Long-term in situ dynamics of the fungal communities in a multi-contaminated soil are mainly driven by plants

Cécile Thion, Aurélie Cébron, Thierry Beguiristain & Corinne Leyval
LIMOS, CNRS UMR 7137, Faculté des Sciences, Lorraine University, Vandoeuvre-lès-Nancy, France

Correspondence: Aurélie Cébron, LIMOS, CNRS UMR 7137, Faculté des Sciences, Lorraine University, BP 70239, 54506 Vandoeuvre-lès-Nancy Cedex, France. Tel.: +33 3 83684296; fax: +33 3 83684284; e-mail: aurelie.cebron@univ-lorraine.fr

Received 20 January 2012; revised 10 May 2012; accepted 10 May 2012. Final version published online 13 June 2012.
DOI: 10.1111/j.1574-6941.2012.01414.x

Editor: Wieetse de Boer

Keywords
fungal community; heavy metals; PAH; polluted soil; rhizosphere effect; thermal desorption.

Abstract
The fungal communities of a multi-contaminated soil polluted by polycyclic aromatic hydrocarbons and heavy metals (NM) were studied within a long-term in situ experiment of natural attenuation assisted by plants. Three treatments were monitored: bare soil (NM-BS), soil planted with alfalfa and inoculated with mycorrhizal fungi (NM-Msm), and soil with spontaneous vegetation (NM-SV). The same soil after thermal desorption (TD) was planted with alfalfa and inoculated with mycorrhizal fungi (TD-Msm). Twice a year for 5 years, the fungal abundance and the community structure were evaluated by real-time PCR and temporal temperature gradient gel electrophoresis targeting 18S rRNA genes. The fungal abundance increased over time and was higher in planted than in bare NM soil and in TD than in NM soil. The Shannon diversity index ($H'$) increased during the first 2 years with the emergence of more than 30 ribotypes, but decreased after 3 years with the selection of a few competitive species, mostly Ascomycetes. $H'$ was higher under complex plant assemblage (NM-SV) than in the NM-BS plots but did not differ between NM and TD soils planted with alfalfa. These results indicated that even in a highly polluted soil, the plant cover was the main driver of the fungal community structure.

Introduction
Due to anthropogenic activities, particularly intensive industrialization, large areas worldwide have been exposed to organic and metallic pollutants during the last two centuries. Polycyclic aromatic hydrocarbons (PAHs), persistent organic compounds resulting from the incomplete combustion of fossil organic matter, are common contaminants of industrial (metallurgy, petrochemical and coke industries) and coal mining wastelands (Wilcke, 2000). In these wasteland soils, PAHs are very often associated with heavy metal (HM) pollution, including Cu, Zn, Pb, As and Ni (Sandrin & Maier, 2003; Amezquita-Allier et al., 2005; Vivas et al., 2008), and can lead to high toxicity and ecotoxicity (Joner et al., 2004; Eom et al., 2007).

Like bacteria, fungi play an essential role in the function of the ecosystem, particularly in the carbon and nitrogen cycles (Wainwright, 1992). They are also involved in PAH biodegradation, the main process by which PAHs are removed from polluted soils. Indeed, a wide range of fungi, including Ascomycetes, Basidiomycetes and Zygomycetes, are known for their ability to degrade PAHs, even very recalcitrant PAHs with high molecular weights, via co-metabolism pathways (Cerniglia, 1997; Peng et al., 2008). Additionally, fungal physiological traits differ from those of bacteria, and it has been shown that the response to environmental conditions (e.g. vegetation, pH, C : N ratio, or water content) could vary between the two kingdoms or even be opposite responses (Williams & Rice, 2007; Houllen et al., 2008; Lauber et al., 2008; Rousk et al., 2010).

However, whereas bacterial diversity in polluted areas has been studied extensively (Chiapusio et al., 2007; Vivas et al., 2008; Cébron et al., 2009), studies focused on the fungal communities in these ecosystems are scarce. Salvo et al. (2005) found a correlation between the abundance of cultivable fungi and the PAH content in harbour sediments. In contrast, the diversity of the cultivable fungi, all identified as imperfect forms of Ascomycetes, decreased with the PAH concentration in an artificially oil-polluted
soil (Obire & Anyanwu, 2009). Van Elsas et al. (2000) focused on the fungal diversity in a soil artificially polluted with crude oil and dibenzothiophene. To our knowledge, however, the global diversity of the fungal communities in the PAH-polluted soils of industrial wastelands has never been studied.

