Journal of Hazardous Materials 186 (2011) 1263–1270

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Lentinus (*Panus*) *tigrinus* augmentation of a historically contaminated soil: Matrix decontamination and structure and function of the resident bacterial community

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ARTICLE INFO

Article history:
Received 10 September 2010
Received in revised form
29 November 2010
Accepted 30 November 2010
Available online 7 December 2010

Keywords:
Lentinus (*Panus*) *tigrinus*
Mycoremediation
Aromatic hydrocarbons
Historical contamination
Biodiversity

ABSTRACT

The ability of *Lentinus tigrinus* to grow and to degrade persistent aromatic hydrocarbons in aged contaminated soil was assessed in this study. *L. tigrinus* extensively colonized the soil; its degradation activity after 60 d incubation at 28 °C, however, was mostly limited to dichloroaniline isomers, polychlorinated benzenes and diphenyl ether while the fungus was unable to deplete 9,10-anthracenedione and 7-H-benz[DE]anthracene-7-one which were the major soil contaminants. Although clean-up levels were limited, both density of cultivable heterotrophic bacteria and richness of the resident bacterial community in *L. tigrinus* microcosms (*Lt*M) increased over time to a significantly larger extent than the respective amended incubation controls (1.9×10^9 CFU g^{-1} *vs.* 1.0×10^9 CFU g^{-1} and 37 *vs.* 16, respectively). Naphthalene- and catechol 2,3-dioxygenase gene copy numbers, however, decreased over time at a higher rate in *Lt*M than in incubation controls likely due to a higher stimulation on heterotrophs than xenobiotics-degrading community members.

1. Introduction

The last decades have witnessed an increasing use of bioremediation approaches to the clean-up of soils contaminated by persistent organic pollutants (POP) as an alternative to physico-chemical techniques [1,2]. The technical feasibility and the degradation efficiency of bioremediation depend on a large number of factors, such as type and bioavailability of contaminants, physico-chemical properties, history of the contaminated matrix, possible presence of co-contaminants [2,3]. The augmentation with ligninolytic fungi (LF) in *ex situ* bioremediation of POP-contaminated soils has been suggested to be more effective than *in situ* techniques under particular circumstances [1,3]. Such cases comprise sceneries where either low contaminant bioavailability or massive presence of compounds with low susceptibility to bacterial degradation or co-contamination with heavy metals negatively affect the degradation activity of both resident and allochthonous bacteria. Due to the structural heterogeneity of lignin, LF have evolved an extracellular degradation machinery that comprises lignin-modifying enzymes (LMEs) with low substrate specificity and operating *via* radical mechanisms. These peculiar properties confer LF the ability to degrade a wide variety of contaminants [1]. LMEs are able to bring about both direct and mediator-assisted oxidation of target contaminants [4]. Moreover, filamentous fungi have the ability to penetrate into soil aggregates acting as dispersion vectors of pollutant-degrading bacteria [5] and exhibit a significant tolerance to heavy metals [6]. Although these organisms have been employed for remediation purposes, little information is so far available on the relationships between augmented LF and the indigenous microbiota and, with few exceptions [7,8], available data are mostly limited to artificially spiked soils [9,10].

Among LF, *Lentinus tigrinus* has been shown to be able to degrade a variety of organic pollutants, such as polycyclic aromatic hydrocarbons [11,12], synthetic dyes [13], chlorinated phenols [14], 2,4,6-trinotrotoluene [15] and asphaltenes from hard coal [16]. These findings associated with its reported ability to produce significant amounts of LMEs on contaminated matrices [17] and to grow in the presence of heavy metals [18] suggest that it might be a promising species in bioremediation. So far, however, the impact of *L. tigrinus* augmentation on the resident microbiota has not been investigated.

Therefore, objectives of the present study were to assess (i) the ability of the *L. tigrinus* CBS 577.79 strain to clean-up a historically contaminated soil and to investigate (ii) time-dependent effect of fungal augmentation on the bacterial community structure and (iii) temporal variation of degradation genes of the resident bacterial community. Mycoaugmentation performances were tested on a contaminated soil from a decommissioned industrial site where

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doi:10.1016/j.jhazmat.2010.11.128

a long-term and diversified production of chemicals had led to the concomitant accumulation of both POP and heavy metals.

2. Materials and methods

2.1. Contaminated soil and fungal strain

The soil from the decommissioned chemical plant (ACNA, Cengio, Italy) contained high concentrations of both heavy metals and aromatic hydrocarbons, the relative identities and concentrations of which have been previously reported [19]. *L. tigrinus* 577.79, from the CBS culture collection (Baarn, NL), was maintained at 4 °C on potato dextrose agar slants and sub-cultured monthly. Inocula were prepared as previously described [20].

2.2. Augmentation conditions

The contaminated soil (40 g) was mixed with milled maize stalks (MMS, 8 g) and placed in 1 L Erlenmeyer flasks. The initial moisture content of the soil/MMS mixture was 7.73%. Inocula were added to each flask so as to obtain a density of 0.4% (w/w) and adjusting the final soil moisture content to 37%. The flasks were incubated at 28 °C for 0, 7, 15, 30, 60 d. Non-inoculated soil and soil/MMS mixture were incubated in parallel and are referred to as non-amended and amended incubation controls (NAIC and AIC, respectively).

