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

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

[a]Department of Microbiology, University of Barcelona, Diagonal 645, E-08028 Barcelona, Spain. vadorllado21@ub.edu, asolanas@ub.edu

[b]Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídenská 1083, 142 20 Prague 4, Czech Republic. covino@biomed.cas.cz

[c]GIRO Joint Research Unit IRTA-UPC, Institute of Research and Technology Food and Agriculture [IRTA], Torre Marimon, E-08140 Caldes de Montbui, Spain. marc.vinas@irta.cat

[d]Department for Innovation in Biological, Agro-Food and Forest systems [DIBAF], University of Tuscia, Via S. Camillo de Lellis snc, 01100 Viterbo, Italy. petrucci@unitus.it, dannib@unitus.it

Corresponding Author:

Marc Viñas Canals

[1]GIRO Joint Research Unit IRTA-UPC, Institute of Research and Technology Food and Agriculture [IRTA], Torre Marimon, E-08140 Caldes de Montbui, Spain.

Tel: [+34] 934674040

FAX: +34934674042

marc.vinas@irta.cat

To be submitted to **Journal of Hazardous Materials**

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.

Keywords: Soil bioremediation, White rot fungi (WRF), creosote, HMW-PAH, Lignin-modifying enzymes [LME], non-ionic surfactant.

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 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 towards 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^{-1}; real acidity [in water], pH 7.5; water holding capacity, 33.7%; total organic carbon, 3.5%; NO_3^-, 15 mg · kg^{-1}; NH_4^+, 1.6 mg kg^{-1}; texture: sand [32%]; silt [28%] and clay [40%].

Ergosterol, 7-dehydrocolesterol, o-terphenyl and methyl 1-[butylcarbamoyl]-2-benzimidazolecarbamate and Brij 30 [dodecyl tetraethylene glycol ether] were purchased from Sigma-Aldrich, Spain. Soybean oil and manganese [II] sulfate [$MnSO_4$] 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

Trametes versicolor ATCC 42530 and *Lentinus 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 ºC 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 4ºC 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 x 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, $MnSO_4$ was added, as a source of bivalent manganese ions, at a rate of [20 mg kg^{-1}]. Fungal cultures were grown for 7 d at 28 ºC 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

146 previously amended by mixing with an additional 10% [w/w] of non-inoculated
147 sterilized LS. All microcosms were incubated at 28 ºC for 60 d in the dark and their
148 moisture contents kept constant by periodic additions of sterile deionized water. All
149 experiments were carried out in three parallel replicates under non-axenic conditions.
150 Non-amended controls were prepared, incubated as above and from here onwards
151 referred to as incubation controls. Subsequent analyses were carried out on the upper
152 soil layer and the results were normalized by taking into account the dilution effect of
153 the LS mixed with the polluted soil.
154
155 ***2.5. Extraction and analysis of organic contaminants***
156 The soil samples were dried for 16 h at room temperature. Total organic extracts
157 [TOE] were extracted and both total petroleum hydrocarbon [TPH] and PAHs were
158 quantified as described elsewhere [7].
159
160 ***2.6. Biochemical determinations***
161 The content of ergosterol, a specific indicator of fungal growth, was determined
162 in soil according to [20]. Prior to the extraction, 7-dihidrocolesterol, dissolved in
163 dichloromethane, was added to each sample as a surrogate internal standard. Finally,
164 samples were analysed as described elsewhere [21].
165 Extracellular ligninolytic enzymes were extracted from soil samples and
166 subsequently assayed as previously reported [19].
167
168 ***2.7 Heterotrophic and hydrocarbon-degrading microbial populations counts***
169 Soil bacterial counts were performed using a miniaturized most probable number
170 [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^{-1} 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 rRNAfragments 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 centred 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^{2+}

