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Environmental metabarcoding reveals contrasting belowground and aboveground fungal communities from poplar at a Hg phytomanagement site

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Abstract

Characterization of microbial communities in stressful conditions at a field level is rather scarce, especially when considering fungal communities from aboveground habitats. We aimed at characterizing fungal communities from different poplar habitats at a Hg-contaminated phytomanagement site by using Illumina-based sequencing, network analysis approach and direct isolation of Hg resistant fungal strains. The highest diversity estimated by the Shannon index was found for soil communities, which was negatively affected by soil Hg concentration. Among the significant correlations between soil OTUs in the co-occurrence network, 80 % were negatively correlated revealing dominance of a pattern of mutual exclusion. The fungal communities associated with *Populus* roots mostly consisted of OTUs from the symbiotic guild, such as members of the *Thelephoraceae*, thus explaining the lowest diversity found for root communities. Additionally, root communities showed the highest network connectivity index, while rarely detected OTUs from the *Glomeromycetes* may have a central role in the root network. Unexpectedly high richness and diversity were found for aboveground habitats, compared to the root habitat. The aboveground habitats were dominated by yeasts from the *Lalaria*, *Davidiella* and *Bensingtonia* genera, not detected in belowground habitats. Leaf and stem habitats were characterized by few dominant OTUs such as those from the *Dothideomycete* class producing mutual exclusion with other OTUs. *Aureobasidium pullulans*, one of the dominating OTUs, was further isolated from the leaf habitat, in addition to *Nakazawaea populi* species, which were found to be Hg resistant. Altogether, these findings will provide an improved point of reference for microbial research on inoculation-based programs of tailings dumps.

Keywords : Hg-enriched tailings dump, Hg resistance, internal transcribed spacer metabarcoding, poplar microbiome, Illumina MiSeq, network.

Introduction

Plants in natural and agricultural settings are colonized by a wide range of microbes on both their outer and inner surfaces [1]. Nevertheless, the scientific understanding of the microbial communities of woody species is quite limited, especially for microbes associated with aboveground tissues. Many tree tissues may indeed represent distinct microbial habitats, such as the rhizosphere, the root and leaf endospheres, the episphere of the phyllosphere and fruits, flowers, leaves, buds, stems, branches or even the trunk [2, 3]. Moreover, some of the microorganisms that colonize these habitats establish strong links with their host, whose disruption may result in loss of host fitness [4]. For instance, a certain groups of mycorrhizal fungi that form symbiotic association with the root system, are of a importance for tree nutrition and health, as they play key roles in the carbon and nitrogen cycles [5]. In the phyllospheric habitat, archaea, bacteria, filamentous fungi and yeasts have been identified , although less work has been done on the two latter groups of microorganisms [6]. Studies are increasingly indicating that fungi influence the fitness of their host plants, either negatively by acting as pathogens [7], or positively by increasing the stress tolerance of the plant [8], shaping insect herbivory [9] or reducing the infection of plant tissues by pathogens [10].

Trees, as perennials, are made of a variety of habitats that may host contrasted microbial communities, which composition and diversity may vary with season, age, species, climatic conditions. Therefore, they are relevant models for studying the structure and composition of microbial communities, but until recently, our knowledge has been limited due to the difficulty of adequately describing microbial communities with classical culture-dependent methods. The recent development of massively parallel 454 pyrosequencing [11, 12] and ion torrent sequencing [13], combined with DNA multiplexing, provides an opportunity to explore parts of the microbiome that are otherwise unreachable through culture-dependent approaches [14]. Microbiomes associated with belowground and aboveground tree habitats have been screened

at a large spatial scale using these technologies in some cases, and several studies revealed the potential of the host tree genotype and environmental conditions to induce the establishment of specific microbial communities [11, 15–17]. More recently, new high-throughput technologies, such as the Illumina sequencing platform, have become available, showing a far greater sequencing capacity, producing millions of sequences, which leads to much greater depth of coverage of microbial [18]. It is now possible to examine the full extent of the richness and diversity exhibited by microorganisms in different habitats. However, the quantity of data reaches a critical point at which previous approaches are insufficient to decipher the structure of complex microbial communities. Network analysis of significant taxon co-occurrence patterns may help to decipher the structure of complex microbial communities among various habitats. Software such as CoNet has been developed and optimized specifically to detect significant non-random patterns of co-occurrence (co-presence and mutual exclusion) using a Reboot method to determine the significance of each associations in the network [19]. Network analysis requires lot of samples replication to obtained strong statistical analysis leading to trustable correlations. This computation method combined with adapted experimental design and with the tremendous amount of metadata obtained by high throughput sequencing technologies gives us the opportunity to explore communities with a new global tool to revealed OTUs importance in the community.