The natural attenuation process consists of monitoring the removal of pollutants from soils by natural processes. Natural attenuation can be assisted by the use of plants to increase the biodegradation rates. Indeed, plants, through the rhizosphere effect, are known to influence the microbial biomass and its activity in the vicinity of their roots (Hiltner, 1904). The extent of the rhizosphere effect on PAH fate seems to depend on many factors, including the plant biomass or species (Parrish et al., 2004; Liste & Prutz, 2006). The symbiosis with arbuscular mycorrhizal (AM) fungi was also shown to positively influence the dissipation of PAHs by the plant rhizosphere (Joner & Leyval, 2003; Verdin et al., 2006). For both fungi and bacteria, this effect can be quantitative and/or qualitative, leading to changes in the community structure and diversity (Marsal Gomes et al., 2003; Broeckling et al., 2008; Curlevski et al., 2010). However, very little is known about the rhizosphere effect on the fungal communities in polluted environments.

The remediation of PAH-polluted soils by biological processes is not always feasible, often because the concentrations of pollutants are too high or because of bioavailability issues. Alternatively, physicochemical treatments can be applied, such as thermal desorption (TD) where the soil is heated to 500°C to eliminate organic pollutants (Biache et al., 2008). While this treatment removes PAH very efficiently, it does not reduce the HM contamination, and it drastically affects the soil characteristics (e.g. soil structure, organic matter and nutrients) (Biache et al., 2008). The impact of such remediative treatment on the soil microbial diversity and function and whether soil characteristics can be restored with time is poorly understood. Cébron et al. (2009) have studied the bacterial communities in the soil from an industrial wasteland with and without treatment by thermal desorption and found that bacterial community structure and diversity evolved differently over time. Even if complex microbial communities recolonized the TD-treated soil, the microbial enzymatic activities remained quite low even 4 years after the TD treatment (Cébron et al., 2011a).

In this study, we investigated the fungal community in a highly PAH- and HM-polluted soil and in the same soil after TD treatment; both soils were monitored within a long-term in situ experiment of natural attenuation assisted by plants (Ouvrard et al., 2011). The 18S rRNA gene copy number, quantified by real-time PCR using previously described fungal-specific primers (Lueders et al., 2004), was chosen as an indicator of the fungal abundance (Smith & Osborn, 2009). The same primers were used to perform temporal temperature gradient gel electrophoresis (TTGE), providing the DNA fingerprints frequently used for studying bacterial and/or fungal global diversity and community structure (Cébron et al., 2009; Ros et al., 2010). We aimed to (1) follow the dynamics of the fungal density and community structure in these soils during 5 years of monitoring, (2) characterize the dominant fungal ribotypes and (3) assess the impact of plant cover and TD treatment on the fungal community.

Materials and methods

In situ experimental device and sampling

The experimental device, installed at the site of an industrial wasteland (Homécourt, Meurthe-et-Moselle, France) within the facilities supported by the GISFI (www.gisfi.fr), was previously described by Cébron et al. (2009, 2011a) and Ouvrard et al. (2011). Briefly, steel tanks (3 × 2 × 0.4 m, length × width × height) equipped with water collection systems were filled with HM- and PAH-polluted soil (NM soil) from a former coking plant site (Neuves-Maisons, Meurthe-et-Moselle, France). At the initiation of the experiment (T0, September 2005), three treatments with four replicates were applied to the NM soil: NM-BS, bare soil where vegetation was manually prevented; NM-SV, soil with spontaneous colonization by endemic plant species; and NM-Msm, where alfalfa (Medicago sativa var. Europe) was sown (40 g seeds per plot) and AM fungi (Glomus mosseae and Glomus intraradices) were inoculated using 250 and 170 g m⁻² of commercial inoculum supplied by the Institut für Pflanzenkultur (Solkau, Germany) as a mixture of propagules in sand and vermiculite substrates, respectively. Four additional plots, TD-Msm, were filled with TD soil: NM soil treated by thermal desorption 6 months before T0, sown with alfalfa and inoculated with AM fungi as described above. Each autumn, the alfalfa shoot biomass was harvested for analyses; thus, less plant litter could accumulate on the NM-Msm and TD-Msm plots than on the NM-SV plots. Every spring and autumn after the initiation of the experiment, samples were taken of soils and plants. Six subsamples per plot were collected with a hand auger and mixed into a single composite sample per plot; the composite sample was sieved at 5 mm. A portion of these soil samples was dried for soil characteristic analyses, and aliquots were stored at −20°C until DNA extraction. We analysed samples at T0, T3 (May 2007), T5 (May 2008), T6 (September 2008), T7 (May 2009), T8 (September 2009), T9 (May 2010) and T10 (September 2010).
Soil characteristics and plant parameters