196 ions, they did not significantly differ from their respective incubation control where a
197 high stimulatory effect of SO on CHB had been observed [Fig. 1A]. This positive
198 impact of LS and SO supplementation on CHB was confirmed by qPCR analyses of the
199 *16S rRNA* gene [Fig. 1B]. It is noteworthy that bioaugmentation treatments did not exert
200 a depressive effect either on 16S rRNA gene copy number or on CHB; conversely, the
201 former parameter was found to be significantly higher in LS-amended microcosm
202 colonized by *T. versicolor*.
203 A different behavior was observed with the cultivable hydrocarbon degrading
204 bacteria [CHDB] from MPN assays. The high population density present in the initial
205 contaminated soil [$2.3 \cdot 10^6$ MPN g^{-1} soil] was not significantly affected in incubation
206 controls with the notable exception of those added with Brij 30 where a striking
207 depressive effect was evident after 60 d incubation [$1.2 \cdot 10^3$ MPN g^{-1} soil] [Fig. 1C].
208 The negative effect of this surfactant on CHDB was also confirmed in all biostimulated
209 and *T. versicolor*-augmented microcosms, regardless of the presence or the absence of
210 Mn^{2+} ions. Conversely, SO had a significantly stimulatory effect on CHDB, in both
211 biostimulated and bioaugmented microcosms with respect to the respective incubation
212 controls [Fig. 1C]. Interestingly, the addition of LS significantly attenuated the
213 aforementioned toxicity effects of Brij 30.
214 The initial soil, arising from a 180 d biopiling treatment exhibited a rather high
215 CHDB/CHB ratio amounting to 20% and it decreased significantly in the non-amended
216 control after 60 d incubation [6.6%]; a dramatic decrease in this ratio was observed in
217 incubation controls added with either SO or Brij 30 [0.73 and 0.01%, respectively] [Fig.
218 1D]. In the presence of the former MA, in LS-amended microcosms inoculated with
219 either *T. versicolor* or *L. tigrinus*, the CHDB/CHB ratios were higher than the
220 respective incubation control [3.8 and 12%, respectively, *vs.* 0.73%]; in the same

bioaugmented microcosms, subjected to the concomitant SO and Mn^{2+} 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 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+}-

246 supplemented microcosms, laccase activity was significantly higher than in incubation
247 controls. Best laccase activity [6.77 IU · g^{-1} soil] was found in *T. versicolor* microcosms
248 undergoing simultaneous supplementation with LS, Mn^{2+} ions and Brij 30; regardless of
249 the type of supplements, laccase activity was generally higher in *T. versicolor* than in *L.*
250 *tigrinus* microcosms [Table 1].
251 In both bioaugmented microcosms, MnP activity appeared to be boosted by the
252 presence of either Mn^{2+} ions or Brij 30; however, when the two additives were
253 combined the stimulatory effect was markedly lower for both fungi.
254
255 ***3.2. Degradation of contaminants in soil***
256 In the soil under study, derived from a previous 180-d-long biopiling treatment,
257 the residual total petroleum hydrocarbon [TPH] and overall PAH contents amounted to
258 2815 and 389 mg · kg^{-1}, respectively [Table 2]. Best TPH removals, ranging from 71%
259 to 73%, were achieved in biostimulated microcosms supplemented with MAs and Mn^{2+}
260 ions. In any case, regardless of the supplementation, the residual TPH contents in
261 biostimulated microcosms were significantly lower than those found in respective
262 incubation controls [Table 2].
263 Conversely, bioaugmented microcosms failed to significantly lead to a lower
264 TPH contents than respective incubation controls with the only exceptions of Mn^{2+}-
265 supplemented *L. tigrinus* microcosms; among them, the most effective was the
266 microcosm concomitantly supplemented with SO and Mn^{2+} ions leading to a 61.2%
267 TPH removal.
268 With regard to the PAH fraction, the soil at start mainly contained 4- and, to a
269 lesser extent, 5-ring compounds. Among the former, the most abundant were
270 fluoranthene [FLT] and pyrene [PYR] [83 and 84 mg · kg^{-1} soil, respectively] while the

most representative 5-ring compounds included benzo[*b*]-fluoranthene [BbF] and benzo[k]fluoranthene [BkF] [57 and 38 mg kg^{-1} 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 towards 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. 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 ICSO and ICBR, 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 towards 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 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-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 allochthnous 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 towards augmented WRF had been frequently reported [35-37].