2.3. Extraction and analysis of contaminants

Sixty-d-old fungal microcosms and respective incubation controls were subjected to Sohxlet extraction with hexane-acetone (3:1, v/v) for 12 h [21]. Two internal standards (*i.e.*, 2,4,6-trichloroaniline and anthracene-d10) were added to the samples prior to extraction and extracts analysed by gas chromatography–mass spectrometry (GC–MS) as previously described [20].

2.4. Biochemical determinations

Extracellular ligninolytic enzymes were extracted from soil samples and assayed as previously reported [20]. Endo-β-1,4-glucanase, endo-β-1,4-xylanase activities and extracellular soluble protein were determined as reported by Sampedro et al. [17]. All activities were expressed in international units (IU), defined as the amount of enzyme producing 1 μmol of product per minute under the assay conditions.

2.5. Determination of mycelial growth and viable heterotrophic bacteria

Ergosterol was extracted and quantified by HPLC as reported elsewhere [21]. Bacterial counts were performed on Plate Count Agar (Oxoid) as previously described [20].

2.6. DNA extraction and PCR amplification

Total community DNA was extracted from 250 mg of soil using the Power Soil DNA Extraction Kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer's instruction. The variable V3 region of 16S rDNA was amplified by PCR with the primers 341F (with an additional 40-nucleotide GC-rich sequence) and 534R [22]. The 16S rRNA gene was amplified from 10 ng of DNA in a PCR reaction with 0.4 μM of each primers, using the illustra™ HotStart Master Mix (GE Healthcare, Little Chalfont UK). PCR amplification was performed in a thermal cycler (Bio-Rad Laboratories, Hercules, CA) as previously reported [23]. PCR products from three parallel amplifications were concentrated with a Microcon filter (Millipore, Bedford, MA), separated in 1.5% (w/v) agarose gel and stained by ethidium bromide.

2.7. DGGE analyses

The INGENYphorU-2 system for DGGE (Ingeny International BV, Goes, NL) was used. Samples were loaded onto 6% polyacrylamide-bisacrylamide (37.5:1.0) gels with denaturing gradients from 40 to 60% (100% corresponds to 7 M urea and 40% [v/v] deionized formamide). Gels were run at 100 V in 0.5× TAE (20 mM Tris acetate, 10 mM sodium acetate, 0.5 mM Na-EDTA at pH 7.4) at 60 °C for 16 h and stained with SBYR Gold (Invitrogen, Carlsbad, CA) in 1× TAE for 45 min. DGGE banding patterns were digitized and processed using the Quantity-one analysis software (Bio-Rad Laboratories). Richness (S) was determined from the number of bands in each lane while the Shannon-Weaver index (H) was calculated from $H = \Sigma(n_i/N)\log(n_i/N)$, where n_i is the peak height of a band and N is the sum of all peak heights in a lane. An unweighed pair group method with arithmetic means dendrogram was generated from a similarity matrix based on common band positions between lanes and calculated using the Dice's coefficient [24].

2.8. Phospholipid fatty acids analysis (PLFA)

PLFA extraction and subsequent analyses were conducted as already reported [17]. Fungal biomass was quantified based on 18:2ω6,9 content while bacterial biomass was quantified as a sum of i14:0, i15:0, a15:0, 16:1ω7t, 16:1ω9, 18:1ω7, 10Me-16:0, i17:0, a17:0, cy17:0, 17:0, 10Me-17:0, 10Me-18:0 and cy19:0 [8]. Terminally branched fatty acids (*a*,*i*) were used as markers for Gram+ bacteria; the methyl branching in the middle of the fatty acid molecule (*i.e.*, 10Me16:0, 10Me17:0 and 10Me18:0) for actinomycetes; the cyclopropyl branched fatty acids (*i.e.*, cy17:0 and cy19:0) and 18:1 ω7 for Gram− bacteria [8]. The cy/pre ratio, was used as an index of physiological and nutritional stress in bacterial community [25].

2.9. Quantitative real-time PCR (qPCR) assays

qPCR was performed on an iCycler IQ (BioRad, Hercules, CA) using the SYBR Green JumpStart™ Taq ReadyMix™ (Sigma, Milan, Italy) following the manufacturer's instruction and according to MIQUE guidelines [26]. Amplification was carried out in a mixture containing 12.5 μL of 2× SYBR Green JumpStart Taq mix, 2.5 μL of each primer at the concentration reported (Table S1, supplementary material) and 7.5 μL of template DNA.

