



## Comparative assessment of bioremediation approaches to highly recalcitrant PAH degradation in a real industrial polluted soil

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### HIGHLIGHTS

- ▶ A diversified approach throughout bioremediation assays on creosote polluted soil is provided.
- ▶ Best hydrocarbon depletion rates are achieved when lignocellulosic substrate is amended.
- ▶ Antagonistic effects exerted by native soil microbiota toward augmented white-rot fungi are reported.
- ▶ Nonionic surfactant Brij 30 hampers 4-ring PAHs degradation due to toxicity over PAH-degraders.
- ▶ Colonization tests on active autochthonous soil microbiota are crucial for fungal bioaugmentation.

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### ABSTRACT

High recalcitrant characteristics and low bioavailability rates due to aging processes can hinder high molecular weight polycyclic aromatic hydrocarbons (HMW-PAHs) bioremediation in real industrial polluted soils. With the aim of reducing the residual fraction of total petroleum hydrocarbons (TPH) and (HMW-PAHs) in creosote-contaminated soil remaining after a 180-d treatment in a pilot-scale biopile, either biostimulation (BS) of indigenous microbial populations with a lignocellulosic substrate (LS) or fungal bioaugmentation with two strains of white-rot fungi (WRF) (*i.e.*, *Trametes versicolor* and *Lentinus tigrinus*) were comparatively tested. The impact of bivalent manganese ions and two mobilizing agents (MAs) (*i.e.*, Soybean Oil and Brij 30) on the degradation performances of biostimulated and bioaugmented microcosms was also compared. The results reveal soil colonization by both WRF strains was clearly hampered by an active native soil microbiota. In fact, a proper enhancement of native microbiota by means of LS amendment promoted the highest biodegradation of HMW-PAHs, even of those with five aromatic rings after 60 days of treatment, but HMW-PAH-degrading bacteria were specifically inhibited when non-ionic surfactant Brij 30 was amended. Effects of bioaugmentation and other additives such as non-ionic surfactants on the degrading capability of autochthonous soil microbiota should be evaluated in polluted soils before scaling up the remediation process at field scale.

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### 1. Introduction

Creosote is a technical wood preservative preparation mainly composed of a mixture of PAHs [approx. 85%] [1] and it has been extensively used worldwide by the wood industry, thus resulting in the pollution of areas proximal to sites of creosote manufacture and use [2].

Bioremediation offers an environmentally friendly and cost-competitive approach for the clean-up of contaminated sites, although natural biodegradation may be slower than other technologies [3]. Unfortunately, on aged historically polluted soils, bioremediation may be strongly constrained by many limiting factors; among them, bioavailability of pollutants and degradation competence of the indigenous microbiota are of primary importance [4]. With regard to the former, a rapid biodegradation of contaminants has been frequently observed in artificially spiked and recently polluted soils; this highlights the need of using actual industrial soils in order to transfer lab-scale experiments to the field-scale.

In a previous work, the application of a dynamic biopile as a remediation strategy for a creosote-contaminated soil led to an

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almost quantitative depletion of 3 ringed PAH fraction [98%] while some HMW-PAHs [4- and 5- ringed ones] were either partially or not degraded at all [5]. These findings have been frequently reported in biostimulation approaches to PAH-contaminated soils and mainly ascribed to both low contaminant bioavailability and lack of catabolic abilities in the indigenous soil microbiota [6–8].

Bioaugmentation with white-rot fungi [WRF] might be a useful approach to overcome the aforementioned limitations associated with the use of biostimulation to PAH-contaminated soils. These organisms, in fact, are efficient degraders of a wide range of organopollutants sharing structural similarities to lignin due to the high non-specificity of their extracellular lignin-modifying enzymes [LMEs] [9]. The most characterized LMEs, namely manganese-peroxidase [MnP], lignin peroxidase [LiP] and laccase have been shown to oxidize PAHs under *in vitro* conditions [10,11]. Since these enzymes operate in the extracellular environment via the production of radical species and highly diffusible oxidants, such as manganic chelates, the degradation efficiency of WRF does not depend on contaminant uptake, which is the rate-limiting step for bacteria [12]. The use of WRF in soil, however, requires the concomitant addition of lignocellulosic materials in order to improve their competition ability with the resident microbiota and, in this respect, inocula formulation has been shown to be important [13,14].

One of the major determinants for the high PAH recalcitrance to biodegradation in soil derives from their high hydrophobicity and tendency to tightly adhere to organic soil colloids thus resulting in low bioavailability. In this specific respect, the use of mobilizing agents [MAs] to enhance mass transfer rate and bioavailability of PAHs has received considerable attention. Among them, nonionic polyoxyethylene-based MAs were found beneficial for the bioremediation of PAHs due to their low toxicity [15]. In addition, plant oils, such as soybean oil, were found to be excellent alternatives to MAs, since, in addition to exhibiting molar solubility ratios toward PAHs which were similar to those of conventional surfactants, they were cost-effective [16].

The main objective of this study was to comparatively evaluate the impact of single or combined supplements on both a biostimulation and a bioaugmentation approach to a PAH-contaminated soil in terms of biodegradation outcomes and evolution of the resident microbiota. The supplements under study included two MAs [i.e., Brij 30 and soybean oil], wheat straw and bivalent manganese ions either alone or in combination. Regardless of the treatment typology, treatments were investigated under unsaturated solid-phase conditions due to their resemblance with those adopted at the field-scale. The selected matrix derived from a 180-d treatment in a pilot-scale biopile and, thus, was characterized by the presence of a highly recalcitrant PAH fraction. To perform the bioaugmentation approach, two allochthonous WRF strains, namely *Trametes versicolor* ATCC 42530 and *Lentinus tigrinus* CBS 577.79, were selected due to their previously reported PAH-degrading efficiencies and competition abilities with soil microbiota [17,18].