The visually observed antagonistic effect of the native mycobiota towards both bioaugmented WRF was confirmed by both ergosterol and ITS qPCR data [Fig. 2A-B]. In biostimulated microcosms, both number of copies of the ITS 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 towards 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 monooxigenase/epoxide hydrolase system [39]. An additional reason might stem from the above reported strong competitive action

371 mainly exerted by the indigenous mycobiota which led to a limited soil colonization by
372 both *T. versicolor* and *L. tigrinus*.
373 The sole addition of Mn^{2+} did not exert relevant effects on the microcosms under
374 study with the only exception of a stimulation of MnP production in bioaugmented
375 ones. Although MnP has been shown to oxidize a variety of PAHs, the increase in its
376 activity in the aforementioned microcosms did not lead to improved contaminant
377 depletion; this was confirmed by PCA analysis showing that MnP activity was among
378 the least influential variables. Conversely, combination of Mn^{2+} with MAs led to
379 interesting effects. In particular, in biostimulated microcosms, 5-ring PAHs and TPH
380 depletions were positively affected by the concomitant presence of MAs and Mn^{2+} ions,
381 regardless of the MA type [Table 2]. This fact was confirmed by the diagonally opposite
382 position of PCA scores of these treatments with respect to the loadings plot of BbF, BkF
383 and BaP residual contents [Fig. 3].
384 The very clear toxic effect of Brij 30 on the CHDB populations [Fig. 1C] was
385 concomitant with lower 4-ring PAHs degradation in most of the microcosms where this
386 surfactant had been added, suggesting that bacterial populations might be of paramount
387 importance in the biodegradation of this relatively more accessible PAH fraction. This
388 can be desumed by the absence of any significant inhibition of the fungal growth in Brij
389 30-containing microcosms [Fig. 2A-B]. Interestingly, the inhibitory effect of Brij 30 on
390 4-ring PAHs degradation was also observed in incubation controls [Table 2]. These
391 findings confirm that the assessment of surfactant toxicity towards indigeous microbiota
392 is a crucial step in the selection of appropriate MAs for bioremediation purposes [40].
393 It is noteworthy that such high depletion extent on 5-ring PAHs had not been
394 previously observed with this soil [33] and that they were obtained by relying on the
395 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.

Acknowledgements

This study was financially supported by the Spanish Ministry of Science and Technology [CTM2007-61097/TECNO] and by the Spanish Ministry of Environment [094/PC08/3-01.1].

REFERENCES

[1] S.W. Rogers, S.K. Ong, T.B.Moorman, Mineralization of PAHs in coal-tar impacted aquifer sediments and associated microbial community structure investigated with FISH, Chemosphere 69 (2007) 1563-1573.
[2] J.G. Mueller, P.J. Chapman, P.H. Pritchard, Action of a fluoranthene-utilizing bacterial community on polycyclic aromatic hydrocarbon components of creosote, Appl. Environ. Microbiol. 55 (1989) 3085-3090.
[3] M. Alexander, Biodegradation and bioremediation. Academic Press, London, 1999.
[4] R. Boopathy, Effect of food-grade surfactant on bioremediation of explosives-contaminated soil, J. Hazard. Mater. 92 (2002) 103-114.
[5] E. Realp, J.A. Doménech, R. Martinez-García, C. Restrepo, S. Lladó, M. Viñas, A.M. Solanas, Ensayo piloto de biorremediación por la tecnología de la biopila dinámica para la descontaminación de suelos contaminados por creosotas provenientes de las actividades dedicadas a la preparación de la madera. Residuos: Revista técnica 102 (2008) 38-49.
[6] F. Fernandez-Luqueno, C. Valenzuela-Encinas, R. Marsch, C. Martinez-Suarez, E. Vazquez-Nunez, L. Dendooven, Microbial communities to mitigate contamination of PAHs in soil-possibilities and challenges: a review, Environ. Sci. Pollut. Res. Int. 18 (2011) 12-30.
[7] S. Llado, N. Jimenez, M. Viñas, A.M. Solanas, Microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil, Biodegradation 20 (2009) 593-601.
[8] F. Reichenberg, U.G. Karlson, O. Gustafsson, S.M. Long, P.H. Pritchard, P. Mayer, Low accessibility and chemical activity of PAHs restrict bioremediation and risk of exposure in a manufactured gas plant soil, Environ. Pollut. 158 (2010) 1214-1220.
[9] S.B. Pointing, Feasibility of bioremediation by white-rot fungi, Appl. Microbiol. Biotechnol. 57 (2001) 20-33.
[10] A. Majcherczyk, C. Johannes, A. Hüttermann, Oxidation of Polycyclic Aromatic Hydrocarbons [PAH] by laccase of *Trametes Versicolor*, Enzyme Microb. Technol. 22 (1998) 335–341.