Within the growing environmental pollution paradigm, poplar is a keystone tree used as feedstock for biofuel production and as a biological tool for phytoremediation and revegetation [20]. The term phytoremediation refers to the use of plants and associated microorganisms to eliminate, attenuate or restrain environmental damage or threats posed by a contaminant. Hg is a contaminant classified within the quantitatively most important pollutant groups known as trace elements (TEs). It is highly persistent in the soil environment and is classified as a “priority hazardous substance” by the Agency for Toxic Substances and Disease Registry

(ATSDR) due to its toxicity, mobility, and long residence time in the atmosphere *(Available online http://www.atsdr.cdc.gov/SPL/index.html).* In a previous paper, we focused on characterizing the microbial communities that had naturally recolonized the sediment of a chlor-alkali tailings dump after sediment deposition had ceased. We further demonstrated that most of the Hg detected in the aboveground parts of *Salicaceae* trees collected at that site had entered the poplar leaves through exclusively through an atmospheric pathway [21]. However, the role of aboveground and belowground microbial populations in Hg-contaminated environments remains unknown, and there is a need for a holistic ecosystem-level understanding of microbial communities associated with poplar [22].

In this study, we collected soil and tree samples from the belowground and aboveground habitats of poplars grown as a short-rotation coppice (SRC) plantation at a Hg-contaminated site, and performed isolation of fungal strains. We combined fungal community analyses using Illumina-based sequencing with network analysis to investigate the composition and assembly of fungal communities in these samples. We expected that we would observe clear differences in the relative abundance and composition of fungal groups across poplar habitats that may improve our understanding of the microbial ecology of these environments. Providing key information on the fungal communities of belowground and aboveground will hopefully be of use for practitioners of bioremediation approaches who often lack of important information such as the effect of the pollutants (in this case heavy Hg) on the microbial communities that surround the flora and fauna. This knowledge may benefit efforts to mitigate the environmental impact of tailing management facilities.

Materials and Methods

Site description and sampling design

The location and history of the site have been fully described elsewhere [13, 23] briefly, the site investigated in the current study was exploited as a sediment storage area from the 1950s to 2003. The sediments originated from effluents produced during electrolytic processes associated with a Hg cell chlor-alkali process. A poplar monoclonal plantation of the cultivar Skado (*P. trichocarpa* x *P. maximowiczii*) was implemented in 2011 as a short-rotation coppice (SRC, 2200 stems/ha). The experimental design thus guaranteed minimum host variation to focus on interactions between microorganisms in various tree habitats. Sampling was carried out in summer 2014, consisting of collecting soil, root, stem and leaf samples from six random trees, selected in three replicated plots (2 trees per plot). Soil samples composed of bulk soils from under the canopy of the trees were sieved to <4 mm. After the removal of litter, the roots were collected from the upper 20 cm layer of soil from under the canopy of the trees. They were separated from the soil via 2 ultra-pure water baths, and the smallest roots were selected and separated from larger roots by cutting them with a scalpel. Branch samples were collected from poplar branches of axe 2 (0.8 to 1.2 cm diameter) at an ca. 5 m height, corresponding to the half-crown of the poplar. Leaf samples were composed of 3 leaves collected from the above branches. All samples were obtained over a one-day period to reduce any heterogeneity imparted by climatic conditions. The samples were either freeze-dried and stored at -20°C for molecular analysis or dried at ambient temperature (24°C ± 1) for physico-chemical analyses. Thus, we considered the belowground and aboveground habitats to include both endophytic and epiphytic fungi.

Molecular methods

Within 2 weeks after sampling, the stored samples were freeze-dried (RP2V, Group S.G.D. France) and ground into a homogenous powder in a Mixer Mill for 3 min at 30 Hz (model MM400; Retsch Inc., Newtown, Pennsylvania, USA). Environmental DNAs were then extracted using a modified hexadecyltrimethylammonium bromide (CTAB)

chloroform/isoamyl alcohol protocol [24, 25] for root, stem and leaf samples, while environmental DNA from the soil samples was extracted with the PowerSoil DNA isolation Kit following the manufacturer's instructions (MoBio Laboratories, Inc., Carlsbad, CA USA). A purification step was added to all samples using the Power Clean® Pro DNA Clean-Up kit (MoBio Laboratories, Inc., Carlsbad, CA USA) to improve the quality of the isolated DNA. DNA quality and quantity were assessed via agarose gel electrophoresis and with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) using an FLX-Xenius spectrofluorometer (SAFAS, Monaco). Equimolar DNA pools were produced and adjusted to 10 ng/µl. Sequencing of the fungal ITS1 region [17, 26] was performed with the Illumina MiSeq platform (Microsynth AG, Switzerland). PCR amplification of the partial ITS gene was performed using the fungi-specific primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) [27]. These primers target a short portion of the fungal ITS region, resulting in an amplicon of small size (~ 300 bp) appropriate for Illumina sequencing.