## 2. Materials and methods

### 2.1. Soil and materials

The aged creosote-contaminated soil was collected from a pilot-scale biopile where it had undergone a remediation treatment for 180 d, in a wood preservative industrial facility near Barcelona [Spain], as described elsewhere [5]. Its main properties were as follows: total petroleum hydrocarbons [TPH] content, 2815 mg kg<sup>-1</sup>; real acidity [in water], pH 7.5; water holding capacity, 33.7%; total organic carbon, 3.5%; NO<sub>3</sub><sup>-</sup>, 15 mg kg<sup>-1</sup>; NH<sub>4</sub><sup>+</sup>, 1.6 mg kg<sup>-1</sup>; texture: sand [32%]; silt [28%] and clay [40%].

Ergosterol, 7-dehydrocholesterol, o-terphenyl and methyl 1-[butylcarbamoil]-2-benzimidazolecarbamate and Brij 30 [dodecyl tetraethylene glycol ether] were purchased from Sigma-Aldrich, Spain. Soybean oil and manganese [II] sulfate [MnSO<sub>4</sub>] were purchased from Sigma-Aldrich, Italy. Solvents were purchased from Scharlab S.L. [Barcelona, Spain]. Solvents and other chemicals and reagents were of the highest purity available. PAH standards for quantification in GC-FID were obtained from Dr. Ehrenstorfer GmbH [Augsburg, Germany].

### 2.2. Microorganisms

*T. versicolor* ATCC 42530 and *L. tigrinus* CBS 577.79, obtained from the American Type Culture Collection [Manassas, USA] and the Centraalbureau voor Schimmelcultures [Utrecht, NL], respectively, were maintained and periodically sub-cultured on potato dextrose agar [Difco, Detroit, MI] slants. Fungal mycelial suspensions were prepared as previously described [19].

### 2.3. Soil pre-treatment and inocula preparation

Previously sterilized [121 °C for 15 min] MAs [either SO or Brij 30] were added to the soil by spraying and subsequent mixing to reach a final concentration of 4.5% [w/w]. The soil thus pre-treated was kept for 6 d at 4 °C prior to its use [13].

On the other hand, lignocellulosic substrate [LS], made of a wheat straw/wheat bran mixture [80: 20, w/w], and destined to the preparation of the fungal inoculum was transferred to 16 × 3.5 cm test-tubes [17]. Then, its moisture content was adjusted to 70% [w/w] with sterile deionized water prior to sterilization. When required, MnSO<sub>4</sub> was added, as a source of bivalent manganese ions, at a rate of [20 mg kg<sup>-1</sup>]. Fungal cultures were grown for 7 d at 28 °C under stationary conditions and non-inoculated LS, to be used in biostimulation experiments, was incubated in parallel under the same conditions. Regardless of the inoculation, the LS mass amounted to 10% of the total dry weight for each microcosm.

### 2.4. Fungal treatment of contaminated soil

A layer of either MA-supplemented or bulk soil [25 g], the moisture content of which had been previously adjusted to 60% of its water holding capacity [w/w], was added to the test-tubes containing either inoculated or non-inoculated LS. The soil was previously amended by mixing with an additional 10% [w/w] of non-inoculated sterilized LS. All microcosms were incubated at 28 °C for 60 d in the dark and their moisture contents kept constant by periodic additions of sterile deionized water. All experiments were carried out in three parallel replicates under non-axenic conditions. Non-amended controls were prepared, incubated as above and from here onwards referred to as incubation controls. Subsequent analyses were carried out on the upper soil layer and the results were normalized by taking into account the dilution effect of the LS mixed with the polluted soil.

### 2.5. Extraction and analysis of organic contaminants

The soil samples were dried for 16 h at room temperature. Total organic extracts [TOE] were extracted and both total petroleum hydrocarbon [TPH] and PAHs were quantified as described elsewhere [7].

### 2.6. Biochemical determinations

The content of ergosterol, a specific indicator of fungal growth, was determined in soil according to [20]. Prior to the extraction, 7-dihidrocholesterol, dissolved in dichloromethane, was added to

each sample as a surrogate internal standard. Finally, samples were analyzed as described elsewhere [21].

Extracellular ligninolytic enzymes were extracted from soil samples and subsequently assayed as previously reported [19].

### 2.7. Heterotrophic and hydrocarbon-degrading microbial populations counts

Soil bacterial counts were performed using a miniaturized most probable number [MPN] method in 96-well microtiter plates, with eight replicate wells per dilution [22]. Total heterotrophs were counted in tryptone soy broth and aromatic hydrocarbon-degraders were counted in mineral medium BMTM [23] containing a mixture of PAHs as the sole carbon and energy source [22]. Methyl 1-[butylcarbamoyl]-2-benzimidazolecarbamate was added as a fungicide at 20 mg kg<sup>-1</sup> when TSB and BMTM were prepared in order to avoid fungal growth in the MPN plates.

### 2.8. Quantitative PCR assay

Gene copy numbers of eubacterial 16S rRNA and fungal ITS1 rRNA fragments were quantified by quantitative real time PCR [qPCR]. Each sample was analyzed in triplicate by means of three independent DNA extracts as described elsewhere [24]. All results were processed by means of the Agilent MxPro™ QPCR software.

### 2.9. Statistical analysis

Data were subjected to analysis of variance [ANOVA] followed by multiple pair-wise comparisons by the Fisher least significant difference [LSD] test. Data were also mean centered and unit variance-scaled [soft scaling] and then subjected to principal components analysis [PCA] by the use of the Statistica 8.0 software [StatSoft Tulsa, OK, USA].

## 3. Results

### 3.1. Impact of treatment on bacterial and fungal biota

The most evident effect on cultivable heterotrophic bacteria [CHB] was exerted by the presence of LS, which led to an invariably higher increase in their concentrations in biostimulated microcosms. Although high CHB densities were found in *T. versicolor*- and *L. tigrinus*-augmented microcosms amended with SO alone or combined with Mn<sup>2+</sup> ions, they did not significantly differ from their respective incubation control where a high stimulatory effect of SO on CHB had been observed [Fig. 1A]. This positive impact of LS and SO supplementation on CHB was confirmed by qPCR analyses of the 16S rRNA gene [Fig. 1B]. It is noteworthy that bioaugmentation treatments did not exert a depressive effect either on 16S rRNA gene copy number or on CHB; conversely, the former parameter was found to be significantly higher in LS-amended microcosm colonized by *T. versicolor*.