460 [11] G. Eibes, T. Cajthaml, M.T. Moreira, G. Feijoo, J.M. Lema, Enzymatic
461 degradation of anthracene, dibenzothiophene and pyrene by manganese
462 peroxidase in media containing acetone, Chemosphere 64 (2006) 408–414.
463 [12] S.M. Bamforth, I. Singleton, Bioremediation of polycyclic aromatic hydrocarbons:
464 current knowledge and future directions, J. Chem. Technol. Biotechnol. 80
465 (2005) 723-736.
466 [13] V. Leonardi, M.A. Giubilei, E. Federici, R. Spaccapelo, V. Sasek, C. Novotny, M.
467 Petruccioli, A. D'Annibale, Mobilizing agents enhance fungal degradation of
468 polycyclic aromatic hydrocarbons and affect diversity of indigenous bacteria in
469 soil. Biotechnol. Bioeng. 101 (2008) 273-285.
470 [14] A. Zeddel, A. Majcherczyk, A. Hüttermann, Degradation of polychlorinated
471 biphenyls by white−rot fungi *Pleurotus ostreatus* and *Trametes versicolor* in a
472 solid state system, Toxicol. Environ. Chem. 40 (1993) 255-266.
473 [15] H. Singh, Mycoremediation: Fungal Bioremediation. John Wiley and Sons, Inc.
474 New Jersey, 2006.
475 [16] C.L. Yap, S. Gan, H.K. Ng, Application of vegetable oils in the treatment of
476 polycyclic aromatic hydrocarbons-contaminated soils, J. Hazard. Mater. 177
477 (2010) 28-41
478 [17] S. Covino, M. Cvancarova, M. Muzikár, K. Svobodová, A. D'Annibale, M.
479 Petruccioli, F. Federici, Z. Kresinová, T. Cajthaml, An efficient PAH-degrading
480 *Lentinus* [*Panus*] *tigrinus* strain: Effect of inoculum formulation and pollutant
481 bioavailability in solid matrices, J. Hazard. Mater. 183 (2010) 669-676.
482 [18] E. Borràs, G. Caminal, M. Sarrà, C. Novotný, Effect of soil bacteria on the ability
483 of polycyclic aromatic hydrocarbons [PAHs] removal by *Trametes versicolor*
484 and *Irpex lacteus* from contaminated soil, Soil Biol. Biochem. 42 (2010) 2087-
485 2093.
486 [19] A. D'Annibale, M. Ricci, V. Leonardi, D. Quaratino, E. Mincione, M. Petruccioli,
487 Degradation of aromatic hydrocarbons by white-rot fungi in a historically
488 contaminated soil, Biotechnol. Bioeng. 90 (2005) 723-731.
489 [20] M.W. Davis, R.T. Lamar, Evaluation of methods to extract ergosterol for
490 quantitation of soil fungal biomass, Soil Biol. Biochem. 24 (1992) 189-198.
491 [21] K.M. Brumfield, J.V. Moroney, T.S. Moore, T.A. Simms, D. Donze, Functional
492 Characterization of the *Chlamydomonas reinhardtii ERG3* Ortholog, a Gene
493 Involved in the Biosynthesis of Ergosterol. PLoS ONE 5(1) (2010) e8659.
494 doi:10.1371/journal.pone.0008659.
495 [22] B.A. Wrenn, A.D. Venosa, Selective enumeration of aromatic and aliphatic
496 hydrocarbon degrading bacteria by a most-probable-number procedure, Can. J.
497 Microbiol. 42 (1996) 252-258.
498 [23] A. Abalos, M. Viñas, J. Sabate, M.A. Manresa, A.M. Solanas, Enhanced
499 biodegradation of Casablanca crude oil by a microbial consortium in presence of
500 a rhamnolipid produced by *Pseudomonas aeruginosa* AT10, Biodegradation 15
501 (2004) 249-260.
502 [24] F.X. Prenafeta-Boldú, M. Guivernau, G. Gallastegui, M. Viñas, G. Sybren de
503 Hoog, A. Elías, Fungal/bacterial interactions during the biodegradation of TEX
504 hydrocarbons (toluene, ethylbenzene and *p*-xylene) in gas biofilters operated
505 under xerophilic conditions, FEMS Microbiol. Ecol. 80 (2012) 722-734.
506 [25] J.F. MacGregor, T. Kourti, Statistical process control of multivariate processes.
507 Contr. Engin. Pract. 3 (1995) 403-414.