Soil characteristics, as previously described in Cébron et al. (2009, 2011a) and Ouvrard et al. (2011), are shown in Table 1 for T0 and T10 sampling dates. The PAH concentration was much lower after the TD treatment, but the HM levels were still very high. The alfalfa shoot biomass (approximately 1700 g dry wt plot⁻¹) was identical during the first 2 years (2006–2007) of the experiment. However, during the last 3 years (2008–2010) it was significantly higher in the TD-Msm plots than in the NM-Msm plots, with 2000 and 400 g dry weight plot⁻¹ at T10 in the TD-Msm plots and NM-Msm plots, respectively (Ouvrard et al., 2011). Moreover, the abundance of mycorrhizal roots, estimated by microscopically examining the trypan-stained root pieces (Koske & Gemma, 1989), was higher in the TD-Msm plots than in the NM-Msm plots (means of 90% and 20%, respectively, during the 2009–2010 period). In the NM-SV plots, the plant diversity increased during the first 2 years with the gradual colonization of the NM soil (Dazy et al. 2008); thereafter, the plant communities exhibited a loss of species richness and diversity, explained by the establishment of highly competitive invasive plants: goldenrod (Solidago canadensis) and cinquefoil (Potentilla reptans) (Ouvrard et al., 2011).

DNA extraction

DNA was extracted from 0.5 g soil using a bead beating-based method as previously described (Cébron et al., 2011b) and eluted in a 50 μL final volume. To prevent coprecipitation of DNA and CaSO₄, formed from the high concentration of SO₄ in both soils (49 and

Table 1. Characteristics of NM and TD soils at T0 and T10 (after 5 years)