The amplifications were carried out with a first step of 95 °C (5 min), followed by 50 cycles of 30 s of denaturation at 95 °C, 30 s at the primer-specific annealing temperature (Table S1, supplementary material) and 30 s of elongation at 72 °C. The final step consisted of 7 min at 72 °C. At the end of the qPCR, a melting curve analysis was performed by measuring the SYBR Green I signal intensities during a 0.5 °C temperature increment every 10 s from 50 °C to 95 °C. The absolute quantification of the target gene copy number was performed using a standard curve for qPCR calibration. Purified 16S rRNA, *nahAc* and *C230* PCR products (QIAquick PCR purification kit, QIAGEN) were cloned into pCR4 vector, using the TOPO TA cloning kit (Invitrogen) following the manufacturer's instruction. After purification (QIAprep Spin Miniprep kit, Qiagen), plasmids were linearized with EcoRI restriction enzyme (Fermentas, Glen Burnie, Maryland). Plasmid DNA concentration was quantified using the Quant-iT™ assay with the Qubit™ fluorometer (Invitrogen). The copy number of standard plasmids was calculated according to plasmid (3956 bp) plus insert (1499, 136 and 379 bp for 16S rRNA, *nahAc* and *C230*, respectively) lengths and assuming an average molecular mass of 660 Da for a base pair [27]. The standard DNA was serially diluted to prepare 10

times dilution series and the quantification cycles (Cq) were measured for every qPCR reaction to create a standard curve. Cq values were defined as the number of cycles where the fluorescence data cross the threshold line, set within the logarithmic increase phase of the acquired fluorescence. The inset in Fig. 4A shows the correlation between Cq values and copy number of 16S rRNA, *nahAc* and *C230* standard DNAs. These curves were used to convert Cq values into copy number of target genes [28]. The specificity of the qPCR assays was confirmed by the occurrence of both single melting peaks and unique bands of the expected size on agarose gels and by DNA sequencing of the PCR amplified target genes.

2.10. Ecotoxicological analyses

AIC and *Lt*M were analyzed for their toxicity by using the Collembola *Folsomia candida* (Willem). Each sample was analyzed both as a whole and after two-fold dilution [20].

2.11. Statistical analysis

PLFA data were also subjected to principal components analysis (PCA) by the use of the Simca-P 8.0 software (Umetrics, Umea, Sweden) and the possible presence of outliers checked as already reported [29].

3. Results and discussion

3.1. Fungal growth and extracellular enzyme production

Although the initial ergosterol concentration in NAIC was not negligible (20.2 mg kg^{-1}) it did not change over time (Fig. 1A). In the amended counterpart (AIC), after a lag phase of 15 d, it rapidly increased on day 30 (112 mg kg^{-1}) and continued to rise reaching 144 mg kg^{-1} on day 60. In agreement with other studies [7,9], our data clearly suggest that the resident fungal community in the ACNA soil was activated by the addition of the sterilized amendant. The ergosterol content in *L. tigrinus* microcosms (*Lt*M) did not vary in the early 15 d; thereafter, it increased over time at an almost linear rate up to the end of the set incubation time reaching about 693 mg kg^{-1} (Fig. 1A). Fungal colonization in *Lt*M was clearly visible after only 20 d from the inoculation; a profuse growth was evident in these microcosms despite the presence of high concentration of toxic metals including arsenic, lead, copper, mercury and chromium [19]. With this regard, *L. tigrinus* carpophores were reported to accumulate toxic metals to a higher extent than other fungi thus indicating a high tolerance of this species to these contaminants [18].

Changes in extracellular protein (EP) content were also investigated over time since it is an indirect index of metabolic activity of