508 [26] K.T. Semple, A.W.J. Morris, G.I. Paton, Bioavailability of hydrophobic organic
509 contaminants in soils: fundamental concepts and techniques for analysis, Eur. J.
510 Soil Sci. 54 (2003) 809-819.
511 [27] S. El Fantroussi, S.N. Agathos, Is bioaugmentation a feasible strategy for pollutant
512 removal and site remediation? Cur. Opin. Microbiol. 8 (2005) 268-275.
513 [28] J.M. Fernández-Sánchez, R. Rodríguez-Vázquez, G. Ruiz-Aguilar, P.J.J. Álvarez.
514 PCB biodegradation in aged contaminated soil: interactions between exogenous
515 *Phanerochaete chrysosporium* and indigenous microorganisms. J. Environ. Sci.
516 Heal. 36 (2001) 1145-1162.
517 [29] J. Hultgren, L. Pizzul, M.P. Castillo, U. Granhall, Degradation of PAH in a
518 creosote-contaminated Soil. A comparison between the effects of willows [*Salix*
519 *viminalis*], wheat straw and a nonionic surfactant, Int. J. Phytorem. 12 (2009)
520 54-66.
521 [30] P. Meysami, H. Baheri, Pre-screening of fungi and bulking agents for contaminated
522 soil bioremediation, Adv. Environ. Res. 7 (2003) 881-887
523 [31] Y. Lu, L. Yan, Y. Wang, S. Zhou, J. Fu, J. Zhang, Biodegradation of phenolic
524 compounds from coking wastewater by immobilized white rot fungus
525 *Phanerochaete chrysosporium*, J. Hazard. Mater. 165 (2009) 1091-1097.
526 [32] M. Walter, L. Boul, R. Chong, C. Ford, Growth substrate selection and
527 biodegradation of PCP by New Zealand white-rot fungi, J. Environ. Manage. 71
528 (2004) 361-369.
529 [33] M. Viñas, J. Sabaté, M.J. Espuny, A.M. Solanas. Bacterial community dynamics
530 and Polycyclic Aromatic Hydrocarbon degradation during bioremediation of
531 heavily creosote-contaminated soil, Appl. Environ. Microbiol. 71 (2005) 7008-
532 7018.
533 [34] C. Axtell, C.G. Johnston, J.A. Bumpus, Bioremediation of soil contaminated with
534 explosives at the naval weapons station Yorktown, J. Soil. Contam. 9 (2010)
535 537-548.
536 [35] R. Canet, J.G. Birnstingl, D.G. Malcolm, J.M. Lopez-Real, A.J. Beck,
537 Biodegradation of polycyclic aromatic hydrocarbons [PAHs] by native
538 microflora and combinations of white-rot fungi in a coal-tar contaminated soil,
539 Bioresour. Technol. 76 (2001) 113-117.
540 [36] N. Magan, S. Fragoeiro, C. Bastos, Environmental factors and bioremediation of
541 xenobiotics using white rot fungi, Mycobiol 38 (2010) 238-248.
542 [37] R. Martens R, F. Zadrazil, Screening of white-rot fungi for their ability to
543 mineralize polycyclic aromatic hydrocarbons in soil, Folia Microbiol. [Praha] 43
544 (1998) 97-103.
545 [38] I.S. Silva, M. Grossman, L.R. Durrant, Degradation of polycyclic aromatic
546 hydrocarbons [2–7 rings] under microaerobic and very-low-oxygen conditions
547 by soil fungi, Int. Biodeter. Biodegr. 63 (2009) 224-229.
548 [39] C.E. Cerniglia, Biodegradation of polycyclic aromatic hydrocarbons,
549 Biodegradation 3 (1992) 351-368.
550 [40] Z. Zheng, J.P. Obbard, Effect of non-ionic surfactants on elimination of polycyclic
551 aromatic hydrocarbons [PAHs] in soil-slurry by Phanaerochaete chrysosporium.
552 J. Chem. Technol. Biotechnol. 76 (2001) 423-429.
553 [41] B. Chen, Y. Wang, D. Hu, Biosorption and biodegradation of polycyclic aromatic
554 hydrocarbons in aqueous solutions by a consortium of white-rot fungi . Journal
555 of Hazardous Materials 179 (2010) 845–851
556 [42] R.A. Kanaly, R. Bartha, Cometabolic mineralization of benzo[a]pyrene caused by
557 hydrocarbon additions to soil, Environ. Toxicol. Chem. 18 (2009) 2186-2190.