A different behavior was observed with the cultivable hydrocarbon degrading bacteria [CHDB] from MPN assays. The high population density present in the initial contaminated soil [2.3 × 10<sup>6</sup> MPN g<sup>-1</sup> soil] was not significantly affected in incubation controls with the notable exception of those added with Brij 30 where a striking depressive effect was evident after 60 d incubation [1.2 × 10<sup>3</sup> MPN g<sup>-1</sup> soil] [Fig. 1C]. The negative effect of this surfactant on CHDB was also confirmed in all biostimulated and *T. versicolor*-augmented microcosms, regardless of the presence or the absence of Mn<sup>2+</sup> ions. Conversely, SO had a significantly stimulatory effect on CHDB, in both biostimulated and bioaugmented microcosms with respect to the respective incubation controls

**Table 1**

Lignin-modifying enzyme activities in soil.

Treatment	Laccase <sup>a</sup> (IU g <sup>-1</sup> )	Mn-Peroxidase <sup>a</sup> (IU g <sup>-1</sup> )
IC	0.12 ± 0.02	N.D. <sup>b</sup>
IC + SO	0.02	N.D.
IC + Br30	0.09 ± 0.00	N.D.
BS-LS	0.80 ± 0.23 aAB	0.11 ± 0.01 aAB
BS-LS + SO	0.60 ± 0.26 aAB	0.02 aA
BS-LS + Br30	0.22 ± 0.10 aA	0.03 aA
BS-LS + Mn <sup>2+</sup>	1.02 ± 0.20 aB*	0.10 ± 0.01 aAB
BS-LS + SO + Mn <sup>2+</sup>	0.40 ± 0.06 aAB	0.07 aAB
BS-LS + Br30 + Mn <sup>2+</sup>	0.57 ± 0.08 aAB	0.26 ± 0.08 aB
TV-LS	2.22 ± 0.50 bAB*	0.71 ± 0.13 bA
TV-LS + SO	5.08 ± 0.63 bB*	0.78 ± 0.03 cA
TV-LS + Br30	0.68 ± 0.13 aA	2.49 ± 1.36 bB*
TV-LS + Mn <sup>2+</sup>	2.07 ± 1.03 aAB*	2.27 ± 0.14 bB
TV-LS + SO + Mn <sup>2+</sup>	1.97 ± 0.67 bAB*	2.69 ± 1.31 aB*
TV-LS + Br30 + Mn <sup>2+</sup>	6.77 ± 1.80 bB*	0.34 ± 0.20 aA
LT-LS	0.93 ± 0.03 aAB	0.89 ± 0.51 bA
LT-LS + SO	0.40 ± 0.07 aA	0.12 bA
LT-LS + Br30	2.18 ± 1.12 aB	2.68 ± 0.42 bAB*
LT-LS + Mn <sup>2+</sup>	1.95 ± 0.51 aB*	7.02 ± 3.43 cB*
LT-LS + SO + Mn <sup>2+</sup>	0.40 ± 0.05 aAB	3.07 ± 1.48 aAB*
LT-LS + Br30 + Mn <sup>2+</sup>	1.42 ± 0.21 aAB*	1.18 ± 0.66 bA

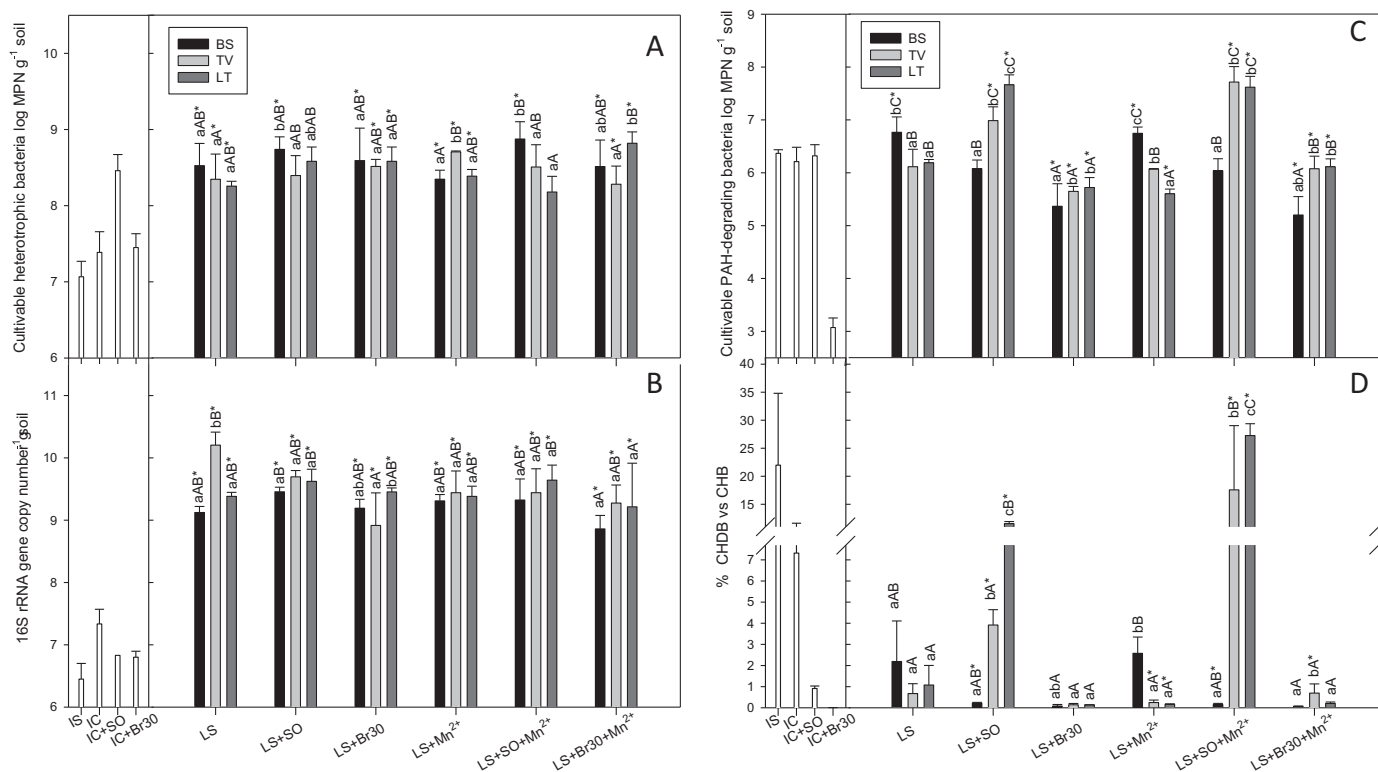
<sup>a</sup>Data are the means of three independent experiments. Same lowercase and uppercase letters indicate lack of statistically significant difference ( $P > 0.05$ ) between microcosms and within each biostimulation or bioaugmentation treatment in the presence of different supplements, respectively. Asterisk represents the occurrence of significant differences between each soil.