<table>
<thead>
<tr>
<th>Agronomical parameters</th>
<th>NM soil</th>
<th>TD soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T0</td>
<td>T10</td>
</tr>
<tr>
<td>N.lo (g kg⁻¹)</td>
<td>2.7 ± 0.1</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>C : N</td>
<td>23.0 ± 1.3</td>
<td>26.7 ± 1.3</td>
</tr>
<tr>
<td>TOC (g kg⁻¹)</td>
<td>62.0 ± 3.6</td>
<td>70.3 ± 5.9</td>
</tr>
<tr>
<td>pH</td>
<td>7.0 ± 0.2</td>
<td>7.6 ± 0.1</td>
</tr>
<tr>
<td>S (g kg⁻¹)</td>
<td>nd</td>
<td>46.3 ± 2.0</td>
</tr>
<tr>
<td>Ca (g kg⁻¹)</td>
<td>41.7 ± 3.4</td>
<td>42.5 ± 2.2</td>
</tr>
<tr>
<td>Mg (g kg⁻¹)</td>
<td>1.01 ± 0.08</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>HM (mg kg⁻¹)</td>
<td>Cadmium (Cd)</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Chromium (Cr)</td>
<td>599 ± 64</td>
</tr>
<tr>
<td></td>
<td>Copper (Cu)</td>
<td>130.8 ± 8.2</td>
</tr>
<tr>
<td></td>
<td>Molybden (Mo)</td>
<td>11.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Nickel (Ni)</td>
<td>97.3 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>Lead (Pb)</td>
<td>481.9 ± 57.6</td>
</tr>
<tr>
<td></td>
<td>Zinc (Zn)</td>
<td>2085.2 ± 229</td>
</tr>
<tr>
<td>PAH (mg kg⁻¹)</td>
<td>Naphthalene</td>
<td>48.7 ± 23.6</td>
</tr>
<tr>
<td></td>
<td>Acenaphthylene</td>
<td>54.3 ± 5.9</td>
</tr>
<tr>
<td></td>
<td>Acenaphthene</td>
<td>61.0 ± 11.0</td>
</tr>
<tr>
<td></td>
<td>Fluorene</td>
<td>52.6 ± 10.9</td>
</tr>
<tr>
<td></td>
<td>Phenanthrene</td>
<td>152.9 ± 25.8</td>
</tr>
<tr>
<td></td>
<td>Anthracene</td>
<td>150.7 ± 29.3</td>
</tr>
<tr>
<td></td>
<td>Fluoranthene</td>
<td>273.9 ± 37.6</td>
</tr>
<tr>
<td></td>
<td>Pyrene</td>
<td>200.1 ± 28.7</td>
</tr>
<tr>
<td></td>
<td>Benzo[a]anthracene</td>
<td>149.1 ± 20.3</td>
</tr>
<tr>
<td></td>
<td>Chrysene</td>
<td>117.9 ± 16.2</td>
</tr>
<tr>
<td></td>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>133.5 ± 15.5</td>
</tr>
<tr>
<td></td>
<td>Benzo[b]fluoranthene</td>
<td>185.8 ± 21.8</td>
</tr>
<tr>
<td></td>
<td>Benzo[k]fluoranthene</td>
<td>69.6 ± 13.4</td>
</tr>
<tr>
<td></td>
<td>Benzo[a]pyrene</td>
<td>158.0 ± 20.4</td>
</tr>
<tr>
<td></td>
<td>Dibenzo[a,h]anthracene</td>
<td>26.6 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>Benzo[g,h,i]perylene</td>
<td>89.6 ± 10.2</td>
</tr>
<tr>
<td></td>
<td>16 PAH US-EPA</td>
<td>1924 ± 258</td>
</tr>
<tr>
<td>nd, not determined.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DNA recovered in the four extractions was similar (i.e. from the same soil sample (NM-SV). The amount of DNA was determined by thermal temporal gradient gel electrophoresis (TTGE) of the 18S rRNA genes. The PCR amplification was performed using the primers Fung5F/FF390R described above, with a GC clamp (5′-CGCCTGCGCCGC GCCCGCGCCGTCCGGCCGCCCCCGCCGC-3′) added to the reverse primer. The PCR were performed in a 50-μL final volume that included 1× PCR Buffer (Invitrogen), 1.25 U recombinant Taq Polymerase (Invitrogen), 1.5 mM MgCl₂, 200 μM of each primer (Eurofins-MWG Biotech), 200 μM each dNTP and 1 μL DNA extract (i.e. 0.02 ng DNA μL⁻¹). The PCR temperature profile comprised of a 7-min initial denaturation step at 94 °C; followed by 40 cycles of 94 °C for 30 s, 50 °C for 45 s and 72 °C for 40 s; and a final step at 72 °C for 10 min. The PCR efficiency and specificity were assessed by agarose (1%) gel electrophoresis. To set the optimal conditions for TTGE, the melting temperatures of the selected Fung5F/FF390R sequences [Fusarium merismoides (AF141950), Sordariomyces sp. (AF292054), Aspergillus nidulans (U77377), Talaromyces flavus (M83262), Phaeotrichum benjamin (AY016348), Saccharomyces cerevisiae (Z75578), Cortinarius iodes (AF026633) and Glomus mosseae (U96139)] were predicted using MACMELT™ software. The temporal gradient and the concentration of urea in the TTGE gel were experimentally adjusted to optimize the electrophoretic separation of the ribotypes. The TTGE gels were composed of 6% w/v acrylamide/bisacrylamide (Sigma™), 5.5 M urea (Euromedex), 2% w/v glycerol and 1.25× TAE (50 mM Tris-acetate, 25 mM acetic acid and 1.25 mM EDTA, pH 8.3) in a final volume of 30 mL. The separation was performed in 1.25× TAE at 145 V with a 1.5 °C h⁻¹ temperature ramp from 51.5 to 60.5 °C. The TTGE gels were stained with SybrGold as described previously (Cébron et al., 2009, 2011a). The TTGE gel images were analysed using QUANTITY ONE 4.0.1 Software (BioRad) to detect the bands and calculate their relative abundance. The equivalence of the bands between the gels was further checked by comparing the DNA sequences within the excised bands (described
below). A matrix of the relative abundance of each ribotype in the samples was then generated. Although the total fungal diversity could not be assessed using TTGE method, a Shannon diversity index ($H'$) was calculated to compare the treatments:

$$H' = - \sum p_i \ln p_i$$

where $p_i$ is the relative abundance of ribotype $i$, and ln is the Napierian logarithm (Hill et al., 2003).

