Journal of Hazardous Materials 283 (2015) 35–43

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Pyrosequencing reveals the effect of mobilizing agents and lignocellulosic substrate amendment on microbial community composition in a real industrial PAH-polluted soil

S. Lladó[a,b], S. Covino[b], A.M. Solanas[a], M. Petruccioli[c], A. D'annibale[c], M. Viñas[d,*]

[a] Department of Microbiology, University of Barcelona, Diagonal 645, E-08028 Barcelona, Spain
[b] Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídenská 1083, 142 20 Prague 4, Czech Republic
[c] Department for Innovation in Biological, Agro-Food and Forest Systems [DIBAF], University of Tuscia, Via S. Camillo de Lellis snc, 01100 Viterbo, Italy
[d] GIRO Joint Research Unit IRTA-UPC, Institute of Research and Technology Food and Agriculture [IRTA], Torre Marimon, E-08140 Caldes de Montbui, Spain

HIGHLIGHTS

- Soil microbial community assessment through classical (MPN) and molecular tools (DGGE and pyrosequencing) is provided.
- A failure of exogenous white rot fungi to colonize the polluted soil is shown by DGGE and pyrosequencing.
- Surfactant Brij 30 hampers 4-ring PAHs degradation due to toxicity over Actinobacteria and Bacteroidetes populations.
- A high prevalence of *Fusarium* and *Scedosporium* populations is revealed during soil bioremediation.
- *Cupriavidus, Mycobacterium and Chithinophagaceae* are potential HMW–PAH degraders in the soil.

ARTICLE INFO

Article history:
Received 3 June 2014
Received in revised form 19 August 2014
Accepted 21 August 2014
Available online 16 September 2014

Keywords:
Soil bioremediation
White rot fungi (WRF)
Creosote
HMW–PAH
Non-ionic surfactant
Next generation sequencing (NGS)

ABSTRACT

Bacterial and fungal biodiversity throughout different biostimulation and bioaugmentation treatments applied to an industrial creosote-polluted soil were analyzed by means of polyphasic approach in order to gain insight into the microbial community structure and dynamics. Pyrosequencing data obtained from initial creosote polluted soil (after a biopiling step) revealed that Alpha and Gammaproteobacteria were the most abundant bacterial groups, whereas *Fusarium* and *Scedosporium* were the main fungal genera in the contaminated soil.

At the end of 60-days laboratory scale bioremediation assays, pyrosequencing and DGGE data showed that (i) major bacterial community shifts were caused by the type of mobilizing agent added to the soil and, to a lesser extent, by the addition of lignocellulosic substrate; and (ii) the presence of the non-ionic surfactant (Brij 30) hampered the proliferation of Actinobacteria (Mycobacteriaceae) and Bacteroidetes (Chitinophagaceae) and, in the absence of lignocellulosic substrate, also impeded polycyclic aromatic hydrocarbons (PAHs) degradation.

The results show the importance of implementing bioremediation experiments combined with microbiome assessment to gain insight on the effect of crucial parameters (e.g. use of additives) over the potential functions of complex microbial communities harbored in polluted soils, essential for bioremediation success.

1. Introduction

Microbial biodegradation is the main process occurring in natural decontamination processes and bioremediation treatments exploiting such microbial activities represent a valuable alternative for the clean-up of matrices contaminated by recalcitrant pollutants, such as polycyclic aromatic hydrocarbons (PAHs) [1]. Furthermore, bacteria, yeasts and filamentous fungi showing remarkable PAH-degrading capabilities are ubiquitous in the terrestrial environment. In spite of the importance of resident microbial communities for success of bioremediation treatments [2], little is known about the shifts occurring in bacterial and fungal populations during reclamation of real historically polluted soils [3–5]. Moreover, when allochthonous microorganisms such

* Corresponding author. Tel.: +34 934674040; fax: +34 934674042.
E-mail addresses: llado@biomed.cas.cz (S. Lladó), covino@biomed.cas.cz (S. Covino), asolanas@ub.edu (A.M. Solanas), petrucci@unitus.it (M. Petruccioli), dannib@unitus.it (A. D'annibale), marc.vinas@irta.cat (M. Viñas).

http://dx.doi.org/10.1016/j.jhazmat.2014.08.065
0304-3894/

as white rot fungi (WRF) are inoculated in the polluted matrix for mycoaugmentation treatments, an in-depth analysis of the cooperative or antagonistic relationships existing between exogenously added fungi and the native microflora is essential [6].