[Fig. 1C]. Interestingly, the addition of LS significantly attenuated the aforementioned toxicity effects of Brij 30.

The initial soil, arising from a 180 d biopiling treatment exhibited a rather high CHDB/CHB ratio amounting to 20% and it decreased significantly in the non-amended control after 60 d incubation [6.6%]; a dramatic decrease in this ratio was observed in incubation controls added with either SO or Brij 30 [0.73 and 0.01%, respectively] [Fig. 1D]. In the presence of the former MA, in LS-amended microcosms inoculated with either *T. versicolor* or *L. tigrinus*, the CHDB/CHB ratios were higher than the respective incubation control [3.8 and 12%, respectively, vs. 0.73%]; in the same bioaugmented microcosms, subjected to the concomitant SO and Mn<sup>2+</sup> ions additions, the same ratio reached high values which were very close to that of the initial soil [Fig. 1D].

Fig. 2 shows that fungal growth, inferred from two independent methods, namely ergosterol quantitation and quantitative PCR analysis of the ITS region, was mostly stimulated by the addition of LS as previously observed for bacteria. This can be desumed by comparing both parameters in incubation controls and respective microcosms, whether they be biostimulated or subjected to bioaugmentation with either *T. versicolor* or *L. tigrinus*. Conversely, no statistical significant differences were found between LS-amended microcosms, irrespective of the presence or the absence of the other additives, such as MAs and/or bivalent manganese ions. With this regard, it has to be highlighted that soil colonization by both *T. versicolor* and *L. tigrinus* mycelia was very fast in the early 15 days of incubation. Thereafter, exogenous fungi were strongly outcompeted by the resident fungal biota, the profuse growth of which was visually evident by the widespread presence of yellow/green spores in augmented microcosms. Thus, the lack of significant differences between bioaugmented and biostimulated microcosms might likely be due to a generalized boosting effect of LS on autochthonous fungi.

In all incubation controls, trace levels of extracellular laccase activity were found while Mn-peroxidase was not detected [Table 1]. A slight increase in the former and the occurrence of the latter was observed in biostimulated microcosms; however, due to the large data variability, both activities therein observed did not significantly differ from incubation controls. For the same



**Fig. 1.** Cultivable heterotrophic bacteria [CHB] [A], 16S rRNA gene copies [B], cultivable PAHs-degrading specialized bacteria [CHDB] [C] and CHDB/CHB percent ratios [D] in the soil bioremediation treatments after 60 d of incubation. Same lowercase and uppercase letters indicate lack of statistically significant difference ( $P > 0.05$ ) between microcosms and within each biostimulation or bioaugmentation treatment in the presence of different supplements, respectively. Asterisk represents the occurrence of significant differences ( $P < 0.05$ ) between each soil treatment and its respective incubation control. <sup>a</sup> IC: Incubation control; BS: Biostimulation; TV: *Trametes versicolor*; LT: *Lentinus tigrinus*; LS: Lignocellulosic substrate; SO: Soybean Oil; Br30: Brij 30;  $Mn^{2+}$ : Manganese ions.

reason, only few bioaugmented microcosms were found to significantly differ from the respective incubation controls; with regard to laccase, in particular, in the majority of bioaugmented and  $Mn^{2+}$ -supplemented microcosms, laccase activity was significantly higher than in incubation controls. Best laccase activity [ $6.77 \text{ IU g}^{-1} \text{ soil}$ ] was found in *T. versicolor* microcosms undergoing simultaneous supplementation with LS,  $Mn^{2+}$  ions and Brij 30; regardless of the type of supplements, laccase activity was generally higher in *T. versicolor* than in *L. tigrinus* microcosms [Table 1].

In both bioaugmented microcosms, MnP activity appeared to be boosted by the presence of either  $Mn^{2+}$  ions or Brij 30; however, when the two additives were combined the stimulatory effect was markedly lower for both fungi.