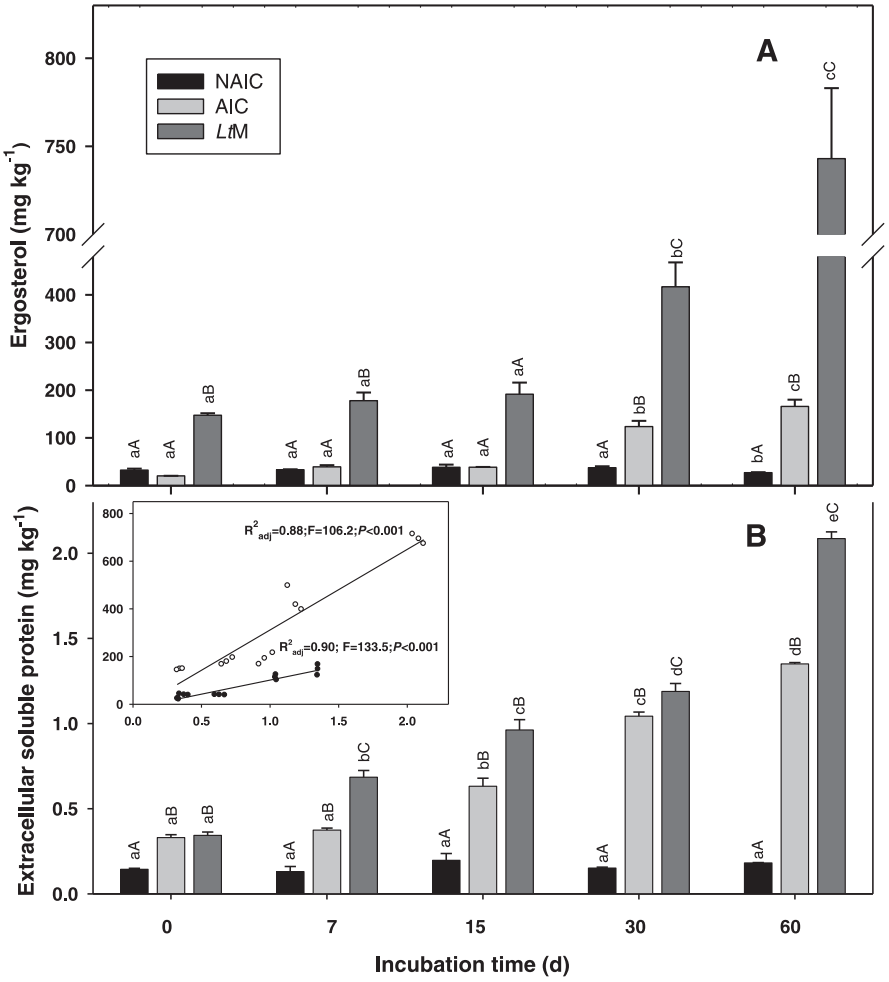

Fig. 1. Time courses of ergosterol (A) and extra-cellular soluble protein (B) contents in non-amended and amended incubation controls (NAIC and AIC, respectively) soil and after incubation at 28 °C with *L. tigrinus* (*Lt*M). Data are the means ± standard deviations of three experiments. Pair-wise comparisons were performed by the Tukey test ($P \leq 0.05$): same lowercase and uppercase letters denote absence of statistical significance between time-dependent changes within the same treatment and between treatments at the same time, respectively. Inset in plot B reports regression lines of ergosterol *vs.* protein contents in AIC (●) and *Lt*M (○) with the following parameters: squared correlation coefficient (R^2_{adj}), *F* value and alpha level (*P*) of the model.

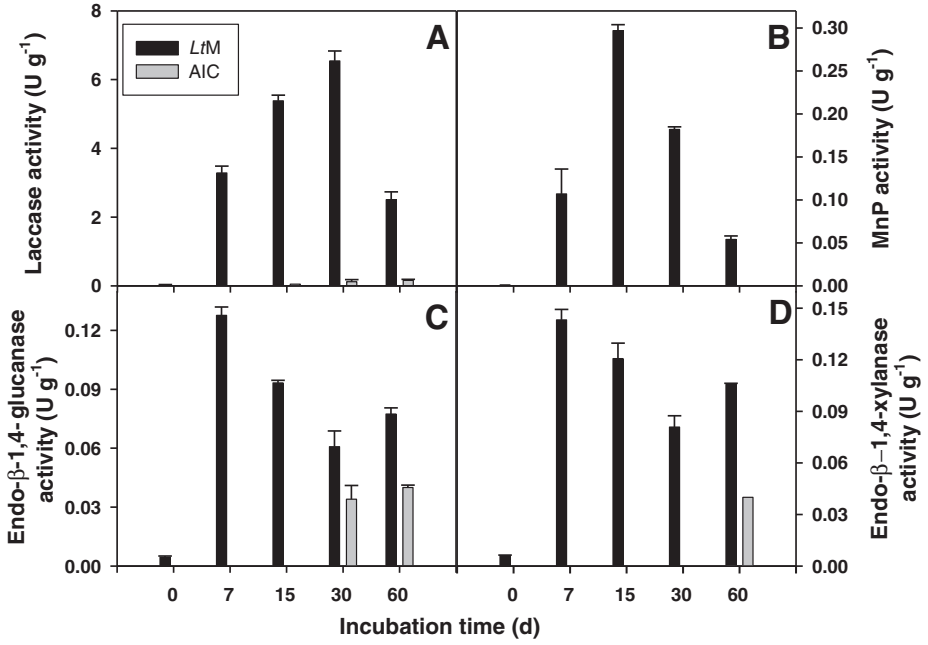

Fig. 2. Time courses of laccase (A), Mn-dependent peroxidase (B, MnP), endo-β-1,4-glucanase (C) and endo-β-1,4-xylanase (D) activities in amended incubation control (AIC) soil and after incubation at 28 °C with *L. tigrinus* (*Lt*M). Data are the means ± standard deviations of three experiments.

fungi in habitats containing lignocellulosic residues. A significant amount of EP, in fact, is secreted as either glycosyl hydrolases and LMEs, the action of which enables fungi to use plant materials as growth substrates. A very low EP content was detected in NAIC at start with no significant changes over time (Fig. 1B). In *Lt*M and, to a lesser extent, in AIC, EP increased from day 15 to day 60 when its respective contents reached about 2.1 and 1.3 g kg^{-1}. Interestingly, ergosterol and EP contents were highly correlated in both *Lt*M and AIC (inset Fig. 1B).

Neither laccase nor MnP activities were detected in NAIC, while only the former enzyme was found in AIC starting from day 15 (Fig. 2A). In *Lt*M, conversely, the onset of both laccase and MnP was observed on day 7, the former peaking on day 30 (about 6.6 U g^{-1} soil) and the latter on day 15 (about 0.3 U g^{-1} soil) (Fig. 2A and B, respectively).

No glycosyl hydrolase activities were detected in NAIC, while both endo-β-1,4-glucanase and endo-β-1,4-xylanase activities were found in AIC where the onset of the former occurred earlier than the latter (30 d *vs.* 60 d, respectively). In *Lt*M, both endo-β-1,4-glucanase and endo-β-1,4-xylanase activities peaked on day 7 (0.13 and 0.14 U g^{-1} soil, respectively) (Fig. 2C and D, respectively).