FIGURE CAPTIONS

Figure 1. Cultivable heterotrophic bacteria [CHB] [A], 16SrRNA 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. [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.

Figure 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.

584 **Figure 3.** Principal component analysis [PCA] of data showing scores [A] and variable
585 loadings [B] plots. Percent variability explained by each principal component [PC] is
586 shown in parentheses after each axis legend. The influence of variables in the model,
587 expressed by the respective variable power equation [25] was as follows: BaA, 0.963;
588 CHR, 0.946; TPH, 0.928; PYR, 0.926; BkF, 0.911; FLT, 0.909; BbF, 0.852; BaP, 0758;
589 CHDB, 0.581; LAC, 0.452; MnP, 0.204; CHDB/CSB, 0.155.

Table 1: Lignin-modifying enzyme activities in soil.

Treatment	Laccase[a] (IU g^{-1})	Mn-Peroxidase[a] (IU g^{-1})
IC	0.12 ± 0.02	N.D.[b]
IC + SO	0.02	N.D.
IC + Br30	0.09 ± 0.00	N.D.
BS-LS	0.80 ± 0.23 aAB	0.11 ± 0.01aAB
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^{2+}	1.02 ± 0.20 aB*	0.10 ± 0.01 aAB
BS-LS + SO + Mn^{2+}	0.40 ± 0.06 aAB	0.07 aAB
BS-LS + Br30 + Mn^{2+}	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^{2+}	2.07 ± 1.03 aAB*	2.27 ± 0.14 bB
TV-LS + SO + Mn^{2+}	1.97 ± 0.67 bAB*	2.69 ± 1.31 aB*
TV-LS + Br30 + Mn^{2+}	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^{2+}	1.95 ± 0.51 aB*	7.02 ± 3.43 cB*
LT-LS + SO + Mn^{2+}	0.40 ± 0.05 aAB	3.07 ± 1.48 aAB*
LT-LS + Br30 + Mn^{2+}	1.42 ± 0.21 aAB*	1.18 ± 0.66 bA

[a]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.