### 3.2. Degradation of contaminants in soil

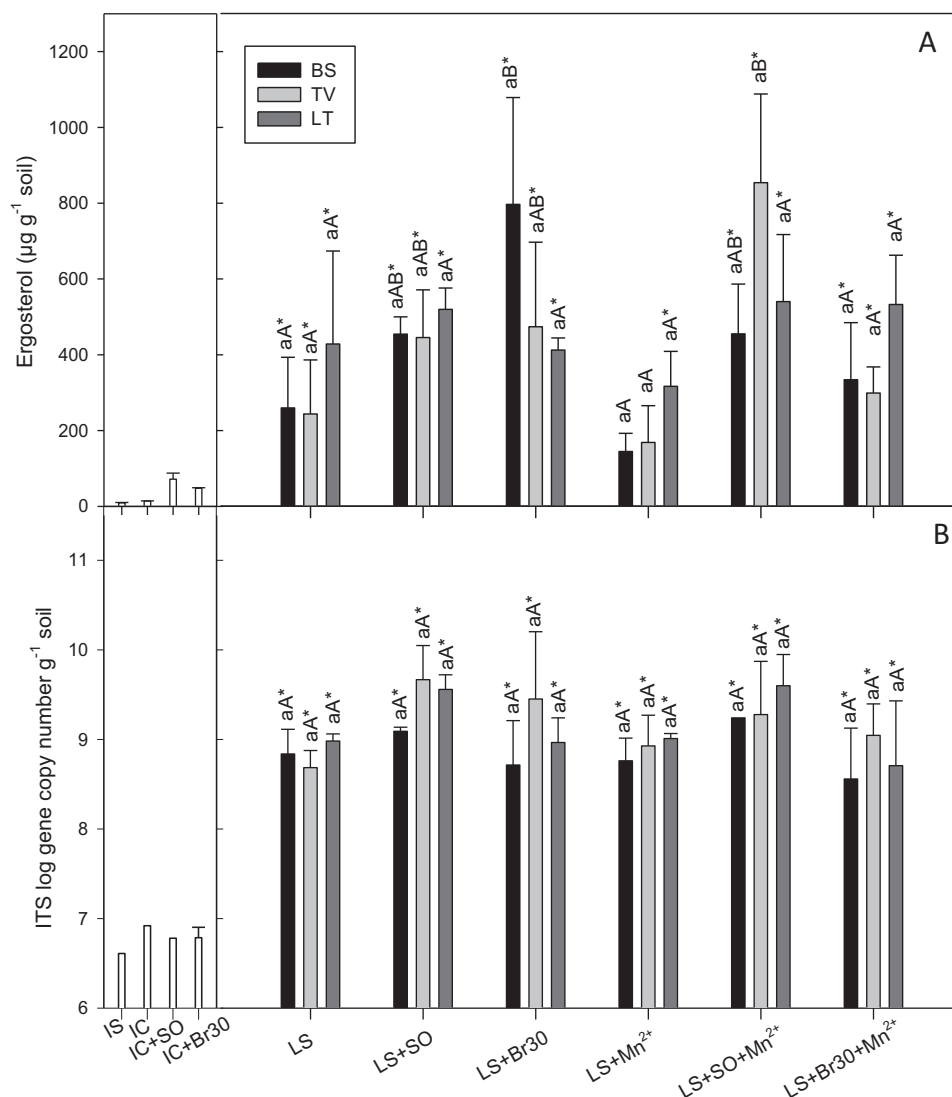
In the soil under study, derived from a previous 180-d-long biopiling treatment, the residual total petroleum hydrocarbon [TPH] and overall PAH contents amounted to  $2815$  and  $389 \text{ mg kg}^{-1}$ , respectively [Table 2]. Best TPH removals, ranging from 71% to 73%, were achieved in biostimulated microcosms supplemented with MAs and  $Mn^{2+}$  ions. In any case, regardless of the supplementation, the residual TPH contents in biostimulated microcosms were significantly lower than those found in respective incubation controls [Table 2].

Conversely, bioaugmented microcosms failed to significantly lead to a lower TPH contents than respective incubation controls with the only exceptions of  $Mn^{2+}$ -supplemented *L. tigrinus* microcosms; among them, the most effective was the microcosm concomitantly supplemented with SO and  $Mn^{2+}$  ions leading to a 61.2% TPH removal.

With regard to the PAH fraction, the soil at start mainly contained 4- and, to a lesser extent, 5-ring compounds. Among the former, the most abundant were fluoranthene [FLT] and pyrene [PYR] [ $83$  and  $84 \text{ mg kg}^{-1} \text{ soil}$ , respectively] while the most representative 5-ring compounds included benzo[b]-fluoranthene [BbF] and benzo[k]fluoranthene [BkF] [ $57$  and  $38 \text{ mg kg}^{-1} \text{ soil}$ , respectively]. It is noteworthy that, unlike that observed for TPH, all treatment typologies were able to yield significantly lower residual PAH contents than those in incubation controls.

The most susceptible PAH compounds to biodegradation were FLT and PYR, the removal extents of which ranged from 63 to 87% and 59 to 83%, respectively, as a function of the treatment typology. Brij 30 appeared to exert a negative impact on biodegradation of both FLT and PYR and this effect was also observed with other compounds such as benzo[a]anthracene [BaA] and chrysene [CHR]. In *L. tigrinus* microcosms, the supplementation of  $Mn^{2+}$  ions alone exerted a positive effect on the degradation of FLT, PYR, BaA and CHR, the residual contents of which were significantly lower than with other supplements [Table 2]. This stimulatory effect of  $Mn^{2+}$  ions, however, was not observed with 5-ring compounds in *L. tigrinus* microcosms; best removal efficiencies toward these compounds, namely benzo[a]pyrene, BbF and BkF were, instead, observed in biostimulated microcosms that underwent concomitant supplementation with  $Mn^{2+}$  and MAs [Table 2].

In order to obtain further insights into similarity of both treatments and variables and respective interplay, a multivariate approach, based on principal component analysis was used. To this aim, those variables [i.e., ergosterol, ITS, CHB and 16S rRNA gene copy number] which had not been shown to be significantly affected by both treatment typology and type of amendant were not recruited within the model.



**Fig. 2.** Ergosterol concentration [A] and ITS region gene copy number quantified by qPCR [B] in the soil bioremediation treatments after 60 d of incubation. Same lowercase and uppercase letters indicate lack of statistically significant difference ( $P > 0.05$ ) between microcosms and within each biostimulation or bioaugmentation treatment in the presence of different supplements, respectively. Asterisk represents the occurrence of significant differences ( $P < 0.05$ ) between each soil treatment and its respective incubation control.