**Phylogenetic analysis**

On each TTGE gel, the differential bands, i.e. the most intense bands and/or the bands that resulted in different patterns among the profiles, were excised and dissolved overnight in 35 μL Tris HCl buffer (10 mM, pH 8.0) at 4°C. The DNA obtained was then amplified with Fung5F/FF390R-GC and run again on TTGE gels to ensure that a single ribotype was present. The individual bands on the second gels were excised, and the DNA amplified using primers without the GC clamp; the PCR products were purified with the High Pure PCR product purification Kit (Roche Applied Science). The purified products were purified with the CLUSTALW tool in BIOEDIT. The phylogenetic tree was constructed using the DNA-DIST, NEIGHBOR, SEQBOOT and CONSENSE tools of PHYLIP 3.65 Software. The sequences of ribotypes 1–16 were deposited in GenBank under the accession numbers JQ423134–JQ423149.

**Statistical analysis**

All the statistical analyses were performed using XL-STAT 2008 Software (Addinsoft) with $\alpha \leq 0.05$ considering the results from the four replicate plots. Two-way analyses of variance (ANOVA) followed by the Newman–Keuls multiple comparison tests were used to evaluate the effects of time, the treatment and their interaction on the dependent factors, i.e. fungal abundance, fungal diversity and the relative abundance of the ribotypes. All ANOVAs were performed separately for the NM soil (treatments NM-BS, NM-SV and NM-Msm), to assess the effect of plant cover, and for the NM-Msm and TD-Msm soils, to assess the effect of the thermal desorption treatment. The principal component analyses (PCAs) were performed using a Pearson correlation matrix of the relative abundance of each ribotypes, independently for each sampling date and considering all treatments together.

**Results**

**The dynamics of fungal abundance**

Fungal abundance was estimated by the real-time PCR quantification of the 18S rRNA genes in the four studied treatments and over time (Fig. 1). Fungal abundance was not significantly different between the two soils (NM-Msm and TD-Msm plots) at the initiation of the experiment (T0). Furthermore, at T0, the three treatments...
The fungal community structure

The fungal community structure was studied using the TTGE fingerprinting method (Fig. 2). Within all the sampling dates and treatments, 45 ribotypes could be distinguished. The TTGE gel pictures and the corresponding PCAs are shown for the sampling times T5 and T6, when the number of bands was the highest, and for T8, when the community had stabilized, i.e. when the structure of the community and the dominant bands was more stable (Fig. 2). The data for the other sampling dates are available in the Supporting Information.

At T0, no TTGE profile could be obtained for the TD-Msm soil because too low a quantity of PCR products was generated with the GC-clamp primers. For all the treatments of the NM soil, the TTGE profiles at T0 were similar and dominated by only two distinct ribotypes (Supporting Information, Fig. S1). The number of ribotypes increased at T3 (1½ years after T0) to an average 7.7 ribotypes per plot (all NM and TD treatments taken together; Fig. S1). At this point, the profiles of the three treatments (BS, SV, Msm) of the NM soil were similar, but the profiles of the TD and NM soils planted with alfalfa and inoculated with AM fungi (Msm) differed. At T5 and T6, the community structure became more complex with the detection of many new ribotypes, and different patterns between the two sampling dates. At T5, the four replicate plots of each treatment showed very heterogeneous profiles (Fig. 2a). Indeed, the heterogeneity of the replicates prevented the discrimination by the PCA between the treatments. In contrast, at T6, the profiles of the treatment replicates became more homogeneous (Fig. 2b), allowing the treatments to be discriminated by the PCA. The bare soil (NM-BS) profile was separated from the planted soils (NM-SV and NM-Msm) on the first axis, explaining 20% of the variation, and the TD and NM soil profiles were clearly separated on the second axis, explaining 14% of the variation. At T8 (Fig. 2c), the structure of the community seemed to stabilize, with the strong dominance of a few ribotypes. In the PCA, the first axis explained 39% of the variation and allowed the discrimination of the type of vegetation, with the NM-Msm sample profiles closer to the TD-Msm sample profiles than to the NM-SV sample profiles; the second axis (12% variation) allowed the discrimination of the bare soil plots (NM-BS) from the plots with vegetation (NM-SV, NM-Msm and TD-Msm). Moreover, this discrimination by the PCA between the bare soil plots and the...
planted plots, and more specifically, between the NM-BS and the NM-SV plots, was possible from T6 to T10 (Fig. S1).