In our previous study [7], a creosote-polluted soil, previously biotreated by dynamic biopiling and characterized by the presence of a highly recalcitrant PAH fraction, was further treated by biostimulation of the indigenous soil microbiota and mycoaugmentation with two WRF. The impact of single or combined soil amendments on both PAH depletion and resident microbial community evolution in the proposed bioremediation approaches were comparatively evaluated [7]. The low WRF colonization rates as well as the effects of soil supplementation with (i) mobilizing agents (MAs) and (ii) a lignocellulosic substrate (LS) on the autochthonous microbial communities were also discussed [7]. On an overall basis, biostimulating the soil by adding sterile LS promoted a significantly higher total petroleum hydrocarbon (TPH) and high molecular weight-PAHs (HMW–PAHs) removal than that achieved by mycoaugmentation with the two WRF. This prompted us to investigate the overall microbial processes occurring in soil during different bioremediation approaches, in order to improve our knowledge about native microbial communities and their role in polluted sites. As far as we know, the present work report for the first time the use of pyrosequencing to study both fungal and eubacterial native populations throughout different bioremediation strategies in a real PAH-polluted soil.

2. Materials and methods

2.1. Soil, materials and microorganisms

A composite sample of an aged creosote-contaminated soil was collected after four months of biopiling at pilot-scale [8] and henceforth referred to as initial soil. Its main properties were as follows [7]: TPH content, 2815 mg kg^{-1}; pH 7.5; water-holding capacity, 33.7%; cultivable heterotrophic bacteria (CHB) and hydrocarbon-degrading bacteria (CHDB) were 1.3×10^7 and 2.3×10^6 MPN g^{-1}, respectively with a CHDB vs CHB ratio of 21%.

Brij 30 (dodecyl tetraethylene glycol ether), soybean oil and manganese (II) sulfate ($MnSO_4$) were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Two different WRF strains, *Trametes versicolor* ATCC 42530 and *Lentinus tigrinus* CBS 577.79, were cultivated to produce inocula as described previously [7].

2.2. Microcosm set-up

Sterile soybean oil (SO) and Brij 30 were used at a final concentration of 4.5% (w/w) for soil pre-treatment as reported elsewhere [9].

The lignocellulosic substrate (LS) composed of a wheat straw/wheat bran mixture (80:20, w/w) was added to 16 cm × 3.5 cm test tubes and sterilized (121 °C for 45 min) [10]. Hence, LS moisture was adjusted to 70% (w/w) with sterile deionized water, while $MnSO_4$ (20 mg kg^{-1}) was added as a manganese source when the treatment required it. Pre-colonized wheat (*Triticum aestivum*) seeds were used as a fungal inoculum for the LS at 5% (w/w) in those treatments where *T. versicolor* and *L. tigrinus* were assayed. Both inoculated and non-inoculated test tubes, the latter being referred to as biostimulation experiments (BS), were incubated for 7 d at 28 °C. Regardless of the treatment, the LS mass amounted to 10% of the total dry weight for each microcosm.

Subsequently, a layer of either mobilizing agent (MA) supplemented or bulk soil (25 g), the moisture content of which had previously been adjusted to 60% of its water-holding capacity (w/w), was added to the test tubes. The soil had been previously

Table 1
Treatment description, molecular analyses carried out and summary of chemical and microbiological results [7].