Around 72% of total variance was explained by the first two components [51.9 and 19.6%, respectively] and no moderate or strong outliers were detected by SPE analysis and Hotelling of scores, respectively [Fig. 3A]. The similarity of behavior in response to treatments of the two variable couples BkF and BbF and FLT and PYR was confirmed by the nearly superimposed loadings while the remaining 5-ring compound BaP was clearly separated by both components [Fig. 3B]. Incubation controls and biostimulated microcosms concomitantly added with MAs and  $Mn^{2+}$  ions were separated from the remaining treatments by the first component [Fig. 3A] and the most influential variables were BaA, CHR, TPH, PYR, BkF and FLT residual contents as ranked by the respective vector lengths [Fig. 3B]. The least influential variables on the PCA model were laccase and MnP activities and the CHDB/CHB ratio. Interestingly, variables CHR, PYR and FLT residual contents were located in the right lower quadrant corresponding to those treatments, namely IC+SO and IC+Br, which had been shown to be the least effective in the degradation of those compounds; the same MA-amended incubation controls that had been shown to yield the lowest ratios between cultivable hydrocarbon-degrading and total heterotrophic bacteria [CHDB/CHB] located in

the diagonally opposed quadrant to those variables [Fig. 3]. The  $Mn^{2+}$ -supplemented *L. tigrinus* microcosms in the presence and in the absence of SO that had been shown to lead to high MnP activities and CHDB/CHB ratios were located in the same quadrant of those variables. Finally, the high degradation abilities toward TPH, BaP and BkF of the biostimulated microcosms, concomitantly added with  $Mn^{2+}$  and MAs, were confirmed by the diagonally opposite position of their relative loadings and scores.

#### 4. Discussion

Bioremediation of aged industrial contaminated soils is still a technical challenge. Two of the most important factors that conspire against biostimulation success in real historically PAH-polluted sites are the low bioavailability of pollutants due to high soil-PAH contact time, a phenomenon referred to as aging [26], and the lack of degradation efficiency in the indigenous microbial community [27].

The creosote-contaminated soil selected in this study had previously undergone a 180-d-long biopiling treatment, which albeit



**Table 2**  
TPH and 4- and 5-ring PAHs concentrations in soil.

Treatment	TPH <sup>a</sup> (mg kg <sup>-1</sup> )	Fluor (mg kg <sup>-1</sup> )	Pyr (mg kg <sup>-1</sup> )	B(a)A (mg kg <sup>-1</sup> )	Chry (mg kg <sup>-1</sup> )	B(b)F (mg kg <sup>-1</sup> )	B(k)F (mg kg <sup>-1</sup> )	B(a)P (mg kg <sup>-1</sup> )
Initial soil	2815 ± 233	83 ± 4	84 ± 4	37 ± 1	68 ± 1	57 ± 2	38 ± 2	22 ± 1
IC	1439 ± 51	30 ± 0.4	35 ± 3	21 ± 0.8	40 ± 6	39 ± 1	24 ± 1	17 ± 1
IC + SO	1395 ± 30	43 ± 2	48 ± 3	19 ± 0.1	47 ± 3	38 ± 1	24 ± 0.3	9 ± 0.3
IC + Br30	1515 ± 179	61 ± 3	70 ± 2	28 ± 0.4	55 ± 3	38 ± 2	25 ± 0.7	14 ± 0.3
BS-LS	1077 ± 242 aB*	17 ± 1 aA*	18 ± 0.7 aA*	15 ± 0.6 aB*	26 ± 1 aAB*	29 ± 3 aB*	18 ± 1 aB*	15 ± 0.7 aC
BS-LS + SO	1098 ± 207 aB*	16 ± 2 aA*	18 ± 2 aA*	14 ± 3 aB*	29 ± 5 aB*	26 ± 2 aAB*	16 ± 2 aAB*	12 ± 1 aB
BS-LS + Br30	1255 ± 68 aB*	28 ± 4 aB*	34 ± 4 aB*	18 ± 2 aC*	35 ± 2 aC*	28 ± 1 aAB*	19 ± 1 aAB*	15 ± 1 aC
BS-LS + Mn <sup>2+</sup>	1106 ± 26 aB*	13 ± 1 aB*	15 ± 2 aB*	12 ± 0.5 aB*	23 ± 0.4 aAB*	25 ± 0.5 aAB*	16 ± 0.4 aAB*	13 ± 0.5 aB*
BS-LS + SO + Mn <sup>2+</sup>	810 ± 27 aA*	13 ± 3 aA*	14 ± 2 aA*	10 ± 0.8 aA*	20 ± 2 aAB*	18 ± 1 aA*	11 ± 0.7 aA*	8 ± 0.2 aA
BS-LS + Br30 + Mn <sup>2+</sup>	766 ± 27 aA*	13 ± 0.2 aA*	16 ± 0.4 aA*	10 ± 0.7 aA*	19 ± 2 aA*	15 ± 1 aA*	10 ± 0.9 aA*	8.5 ± 0.4 aA*
TV-LS	1545 ± 153 bB	14 ± 2 aA*	16 ± 2 aA*	17 ± 0.6 aB*	30 ± 1 bA*	35 ± 2 bB*	23 ± 1 bC*	19 ± 2 aA
TV-LS + SO	1338 ± 204 abA	14 ± 2 aA*	17 ± 3 aA*	17 ± 2 aAB*	33 ± 2 aAB*	32 ± 1 aAB*	21 ± 1 aB*	15 ± 0.4 bA
TV-LS + Br30	1552 ± 29 bB	31 ± 4 aB*	36 ± 5 aC*	21 ± 0.9 aB*	39 ± 0.8 abB*	32 ± 2 aB*	21 ± 0.2 abB*	17 ± 0.1 bA
TV-LS + Mn <sup>2+</sup>	1417 ± 155 bAB	16 ± 3 bA*	17 ± 3 bA*	16 ± 2 bA*	29 ± 4 aA*	32 ± 4 aB*	21 ± 2 bB*	17 ± 2 bA
TV-LS + SO + Mn <sup>2+</sup>	1449 ± 65 cAB	23 ± 4 bAB*	24 ± 4 bB*	16 ± 1 bA*	31 ± 2 cA*	27 ± 2 bA*	18 ± 0.5 bA*	17 ± 4 bA
TV-LS + Br30 + Mn <sup>2+</sup>	1436 ± 60 cAB	28 ± 3 bAB*	30 ± 3 bB*	19 ± 2 bB*	37 ± 2 aB*	31 ± 0.8 bAB*	20 ± 1 bB*	16 ± 1 bA
LT-LS	1396 ± 15 abAB	15 ± 3 aAB*	17 ± 2 aAB*	16 ± 0.5 abB*	31 ± 1 bAB*	32 ± 1 abB*	21 ± 1 bAB*	17 ± 3 aB
LT-LS + SO	1467 ± 170 bAB	20 ± 7 aAB*	22 ± 7 aAB*	16 ± 3 aB*	33 ± 8 aAB*	30 ± 4 aAB*	20 ± 3 aAB*	14 ± 1 abA
LT-LS + Br30	1578 ± 42 bB	32 ± 5 aB*	35 ± 5 aB*	21 ± 1 aC*	43 ± 3 bB*	31 ± 2 bB*	21 ± 1 bAB*	17 ± 0.5 bB
LT-LS + Mn <sup>2+</sup>	1147 ± 53 aAB*	11 ± 2 aA*	13 ± 1 aA*	13 ± 0.7 aA*	24 ± 3 aA*	26 ± 2 aAB*	18 ± 0.1 aAB*	13 ± 0.6 aA*
LT-LS + SO + Mn <sup>2+</sup>	1093 ± 71 bA*	16 ± 4 abAB*	18 ± 3 aAB*	13 ± 2 bA*	25 ± 4 bA*	24 ± 2 bA*	15 ± 3 bA*	11 ± 0.9 aA
LT-LS + Br30 + Mn <sup>2+</sup>	1260 ± 2 bAB*	25 ± 0.1 bAB*	31 ± 3 bB*	19 ± 0.4 bC*	37 ± 0.2 aB*	30 ± 0.2 bB*	21 ± 0.6 bB*	17 ± 0.1 bB