3.2. Organopollutant degradation and detoxification in soil

Several aromatic contaminants were found in NAIC as shown in Table 1. Among them, most abundant were 9,10-anthracenedione (461.4 mg kg^{-1}) and 7-H-benz[DE]anthracene-7-one (918.4 mg kg^{-1}). Several chloro-substituted compounds were also detected the most abundant of which being 2,6- and 2,4-dichloroaniline (77.2 and 35.1 mg kg^{-1}, respectively), 1,2,3,4-tetrachlorobenzene (17.4 mg kg^{-1}) and 2,3,4,5,6-pentachloroaniline (17.4 mg kg^{-1}). Some pollutants, including tetrachlorothiophene, 1,2,45-tetrachlorobenzene, phenanthrene and *N*-phenyl-1-naphthaleneamine were not detected in AIC (Table 1). Other compounds were depleted by different extents, such as 2,6- and 2,4-dichloroaniline (28 and 39%, respectively), while the major contaminants (*i.e.*, 9,10-anthracenedione and benz[DE]anthracene-7-one) were not degraded at all (Table 1). The quantitative or partial removal of the above mentioned contaminants from AIC might be due to both abiotic and biotic factors, the respective contributions of which were not assessed since this was outside the scope of this study.

To evaluate the impact of fungal augmentation on soil decontamination, percent reduction of pollutants were calculated with respect to their residual concentrations in AIC (Table 1). The highest percent reductions were observed for 2.4- and 2.6-dichloroaniline (100 and 82.8%, respectively), diphenylether (78%), 1,2,3,4-tetrachlorobenzene (73.4%) and naphthalene (57.3%). However, as already observed for AIC, the residual concentrations of both 9,10-anthracenedione and benz[DE]anthracene-7-one were not affected by the fungus. The non-susceptibility of these compounds to degradation by white-rot species, such as *Pleurotus*

Table 1
Concentrations of contaminants in non-amended and amended incubation controls (NAIC and AIC, respectively) and percent reductions after 60 d incubation at 28 °C with *L. tigrinus* (*Lt*M).[a]

Contaminant	Concentration (mg kg^{-1}) in:		Percent reduction by *Lt*M[b]
	NAIC	AIC	
Naphthalene	12.9	9.0	57.3
Tetrachlorothiophene	1.3	n.d.[a]	n.d.[a]
1,2,4,5-tetrachlorobenzene	10.3	n.d.[a]	n.d.[a]
2,6-dichloroaniline	77.2	55.1	82.8
2,4-dichloroaniline	35.1	21.4	100
1,2,3,4-tetrachlorobenzene	17.4	3.6	73.5
Diphenylether	31.7	16.0	78.0
Phenanthrene	13.9	n.d.[a]	n.d.[a]
2,3,4,5,6-pentachloroaniline	11.3	17.1	37.2
Diphenylsulphone	18.1	18.0	24.4
9,10-anthracenedione	461.4	462.1	0.0
1-chloro-9,10-anthracenedione	3.3	3.1	6.5
N-phenyl-1-naphthaleneamine	5.8	n.d.[a]	n.d.[a]
1,1-binaphtalene	13.6	6.6	48.5
1-ammino-9,10-anthracenedione	9.0	8.0	3.1
1,2-binaphtalene	12.7	12.7	50.2
7[H]-benz[DE]-anthracen-7-one	918.0	918.0	0.0.

[a] n.d., not detected.
[b] Calculated with respect to residual concentrations of each contaminant in AIC, taken as 100%.