Although TTGE profiles do not allow total fungal diversity characterization, a Shannon diversity index ($H'$) was calculated for each plot and each treatment at every sampling date based on the matrix of the relative abundance of each ribotype, to compare the treatments (Fig. 3). The Shannon diversity index significantly increased ($P < 0.0001$) during the first 3 years; at T6, the index of the NM-BS, NM-SV, NM-Msm and TD-Msm plots reached 1.75, 2.57, 2.27 and 2.43, respectively. This increase was followed by a decline during the last 2 years, with the Shannon diversity index at T10 reaching an average value of 1.67 (no significant difference between the treatments at this time), which was equivalent to the T3 value. In the NM soil, the Shannon diversity index was significantly higher in the plots with spontaneous vegetation compared with the bare soil plots ($P = 0.036$). In contrast, the Shannon diversity index did not differ significantly between the plots planted with alfalfa and inoculated with AM fungi (NM-Msm) and the bare soil plots (NM-BS). The analysis of the effect of interaction between the time elapsed and the treatment ($P = 0.017$) revealed that there was no significant temporal variation of the Shannon index in bare soil.

Identification of dominant fungal ribotypes

The dominant fungal ribotypes were identified by the sequencing of TTGE bands (Table 2). The sequences were used to construct a phylogenetic tree (Fig. 4). In the NM and TD soils, the most dominant ribotypes belonged to the Ascomycota and, more specifically, the Pezizomycotina, Dothideomycetes, Eurotiomycetes, Leotiomycetes and Sordariomycetes. Only three ribotypes could be identified
as Basidiomycota, among which ribotype 4 (the closest match to Clitopilus prunulus) was the dominant one. Moreover, neither Zygomycota nor Glomeromycota sequences could be identified. The relative abundance of the ribotypes varied over time and with plant cover and soil type (Fig. 2). For instance, ribotype 1 (the closest match to Geomyces sp.) and ribotype 3 (the closest match to Penicillium purpureogenum) were dominant at T0, before other ribotypes could be detected, such as ribotype 2 (the closest match to Phoma sp.) or ribotype 6 (the closest match to Meliniomyces variabilis), which were detected at T3. The representation of ribotype 2 continued to increase throughout the first 4 years of the experiment and then significantly declined in both soils ($P = 0.001$). Ribotype 9 (the closest match to Cylphella phora laciniata) was represented to a much higher degree at T5 than at the other sampling dates ($P \leq 0.006$). Vegetation also significantly affected several ribotypes. The dominant ribotype 1 was more abundant in the NM-BS plots than in the NM-Msm ($P = 0.008$) and NM-SV ($P = 0.058$) plots. Some ribotypes were represented more in the NM-SV plots than in the NM-Msm plots, such as ribotypes 2 ($P = 0.061$), 3 ($P = 0.058$), 15 (the closest match to Cyathus striatus, $P = 0.044$) and 13 (the closest match to Fusarium solani, $P = 0.021$). In contrast, some ribotypes, such as ribotypes 10 (the closest match to Thelebolus stercorarius) and 11 (the closest match to Neusporospora crassa), were equally abundant in both the bare and planted soils. Furthermore, some ribotypes were clearly visible in the TD soil but not in the NM soil, i.e. ribotypes 4 ($P = 0.008$), 13 ($P = 0.001$) and 8 (the closest match to Chaetomium elatum, $P = 0.065$), whereas others were better represented in the NM soil, such as ribotypes 1 ($P < 0.0001$) and 6 ($P = 0.004$). In contrast, the relative abundance of ribotypes 2 and 3 was not affected by the soil type.