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

Treatment	TPH[a] (mg ·kg^{-1})	Fluor (mg ·kg^{-1})	Pyr (mg ·kg^{-1})	B(a)A (mg ·kg^{-1})	Chry (mg ·kg^{-1})	B(b)F (mg ·kg^{-1})	B(k)F (mg ·kg^{-1})	B(a)P (mg ·kg^{-1})
InitialSoil	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^{2+}	1106±26 aB*	13±1 abA*	15±2 abA*	12±0.5 aB*	23±0.4 aAB*	25±0,5 aAB*	16±0.4 aAB*	13±0.5 aB*
BS-LS + SO + Mn^{2+}	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^{2+}	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 bAB*	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 bAB*	21±0.2 abB*	17±0.1 bA
TV-LS + Mn^{2+}	1417±155 bAB	16±3 bA*	17±3 bA*	16±2 bA*	29±4 aA*	32±4 bAB*	21±2 bB*	17±2 bA
TV-LS + SO + Mn^{2+}	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^{2+}	1436±60 cAB	28±3 bAB*	30±3 bB*	19±2 bB*	37±2 bAB*	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^{2+}	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^{2+}	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^{2+}	1260±2 bAB*	25±0.1 bAB *	31±3 bB*	19±0.4 bC*	37±0.2 bAB*	30±0.2 bB*	21±0.6 bB*	17±0.1 bB

[a]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.