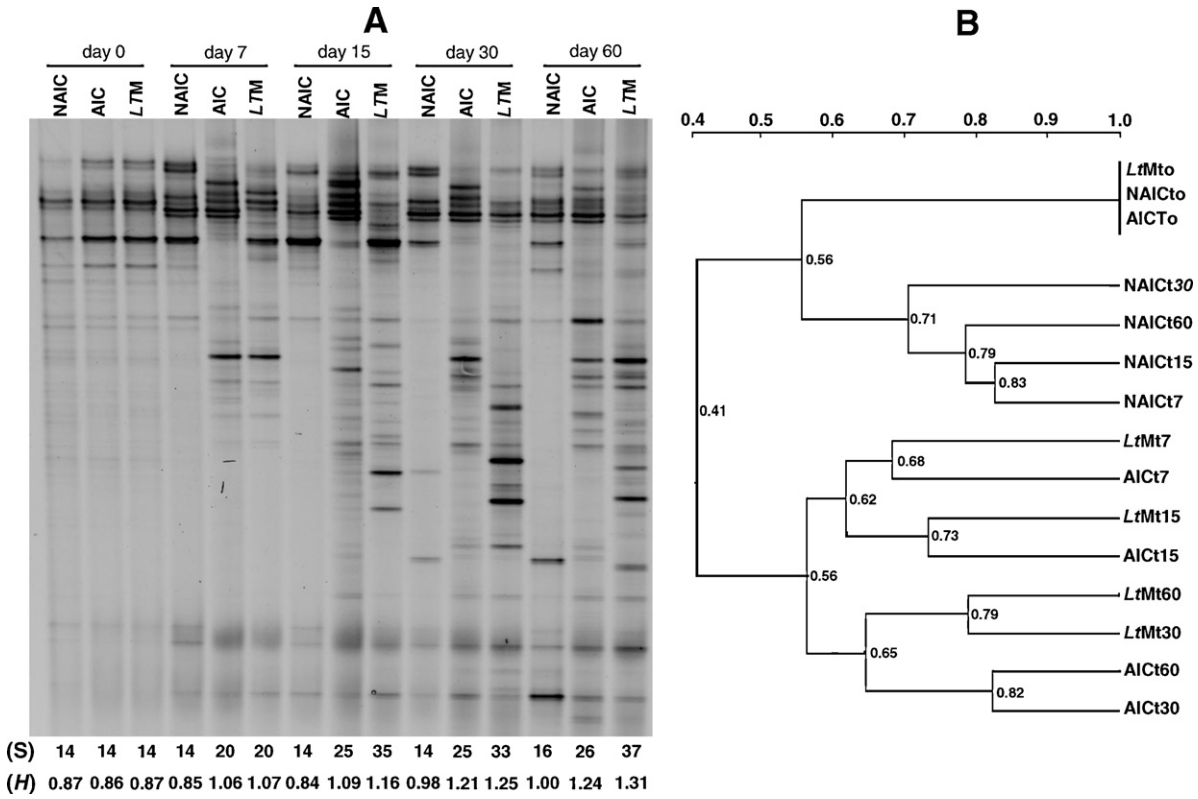

Fig. 3. (A) DGGE analysis of the bacterial communities in the non-amended and amended incubation controls (NAIC and AIC, respectively), *L. tigrinus* microcosms (*Lt*M) at start and after 7, 15, 30 and 60 d incubation at 28 °C and (B) cluster analysis obtained from the DGGE profiles based on the averaged similarity matrix. Numbers on the nodes represent the degree of similarity.

pulmonarius and *Phanerochaete chrysosporium* had been already observed in the ACNA soil [30] and the sole fungus capable to partially degrade these compounds was a *Stachybotrys* sp. strain that had been isolated from the same matrix [16]. It is interesting to note that 9,10-anthracenedione was reported to be a dead-end metabolite in several fungal microcosms [7,9]. The partial removal of polychlorinated anilines observed in both AIC and *Lt*M might be ascribed to the action of LMEs the activities of which were detected in both microcosms. It is worth mentioning that an active cytochrome P-450 monooxygenase was recently detected in the microsomal fraction from *L. tigrinus* liquid cultures conducted in the presence of PAHs [31]; this enzyme might have been involved in the oxidation of certain contaminants such as, for instance, diphenyl ether and phenanthrene.

Toxicity determinations were only limited to those microcosms (*i.e.*, AIC and *Lt*M) where at least a partial depletion of organic contaminants had been observed. AIC at start was highly toxic, resulting in 100% mortality of *F. candida* and retained a high toxicity even when it had been five-fold diluted with a non-contaminated soil (100 and 67% mortality, respectively). Regardless of the dilution, a time-dependent decrease in toxicity in both AIC and *Lt*M was observed. For instance, mortalities of Collembola contacted with non-diluted 30-d-old and 60-d-old AIC and *Lt*M were 73.3% *vs.* 43.3%, respectively, and 46.7% *vs.* 33.3%, respectively. Therefore, fungal augmentation promoted a significantly higher detoxification than the amendment.

3.3. Effect of augmentation on cultivable bacteria and resident community structure

In NAIC, a low and constant density of cultivable heterotrophic bacteria (about 8.1×10^4 CFU g^{-1}) was found within the early 15 d of incubation, then a slight increase was detected in 30- and 60-d-old microcosms (about 3.1×10^5 and 12.9×10^5 CFU g^{-1}, respectively). By contrast, the presence of MMS in AIC led to an abrupt increase in bacterial density which was already evident after 7 d of incubation (from 5.6×10^4 to 1.4×10^8 CFU g^{-1}). Fungal augmentation did not negatively affect cultivable bacteria as inferred by comparisons with counts in AIC and, after 60 d, bacterial density in the former microcosms was significantly higher than in the latter (1.9×10^9 CFU g^{-1} *vs.* 1.1×10^9 CFU g^{-1}, respectively).

It is known, however, that cultivable bacteria comprise only a minor fraction of the resident community [32]. Thus, to provide further insights into the impact of augmentation, a cultivation-independent method, based on DGGE analysis of PCR-amplified 16S rRNA genes, was used. The bacterial community in NAIC was characterized by low richness ($S = 14$) and diversity ($H = 0.87$) that, furthermore, did not significantly vary over time (Fig. 4A). By contrast, S and H markedly increased in AIC and *Lt*M; for instance, after 60 d incubation (26 and 37, respectively, and 1.24 and 1.31, respectively) (Fig. 3A).

Fig. 3B shows the separation of two main clusters with low similarity (0.41), the former including all the NAIC sampling times and both AIC and *Lt*M at start and the latter containing fungal microcosms and respective AIC in the subsequent incubation times. Cluster analysis of DGGE fingerprints also showed that the microbial populations associated with AIC exhibited a larger extent of similarity with fungal augmented soil during the early 15 d of incubation. It is noteworthy that despite a substantial degree of similarity (0.65) was found for 60-d-old AIC and *Lt*M, a very low extent of contaminant reduction was observed in the former; this outlines the importance of the degradation activity of *L. tigrinus*.

