried FAME fraction was dissolved in isooctane containing 0.23 mg ml^{-1} of the 21:0 FAME as an internal standard. The analysis was performed using a Trace Ultra Thermo Scientific gas chromatograph fitted with a 60 m capillary column (Thermo TR-FAME 60 m × 0.25 mm ID × 0.25 μm film), using helium as carrier gas.

The following fatty acids were used as characteristic biomarkers for the bacterial community: i15:0, a15:0, 15:0, i16:0, i17:0, cy17:0, cy19:0, 16:1ω7c, 16:1ω7t, 18:1ω9c and 18:1ω9t [24,26]. The 18:2ω6 fatty acid was used as an indicator for fungal biomass [27,28]. The i15:0, a15:0, i16:0, and i17:0 were used as Gram-positive markers; the Gram-negative fatty acids used were cy17:0, cy19:0, 16:1ω7c, 16:1ω7t, 18:1ω9c and 18:1ω9t [24,26]. The biomarkers used for the actinobacteria were 10Me16:0 and 10Me18:0.

2.5. Protein extraction, separation and proteolytic cleavage

Protein extraction was done in replicated biological samples ($n = 2$), as described by Chourey et al. [13] and Bastida et al. [14,29]. Briefly, soil aggregates were disrupted by vortexing for 2 min in 10 ml of alkaline SDS-buffer (with 50 mM dithiothreitol) added to 5 g of soil. Cell lysis was achieved by placing samples in boiling water bath for 10 min. Protein precipitation were performed using trichloroacetic acid (TCA) followed by three acetone washing steps.

Prior polyacrylamide gel electrophoresis (SDS-PAGE) [30], the protein pellets were dissolved in 10 ml of deionized water using sonication (sonicating water bath, 5 min). The solubilized proteins were mixed with sample buffer [31], incubated for 5 min at 90 °C, and loaded on SDS gels (4% stacking gel, 12% separating gel). After electrophoresis, the gels were stained with colloidal Coomassie brilliant blue. The gel lanes were sliced into three gel pieces, destained and subsequently proteolytically cleaved using 100 ng of trypsin (Sigma, Munich, Germany), overnight at 37 °C.

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