Bioinformatics and statistical analysis of diversity

Sequence de-multiplexing and bioinformatics processing of the datasets were performed using the PIPITS pipeline [28]. PIPITS is an automated bioinformatics pipeline dedicated for fungal ITS sequences which incorporates ITSx to extract subregions of ITS and exploits the latest RDP Classifier to classify sequences against the curated UNITE fungal data set. Briefly, all raw read pairs were joined at the overlapping region and then quality filtered, chimera filtered, singleton filtered, contaminant filtered, merged and clustered into operational taxonomic units (OTUs), defined at 97% sequence similarity. We excluded singleton OTUs to avoid technical artifacts and overestimation of the number of species [29, 30]. The taxonomic assignment of OTUs was performed using the UNITE [31] database at a 97% similarity

threshold. The samples were rarefied to 26 671 sequences. The Shapiro test and the Bartlett test were employed to check the normality and homoscedasticity of the data, respectively. Our data were systematically verified for non-normality and homoscedasticity, and the effects of the compartment on the alpha diversity estimates for the fungal assemblage were examined using the Kruskal-Wallis test. A number of alpha diversity indices (OTU richness, Chao estimation, Shannon diversity index, inverse of the Simpson diversity index, measure of evenness based on the Shannon index and coverage) were calculated using MOTHUR [32]. The coverage calculator returns Good's coverage for an OTU definition. Coverage was calculated as: $C=[1-(n/N)]*100$ (%), where n is the number of OTUs, and N is the number of sequences.

A 2-dimensional non-metric multi-dimensional scaling (NMDS) was calculated using the Bray–Curtis method (k=3) on the basis of standardized (Wisconsin double) and square root transformation of OTU abundance using the “metaMDS” function in the Vegan package in R. We PERformed a single Multivariate ANalysis Of the VAriance (PERMANOVA), run with 1,000 permutations, using the “anosim” function in the Vegan package in R and employed ANalysis Of SIMilarities (ANOSIM) to obtain P-values (i.e., significance levels) and the R value (i.e., the strength of the factors on the samples). These results were paired with a heatmap of Spearman's correlations between the relative abundances created with “heatmap.2” from the gplots package. The numbers of OTUs that were shared between habitats were visualized using Venn diagrams implemented in Mothur with the function “venn”. We considered an OTU to be present in a compartment if that OTU was present in at least 25% of the samples from the habitats. Correlations between the diversity parameters and the measured Hg parameters were calculated based on Spearman’s product moment correlation coefficient (R^2). Riverplots were created with the “riverplot” function in the riverplot R package. Rarefaction curves were generated with the “rarecurve” function of the Vegan package in R. The bioinformatic analysis

was conducted using a computer with the following specifications: Ubuntu, Intel®Core™i7-4790 CPU @ 3.60GHz x8, 16GB RAM.

Network analysis

To construct a network and simplify visualization and interpretation, a separate OTU abundance table was derived using the aforementioned pipeline, but with a different OTU clustering threshold (90%) [33]. Following Weiss and collaborators [34] extremely rare OTUs were filtered out; i.e., for each habitat, all OTUs appearing in less than 25% of samples were discarded, and all OTUs showing a relative abundance of < 0.01% of the total sequences were also discarded. Network construction was performed with the plugin CoNet (v. 1.1.b) [35] in Cytoscape software (v. 3.3.0) [36] following the protocol described by Faust and collaborators [37]. Briefly, for each of the four similarity measures (Bray–Curtis and Kullback–Leibler dissimilarity, Pearson and Spearman correlation), the distribution of all pair-wise scores was computed. Given these distributions, initial thresholds were selected such that each measure contributed 1,000 positive and 1,000 negative edges to the initial network. For each measure and each edge, 1,000 renormalized permutation and bootstrap scores were computed, followed by the measure-specific P-value. Any edges exhibiting scores outside the 95% confidence interval defined by the bootstrap distribution or that were not supported by all measures were discarded as well [34]. The networks were explored and visualized with Cytoscape. Based on the results of Berry and Widder (2014)[38], we have chosen to use the degree as a keystone proxy.