Although DGGE analysis indicates that augmentation with *L. tigrinus* led to an increase in bacterial diversity, it has to be taken into account that this technique only detects members of the dominant populations and does not allow a thorough determination of

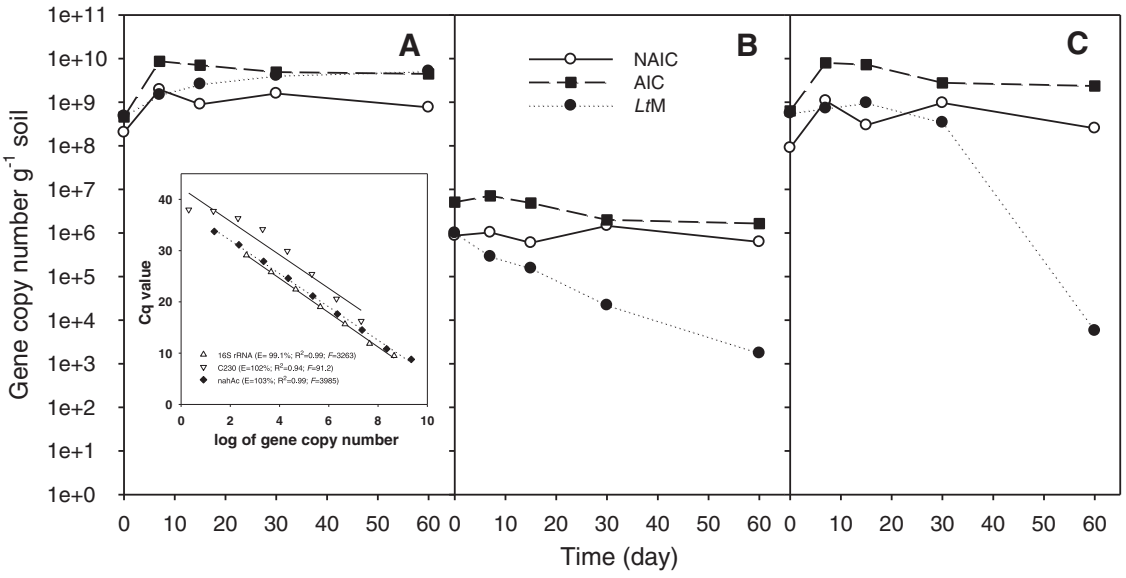

Fig. 4. Semi-logarithmic plots of time dependent changes in the copy number of *16S rRNA* (A) *NahAc* (B), *C230* (C) genes in non-amended and amended incubation controls (NAIC and AIC, respectively) and in *L. tigrinus* microcosms (*Lt*M). The inset in plot A reports standard curves for 16S rRNA, catechol-2,3-dioxygenase (C230) and naphthalene dioxygenase (nahAc) from qPCR amplification assays obtained by plotting the logarithm of the gene copy number *vs.* the quantification cycle (Cq) values. For each standard curve, the following parameters of regression analysis are reported: squared correlation coefficient (R^2) and *F* value. For each model, relative alpha levels were lower than 0.001. These parameters are preceded by the percent amplification efficiency (*E*), calculated by the expression: $E = (10^{-1/\text{slope}}) - 1$.

the full complexity of the system [33]. By contrast, PLFA analysis provides more valuable insights into the viable microbial community because phospholipids are not either found in storage products or in dead cells since the phosphate group is rapidly hydrolyzed upon cell death [34]. Therefore, PLFA data can be used to study changes in the microbial community, the groups of which are identified by specific fatty acids methyl esters (FAMEs). In addition to differentiating taxonomic groups *via* the use of well defined FAMEs, some ratios between specific fatty acids (*e.g.*, cy/pre) have been used to assess the physiological state of microbial communities [25,34].

In all microcosms at start, PLFA analysis showed that Gram− dominated over Gram+ bacteria as indicated by the large relative abundances of both the cyclopropyl-derived (*i.e.*, cy17 and cy19) and the monounsaturated fatty acids 16:1ω7 and 18:1ω7 (Table S2, supplementary material). An abrupt increase of Gram− bacteria in the first sampling time (*i.e.*, 7 d) was detected in both NAIC and AIC followed by a decline in the successive incubation periods. This was followed by a significant shift in the bacterial community where the relative abundances of Gram+ bacteria over the 7–60 d time range increased from 8.2 to 43.4% and from 14.3 to 47.7% in NAIC and AIC, respectively (Table S2, supplementary material). The presence of MMS in AIC, moreover, stimulated the growth of resident fungi as indicated by the marked increase in the relative abundance of the fatty acid 18:2ω6,9 (Table S2, supplementary material). In *Lt*M, the percent abundance of Gram− did not change in the early 7 d; thereafter, the Gram+/Gram− ratio increased in the subsequent incubation times. In the same microcosms, from the 15th d incubation, both 10Me-16:0 and 10Me-18:0 were detected thus suggesting the presence of Actinomycetes which were only observed in 60-d-old AIC. The significant inoculum rate employed in *Lt*M led to a high F/B ratio at start (about 3.1) which steadily declined throughout the incubation reaching a value of 0.67 on day 60 (Table S2, supplementary material). A previous study [7] indicated that augmentation with three fungal species did not significantly affect the concentration of individual PLFAs with the only exceptions of i15:0 and cy19:0 which are characteristic of Gram− and Gram+ bacteria, respectively. Our data, instead, indicate a marked stimulation of Gram+ bacteria upon augmentation with *L. tigrinus*, and are in agreement with another study conducted on creosote-contaminated soils with *Pleurotus ostreatus* [8]. The cy/pre ratio, which has been linked to nutritional stress in bacterial communities [25], was lowest in *Lt*M. In this respect, higher values of this ratio have been suggested to be due to decrease in bacterial growth rates and increase in carbon limitation [34]. This might suggest that community level stresses in *Lt*M were lower than in NAIC and AIC.