<sup>a</sup> Data are the means of three independent experiments. Same lowercase and uppercase letters indicate lack of statistically significant difference ( $P > 0.05$ ) between microcosms and within each biostimulation or bioaugmentation treatment in the presence of different supplements, respectively. Asterisk represents the occurrence of significant differences between each soil.

being efficient, was unable to reduce a residual highly recalcitrant pollutant fraction upon extension of the incubation time.

The failure of the biopile system to proceed further was supposed to be due to either the scant ability of the resident microbiota to deplete high molecular mass PAHs or to their low bioavailability [5].

Thus, the aim of the present study was to assess whether the stimulation of the resident fungal biota or, alternatively, the inoculation of exogenous effective PAH-degrading fungi [17,19] might be able to deplete the aforementioned residual contaminant fraction. Several studies report the ability of a variety of lignocellulosic materials to boost colonization by either resident or exogenously applied fungi in soil [28–30]. Wheat straw was selected due both to its abundance in cell wall polysaccharides and to its wide commercial availability [31] and, in order to avoid artifacts, it was used in sterile form. The further possible impact of supplements, namely bivalent manganese ions and MAs, such as Brij 30 and soybean oil, was also assessed.

Wheat straw is one of the most commonly used LSs in bioaugmentation strategies [31]. These supports can stimulate ligninolytic enzyme production, fungal growth and pollutant degradation [32]. In the present study, LS addition to the soil exerted a notable biostimulation action which was evident on indigenous fungal growth [Fig. 2A and B] and, to a lesser extent, on CHB [Fig. 1A]. Thus, the lignocellulosic mixture and incubation-controlled conditions supported the development of a robust indigenous fungal population in the contaminated soil which had not been observed in the previous biopiling treatment [5] or in other lab-scale studies [31].

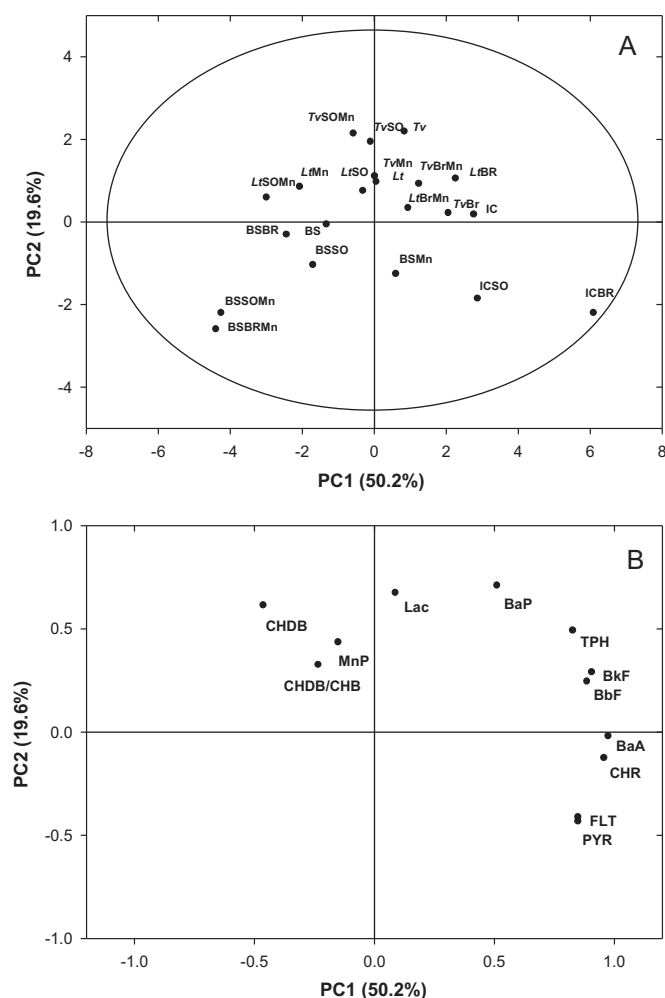
Furthermore, [34] reported that the addition of lignocellulosic amendants able to promote both growth and activity of native populations markedly enhanced the degradation of residual toxic compounds in the soil. Our results confirmed these findings and showed that LS-promoted biostimulation led to better TPH and 5-ring PAHs than those obtained with effective PAH-degrading allochthonous fungal strains [Table 2]. Indeed, as a consequence of the outstanding growth capabilities of indigenous soil mycobiota, the colonization of the upper [soil] layer by the WRF inoculants was clearly hindered after the early two weeks of the incubation. In this respect, marked antagonistic effects exerted by native soil

populations toward augmented WRF had been frequently reported [35–37].