Figure 1

FIGURE 1

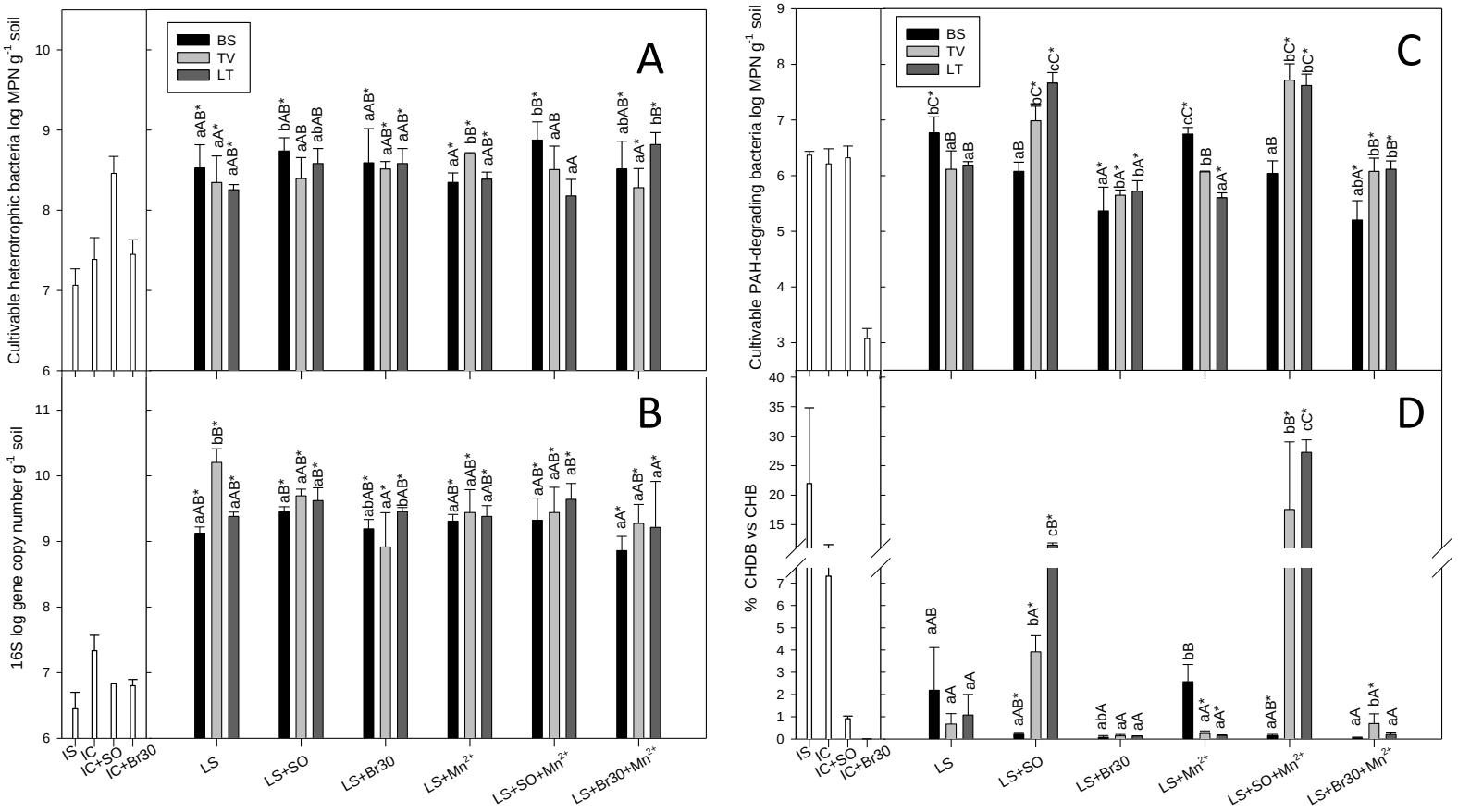

Figure 2

FIGURE 2

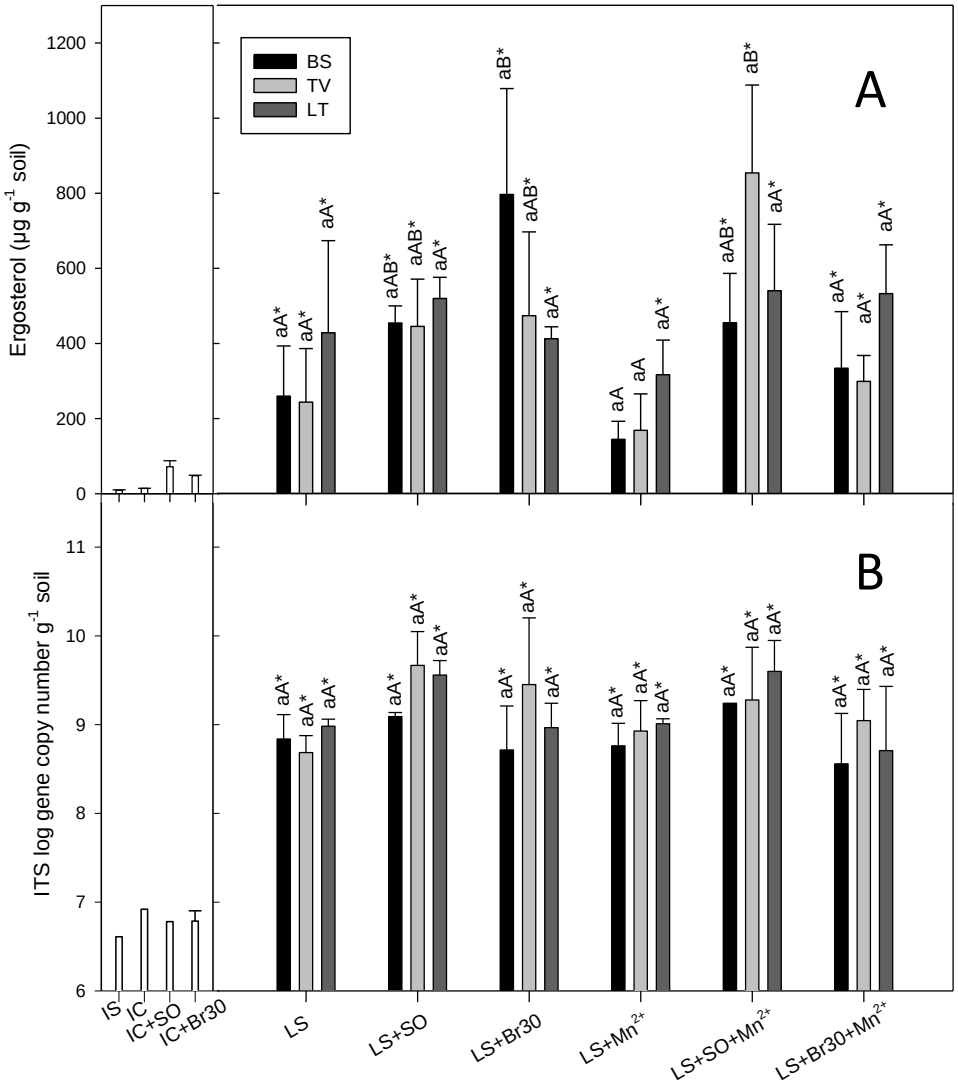

Figure 3

FIGURE 3