Principal component analysis (PCA) performed to analyze PLFA data showed that 83.5% of variability was explained by the first two principal components (51.7% and 31.8%, respectively) (Fig. S1, supplementary material). The observations relative to the various treatment typologies (*i.e.*, NAIC, AIC and *Lt*M) clearly separated along the first principal component and 30- and 60-d-old *Lt*M were located in the upper right quadrant (Fig. S1, supplementary material); the placement of variables Gram− and cy/pre ratio and fungi at the opposite quadrant in the loadings plot suggest that they were inversely related (Fig. S1, supplementary material). In this respect, the most drastic reductions in Gram− and the lowest cy/pre ratios had been observed where the signature PLFA 18:2 ω6,9 was most abundant. Early sampling times of both AIC and NAIC, instead, were located in the lower left quadrant corresponding to variables Gram−, cy/pre ratio and total bacteria (Fig. S1, supplementary material) which were highest in these microcosms.

3.4. Effect of augmentation on copy numbers of catabolic genes

An additional aim of this study was to investigate the time-dependent changes in the populations bearing catabolic genes for enzymes potentially involved in the degradation of POPs in NAIC, AIC and *Lt*M by monitoring the relative variations in copy number by qPCR. The concomitant presence of PAHs and quinone derivatives in the ACNA soil prompted us to select *nahAc* as a bacterial gene target, since it codes for a subunit of the ubiquitous multienzymatic naphthalene dioxygenase (NDO) system, which is a key component of the upper degradation pathway of several contaminants [35]. Despite its high enantio- and regio-selectivity, in fact, NDO catalyzes the *cis*-hydroxylation of a wide range of substrates [36]. A further target was the *C230* gene coding for the catechol-

2,3-dioxygenase, a key enzyme of the lower degradation pathway of several aromatic contaminants. Both *nahAc* and *C230* are highly conserved genes and thus they can be employed as markers to monitor bacterial population involved in POP degradation [35,37]. In order to assess whether possible differences among microcosms in the copy number of the target genes were not simply due to variations in bacterial growth, qPCR experiments were also conducted on the housekeeping *16S rRNA* gene. In *Lt*M, the copy numbers of both *nahAc* and *C230* genes (Fig. 4B and C, respectively) were drastically reduced despite a very slight initial increase in 16S *rRNA* gene therein (Fig. 4A). On the other hand, the copy numbers of *nahAc* and *C230* genes in both NAIC and AIC were rather constant throughout the incubation while the time course of the *16S rRNA* gene in these microcosms suggested an initial increase of the overall bacterial community. The abundance of the *nahAc* gene has been reported to be affected by concentration of target contaminants [27,35] and it has been shown that NDO is induced by a variety of hydroxy-substituted degradation intermediates which are susceptible to oxidation by LMEs [38]. The concomitant depletion of putative NDO substrates (*i.e.*, naphthalene and phenanthrene) and the prompt oxidation of hydroxylated degradation intermediates by *L. tigrinus* LMEs might explain the steeper decrease in the copy number of this gene over time in *Lt*M. With regard to *C230*, the convergence of several degradation pathways of aromatic contaminants providing a constant supply of catechol derivatives [39] might similarly explain the constant levels in *C230* copy number detected in both incubation controls. By contrast, the early onset and the constant presence of LMEs, such as laccase and MnP, might have impaired the accumulation of catechols in *Lt*M due to the high susceptibility of these compounds to the oxidation by those enzymes. An additional explanation for the steeper decreases in the copy number of both *nahAc and C230* might involve a higher stimulation of heterotrophs, due to augmentation, than of xenobiotics-degrading community members. It is worth mentioning that an initial increase of heterotrophic bacterial population without any stimulation of hydrocarbon-degrading bacteria had been observed after augmentation with either *P. ostreatus* [9] or Enzyveba, a stable fungal/bacterial consortium [40].

4. Conclusions

Sequential fungal–bacterial degradation is considered necessary requisite for the clean-up of a soil contaminated by a wide array of xenobiotics. The negative effect on specialized indigenous bacteria, witnessed by the reduction in copy numbers of relevant catabolic genes in *Lt*M, might be the reason why the major contaminants were not depleted although the fungus colonized the soil. The positive impact of *L. tigrinus* augmentation on both density of cultivable heterotrophic bacteria and diversity of the bacterial community appears to be important but not sufficient to reach satisfactory clean-up levels in aged and highly contaminated soils such as the ACNA one.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2010.11.128.

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