**Discussion**

**Influence of plant cover on the fungal communities in multi-contaminated soil**

Our results indicated that vegetation strongly influenced the fungal communities in the highly polluted NM soil. First, the fungal abundance, as determined by real-time PCR targeting fungal 18S rRNA genes, was significantly higher (approximately four-fold during the period from T3 to T10) in the planted soil than in the bare soil, which illustrates the quantitative rhizosphere effect. In agreement with our results, Hannula *et al.* (2010) showed that the fungal biomass, evaluated by total ergosterol measurement, was higher in a potato rhizosphere than in the bulk soil. In contrast, Chen *et al.* (2008) did not observe any difference in fungal biomass, as estimated by phospholipid fatty acid analysis, between soils planted with various legumes and grasses and unplanted soil. At the moment, it is not clear to what extent these differences could be caused by the use of different methods (Joergensen & Wichern, 2008). Moreover, the vegetation significantly affected the fungal community structure, as demonstrated by the TTGE profile analyses. Community fingerprinting techniques such as TTGE do not reveal information on total fungal diversity but rather on the diversity and composition of a well represented subset of fungal ribotypes. In addition, creation of a matrix with band intensity data from TTGE profiles may lead to an underestimation of the Shannon diversity indexes. Despite, these shortcomings, trends in diversity changes revealed by TTGE do indicate responses of the community to changes in environmental conditions (Cébron *et al.*, 2009). Here, we assumed that the biases were similar for all sampling dates and treatments, allowing comparison of the data. From T6 to T10, the PCAs discriminated between the bare and planted soil, illustrating the qualitative rhizosphere effect observed on fungi and bacteria (Marcial Gomes *et al.*, 2003; Phillips *et al.*, 2006; Chen *et al.*, 2008). The dominant ribotypes were identified by the comparison of their sequences with those of referenced species; it should nevertheless be noted that the physiological properties of these ribo-
types may differ from those of the referenced species. Ribotype 1, the closest match to *Geomyces* sp., was the most dominant species in all the NM treatments, and particularly in bare soil, where its relative abundance was higher than that in the planted soil. This genus, commonly found in forest and agricultural soils (De Bellis et al., 2007; Baldrian et al., 2011), was previously detected in extreme environments, notably the polar areas of the Arctic and Antarctica (Kirtsideli, 2007; Tosi et al., 2010) and within a HM-polluted forest soil (Nordgren et al., 1985). These data could suggest that the genus *Geomyces* is greatly adapted to environmental stresses. Moreover, the difference of the fungal communities was more apparent in the PCA when comparing the NM-SV and NM-BS plots, indicating that diversified vegetation more strongly impacts the fungal community structure than does a monoculture of alfalfa. This result was confirmed by the higher Shannon diversity index in the NM-SV plots than in the NM-BS plots. Indeed, several of the ribotypes identified in these more diversified plots, such as ribotypes 2, 3 13 and 15 with the closest match to *Phoma* sp., *P. purpurogenum*, *F. solani* and *C. striatus*, respectively, were better represented in the spontaneously vegetated soil than in the soil planted with alfalfa. Some of these fungal genera were previously detected in soils contaminated with organic and/or metallic pollutants (Verdin et al., 2004; Naranjo Briceno et al., 2007; Hong et al., 2010; Diriginicute-Volodkieni & Peciulyte, 2011) and in rhizospheres (Hamayun et al., 2009; Curlevski et al., 2010; Manici & Caputo, 2010). A more diverse plant community could increase the range of carbon sources for the heterotrophic fungi and thus increase the fungal diversity, because the existence of various niches is a key factor for diversity (Levine & HilleRisLambers, 2009). Cébron et al. (2009) monitored the bacterial diversity in the same experimental device for 2 years and also found that the structure of the bacterial community of NM-SV plots was more different from the community of the NM-BS plots than from the NM-Msm one. It is surprising that no Zygomycete was found. Moreover, no ribotype could be identified as Glomeromycota, even in the vegetated plots where plants were colonized by mycorrhizal fungi. This result could be explained by the fact that we did not identify all the TTGE bands, especially the faintest bands, or because specific primers for the AM fungi should be used instead (Sonjak et al., 2009).
The influence of the thermal desorption treatment