The visually observed antagonistic effect of the native mycobiota toward both bioaugmented WRF was confirmed by both ergosterol and ITS qPCR data [Fig. 2A and B]. In biostimulated microcosms, both number of copies of the ITS region gene and the ergosterol concentrations were not significantly lower than those found in *T. versicolor*- or *L. tigrinus*-augmented ones; conversely, both parameters were significantly higher in biostimulated and bioaugmented microcosms than in incubation controls thus proving the stimulatory effect of LS toward indigenous fungi.

The presence of detectable laccase and MnP activities in biostimulated microcosms clearly showed that the indigenous microbiota included members able of producing both enzymes. In this respect, several yeasts, ascomycetes and other non-basidiomycete soil fungi are capable of producing laccase and other peroxidases [38]. Although ligninolytic enzymes activities in biostimulated microcosms were significantly lower than in bioaugmented ones, the extent of PAH biodegradation were higher in the former than in the latter. With this regard, it is known that non-ligninolytic fungi are able to transform and detoxify PAHs by means of intracellular enzymatic systems, such as the cytochrome P-450 monooxygenase/epoxide hydrolase system [39]. An additional reason might stem from the above reported strong competitive action mainly exerted by the indigenous mycobiota which led to a limited soil colonization by both *T. versicolor* and *L. tigrinus*.

The sole addition of Mn<sup>2+</sup> did not exert relevant effects on the microcosms under study with the only exception of a stimulation of MnP production in bioaugmented ones. Although MnP has been shown to oxidize a variety of PAHs, the increase in its activity in the aforementioned microcosms did not lead to improved contaminant depletion; this was confirmed by PCA analysis showing that MnP activity was among the least influential variables. Conversely, combination of Mn<sup>2+</sup> with MAs led to interesting effects. In particular, in biostimulated microcosms, 5-ring PAHs and TPH depletions were positively affected by the concomitant presence of MAs and Mn<sup>2+</sup> ions, regardless of the MA type [Table 2]. This fact was confirmed by the diagonally opposite position of PCA scores of these treatments with respect to the loadings plot of BbF, BkF and BaP residual contents [Fig. 3].



**Fig. 3.** Principal component analysis [PCA] of data showing scores [A] and variable loadings [B] plots. Percent variability explained by each principal component [PC] is shown in parentheses after each axis legend. The influence of variables in the model, expressed by the respective variable power equation [25] was as follows: BaA, 0.963; CHR, 0.946; TPH, 0.928; PYR, 0.926; BkF, 0.911; FLT, 0.909; BbF, 0.852; BaP, 0.758; CHDB, 0.581; LAC, 0.452; MnP, 0.204; CHDB/CSB, 0.155.

The very clear toxic effect of Brij 30 on the CHDB populations [Fig. 1C] was concomitant with lower 4-ring PAHs degradation in most of the microcosms where this surfactant had been added, suggesting that bacterial populations might be of paramount importance in the biodegradation of this relatively more accessible PAH fraction. This can be desumed by the absence of any significant inhibition of the fungal growth in Brij 30-containing microcosms [Fig. 2A and B]. Interestingly, the inhibitory effect of Brij 30 on 4-ring PAHs degradation was also observed in incubation controls [Table 2]. These findings confirm that the assessment of surfactant toxicity toward indigenous microbiota is a crucial step in the selection of appropriate MAs for bioremediation purposes [40].

It is noteworthy that such high depletion extent on 5-ring PAHs had not been previously observed with this soil [33] and that they were obtained by relying on the stimulation of autochthonous fungal and bacterial populations. In this respect, it has been previously described the importance of specific PAH biosorption mechanisms of fungal biomass [41] which might also be an important process concomitantly with biodegradation in the polluted soil. In fact, biosorption process should also be considered in further studies focused on the biostimulation of autochthonous mycobiota in PAH-polluted soils.

Conversely, very curious is the case of the SO-amended incubation control, where the surfactant addition promoted unexpected levels of benzo[a]pyrene degradation and led to a stimulation of the bacterial heterotrophic population that was one order of magnitude higher than in the same soil with Brij 30 or with no MA addition. It is known that accessible carbon sources as SO, can enhance the cometabolism required for the bacterial benzo[a]pyrene degradation [42] but is very interesting how specific this effect was in this case, because no other PAH was affected in the same way as benzo[a]pyrene; further research is needed to better ascertain this apparent SO-promoted and contaminant-specific co-metabolic mechanism.

## 5. Conclusions

The present study documented that a highly recalcitrant TPH and HMW-PAHs fraction, remaining in an actual creosote-polluted soil after a 180-d pilot-scale biopiling treatment, might be significantly degraded by a biostimulation approach based on LS addition. Degradation results might be further boosted by the presence of a concomitant mobilizing agent and  $Mn^{2+}$ .

In this respect, possible mycoaugmentation approaches, which strictly require the concomitant LS addition with fungal inoculants might fail due to the LS-promoted growth of indigenous fungal and bacterial populations as it was clearly observed in this study. Thus, the implementation of bioremediation technologies, based on exogenous inoculants, strictly requires a lab-scale assessment of interactions between indigenous microbiota and the selected allochthonous species.

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