Treatment[a]	DGGE 16S MPN[b]	Pyrosequencing 16S/ITS[b]	TPH[c]	4-Ring PAHs	5-Ring PAHs	CHDB[d]	qPCR[e] 16S	qPCR ITS
Initial soil	+	+	2815 ± 233	272 ± 10	117 ± 4	6.37 ± 0.07 (21%)	6.45 ± 0.25	6.56
IC	+	+	1439 ± 51	96 ± 10.2	80 ± 3	6.21 ± 0.01 (6.9%)	7.34 ± 0.23	6.92
IC + SO	+	+	1395 ± 30	157 ± 8.1	71 ± 2	6.32 ± 0.02 (0.9%)	6.83	6.78
IC + Br30	+	+	1515 ± 179	214 ± 8.4	77 ± 3	3.07 ± 0.06 (0.004%)	6.80 ± 0.1	6.74 ± 0.12
BS-LS	+	–	1077 ± 242	76 ± 3.3	62 ± 5	6.76 ± 0.16 (2.2%)	9.12 ± 0.1	8.79 ± 0.27
BS-LS + SO	+	–	1098 ± 207	77 ± 12	55 ± 6	6.07 ± 0.05 (0.2%)	9.46 ± 0.07	9.04 ± 0.05
BS-LS + Br30	+	–	1255 ± 68	115 ± 12	62 ± 3	5.36 ± 0.07 (0.08%)	9.19 ± 0.14	8.67 ± 0.5
BS-LS + Mn^{2+}	+	–	1106 ± 26	63 ± 3.9	54 ± 1	6.74 ± 0.26 (2.6%)	9.31 ± 0.1	8.71 ± 0.25
BS-LS + SO + Mn^{2+}	+	+	810 ± 27	56 ± 7.8	37 ± 2	6.03 ± 0.01 (0.15%)	9.32 ± 0.34	9.24
BS-LS + Br30 + Mn^{2+}	+	+	766 ± 27	58 ± 3.3	33 ± 2	5.20 ± 0.22 (0.05%)	8.86 ± 0.22	8.51 ± 0.57
TV-LS	–	–	1545 ± 153	77 ± 5.6	78 ± 6	6.11 ± 0.02 (0.62%)	10.2 ± 0.21	8.64 ± 0.19
TV-LS + SO	–	–	1338 ± 204	81 ± 9	68 ± 0.1	6.98 ± 0.18 (3.8%)	9.70 ± 0.1	9.62 ± 0.64
TV – LS + Br30	–	–	1552 ± 29	127 ± 10.7	70 ± 3	5.65 ± 0.09 (0.14%)	8.92 ± 0.52	9.41 ± 0.21
TV-LS + Mn^{2+}	–	–	1417 ± 155	78 ± 12	70 ± 8	6.06 ± 0.21 (0.24%)	9.44 ± 0.35	8.88 ± 0.34
TV-LS + SO + Mn^{2+}	+	+	1449 ± 65	94 ± 11	62 ± 3	7.71 ± 0.06 (16.8%)	9.44 ± 0.38	9.23 ± 0.59
TV-LS + Br30 + Mn^{2+}	–	–	1436 ± 60	114 ± 10	67 ± 3	6.07 ± 0.05 (0.65%)	9.27 ± 0.29	9.00 ± 0.35
LT-LS	–	–	1396 ± 15	79 ± 6.5	70 ± 2	6.19 ± 0.37 (1.05%)	9.38 ± 0.06	8.94 ± 0.08
LT-LS + SO	–	–	1467 ± 170	91 ± 14	64 ± 9	7.66 ± 0.17 (12%)	9.62 ± 0.19	9.51 ± 0.16
LT-LS + Br30	–	–	1578 ± 42	131 ± 14	70 ± 3	5.72 ± 0.20 (0.14%)	9.45 ± 0.06	8.92 ± 0.28
LT-LS + Mn^{2+}	–	–	1147 ± 53	61 ± 6.7	58 ± 2	5.60 ± 0.03 (0.16%)	9.38 ± 0.16	8.96 ± 0.06
LT-LS + SO + Mn^{2+}	+	+	1093 ± 71	72 ± 13	51 ± 2	7.62 ± 0.24 (27.6%)	9.64 ± 0.24	9.55 ± 0.35
LT-LS + Br30 + Mn^{2+}	–	–	1260 ± 2	112 ± 3.7	69 ± 0.2	6.11 ± 0.01 (0.19%)	9.21 ± 0.7	8.66 ± 0.72

[a] IC, incubation control; BS, biostimulation; TV, *Trametes versicolor*; LT, *Lentinus tigrinus*; LS, lignocellulosic substrate; SO, soybean oil; Br30, Brij 30; Mn^{2+}, manganese ions.
[b] Samples that underwent MPN–DGGE and/or pyrosequencing processing are displayed with a+ symbol.
[c] All concentrations are expressed as mg kg^{-1} of dry soil and data are the means of three independent experiments.
[d] Cultivable PAHs-degrading specialized bacteria (CHDB), expressed as Log MPN g^{-1} soil and CHDB/CHB percent ratios; data are the means of three independent experiments.
[e] *16SrRNA* and ITS region gene copies quantified by qPCR, expressed as Log gene copies g^{-1}; data are the means of three independent experiments.