Our results highlighted differences in the fungal abundance and community structure between the NM and TD soils. Whereas no significant difference was found at T0, the fungal abundance, i.e. the 18S rRNA gene copy number g⁻¹ soil, was 1.7-fold higher in the TD soil than in the NM soil in the period from T3 to T10. The thermal desorption treatment not only drastically depleted PAH from this wasteland soil but also modified the physico-chemical characteristics, e.g. the porosity, the N content (and thus the C : N ratio), and the Ca, Mg and S contents (Table 1; Ouvrard et al., 2011), and did not lower the HM concentrations, which remained very high. Biache et al. (2008) showed that thermal desorption did not mobilize the metal elements such as Zn and Fe from the most available compartments. However, the effect on the fungal abundance cannot only be attributed to the removal of the PAH. The alfalfa biomass was significantly higher in the TD-Msm plots than in the NM-Msm plots; the better alfalfa growth in TD-Msm treatment may have increased the input of available carbon substrate into the rhizosphere, promoting fungal growth. Alternatively, the PCAs, with the exception of those at T3 and T6, did not discriminate between the TD-Msm and NM-Msm plots, suggesting that the fungal community structure was driven primarily by the vegetal cover and not by the soil characteristics in the present experiment. It is interesting to note that the bacterial community differed strongly between the TD and NM soils during the first 2 years of the experiment, despite an equivalent alfalfa biomass (Cébron et al., 2009). Nevertheless, the relative abundance of some fungal ribotypes was affected by the soil type. Ribotype 4, the closest match to the Agaricomycete C. prunulus, was favoured in the TD soil, where it was very often among the three dominant ribotypes. To our knowledge, this fungus has not yet been reported in a polluted environment. At first glance, this result contradicts with that of Lauber et al. (2008), who found that the relative abundance of Agaricomycetes negatively correlated with the C : N ratio. However, the organic matter in the TD soil is more condensed than that in the NM soil (Biache et al., 2008), which decreases its lability and bioavailability and increases its recalcitrance to biodegradation, in concordance with the lower respiration rate and enzymatic activities (e.g. arylamidase, lipase or phosphatase) reported in the same soil (Cébron et al., 2011a). Thus, the biodegradable carbon sources in this soil could mainly be derived from its plants. In fact, only three ribotypes found in both the NM and TD soils were related to Basidiomycetes, others belonged to the Ascomycetes, of the Dothideomycyte, Eurotiomycyte, Leotiomyocyte and Sordariomycete classes. This was quite surprising, considering the high potential of Basidiomycetes for PAH degradation (Gramss et al., 1999; D’Annibale et al., 2005). It is interesting to note that in the few previous studies of fungi in polluted environments, Ascomycetes of the same classes, and belonging to Penicillium, Phoma, Aspergillus, Fusarium, Cladosporium and Trichoderma genera, were also the dominant ribotypes (Van Elsas et al., 2000; Salvo et al., 2005; Naranjo Bricen˜o et al., 2007; Obire & Anyanwu, 2009). Such results may suggest that Basidiomycetes are less competitive than Ascomycetes in polluted environments. The relative abundance of ribotypes 8 and 13 (the closest match to the imperfect fungi C. elatum, known as a cellulytic brown-rot fungus (Violi et al., 2007), and F. solani, respectively) were also higher in the TD soil than in the NM soil. In contrast, ribotype 1 (the closest match to Geomyces sp.) was significantly favoured in the NM soil, indicating that it is highly competitive in this extremely contaminated soil relatively to other taxa.

Conclusions

This study permitted the structure of the fungal community in a polluted soil and the re-colonization of a TD-treated soil to be monitored for 5 years. At the beginning of the experiment (T0), only two fungal taxa were detected by TTGE, a ‘geometric’ distribution typical of disturbed communities, i.e. a very small number of dominant species (Zak, 1992). The fungal species that emerged during the colonization phase, from T0 to T6, could have been (1) indigenous fungi previously present but very rare and not detectable by the TTGE technique and/or (2) exogenous fungi freshly introduced from the surrounding area (Malloch & Blackwell, 1992). At T6, the fungal community comprised many species with a few dominant ones, corresponding to a ‘log-normal’ distribution (Zak, 1992). From T6 to T10, few taxa were selected as a function of soil characteristics and mostly of the vegetal cover (no vegetation, a single plant species or a diverse vegetal community), which was the main driver of the fungal community dynamics even in this highly contaminated environment. At T10, the communities seemed to have stabilized, displaying the intermediate ‘log-series’ distribution that included a small number of dominant species adapted to the high levels of organic and metallic pollution, mostly Ascomycetes, and a relatively large number of rarer species (Zak, 1992). This type of species distribution has been previously observed in the bacterial and fungal communities of polluted areas (Van Elsas et al., 2000; Andreoni et al., 2004; Vivas et al., 2008). The positive effect of plants, especially in multispecies assemblage, on the fungal abundance and diversity could provide a significant advantage to natural attenua-
Dynamics of the fungal communities in polluted soils

References


Thion C, Beguiristain T, Ceborn A & Leyval C (2012) PAH biotransformation and sorption by Fusarium solani and


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Structure of the fungal community for the four treatments.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.