Hg analysis in the substrate and biomass

Hg was measured in the soil and poplar samples with an AMA-254 cold vapor atomic absorption (CV-AAS) Hg analyzer (Altec Co., Czech Republic), using the standard conditions

recommended by the manufacturer (120 s drying, 150 s heating, 45 s cooling). The validity of the analytical method was checked using the certified reference material (CRM) Oriental Basma Tobacco Leaves (INCT-OBTL-5), with a certified Hg content of 20.9 ± 1.3 ng/g DM [39], and quality controls were regularly performed as described elsewhere [23].

Hg resistant yeast isolation and Hg resistance

Leaves were collected from the field experimental site described above during summer 2015 and 2016, and immediately brought to the laboratory. The yeast were isolated using an enrichment technique on a malt extract medium adapted from a previously described method [40]. Briefly, intact leaves were incubated for 48 hr at 25°C and 200 rpm in an enrichment medium (at pH 3.7 adjusted with lactic acid) containing 30 g/l malt, 5 g/l peptone, 5 ml/l filtered leaf extract, and increasing amounts of $HgCl_2$ at final concentrations of 0, 2, 5, 10 or 20 µM. Hundred µl of leaf samples were then plated on malt extract agar (12 g/l malt) and PDA (Potato Dextrose Agar, sigma) media, supplemented with the corresponding $HgCl_2$ concentrations. The number of growing yeast was expressed in colony forming units (CFU) per ml. The strains growing at 10 µM Hg were purified, and resuspended in liquid malt extract or PDA media supplemented with 35% v/v glycerol and maintained at -80°C for further analysis.

Isolated strains were grown in 8 ml of growth medium for 48h at 27°C on a shaker table (200 rpm). After centrifugation, DNA was extracted from the pellet with the EZNA Bacterial DNA kit (OMEGA bio-tek, USA) in accordance with the manufacturer's instructions. The D1/D2 domain of LSU rRNA was amplified using a PCR with the universal primers ITS-1 (59-TCCGTAGGTGAACCTGCG-39) and NL-4 (59-GGTCCGTGTTTCAAGACGG- 39) [41]. All the ITS PCR products were sequenced by pyrosequencing 454 (Genewiz Beckman Coulter Cenomics, United Kingdom). DNA sequences were edited with BioEdit software and screened

against the GenBank database using BLASTn tool of the NCBI site (http://www.ncbi.nlm.nih.gov/).

Minimal inhibitory concentrations (MIC) for Hg were determined for each isolated strains. Microtitration plates (96 wells) were prepared using two-fold dilutions of Hg in YPD liquid medium, from a starting concentration of 256 µM down to 0 µM. Growth was measured by spectrophotometry at DO_{595} after 24h and 48h of incubation at 25°C.

Results

Illumina MiSeq sequencing revealed high diversity of the leaf microbiome

Following total genomic DNA extraction from soil and poplar samples, amplicons of the ITS1 region were generated, and a total of 8,345,173 paired-end reads were obtained through Illumina MiSeq sequencing (Table S1). Among the 24 samples from each habitat, those exhibiting a low sequence count were eliminated from the rest of the analysis. Thus, a total of 7,519,254 filtered and non-chimeric fungal sequences constituted our final processed dataset, representing 90% of the initial post-sequencing reads, spread among 6,100 non-singleton OTUs defined by representative DNA sequences with sizes of 101 to 363 bp (mean = 181.9 bp). After subsampling, our dataset contained 26,672 reads per sample, distributed in 5,565 non-singletons OTUs.

Rarefaction curve analysis, which assesses OTUs richness as a result of sampling, showed that all samples approached an asymptote, revealing that the overall fungal diversity was well represented (Fig. S1). Moreover, the measured Good's coverage values (an estimator of completeness of sampling) were greater than 99% for each sample type (Table S1) and in every sample, highlighting good overall sampling. Coverage, richness, and diversity, estimates were

calculated for each dataset (Table 1). The Chao1 estimator of Mothur, indicated good sample OTUs richness throughout. The Shannon and Simpson diversity indices, measurements of overall diversity, indicated a diverse microbiota. More specifically, the diversity and richness estimates were always significantly higher in the soil samples, followed by the leaf, stem and root samples (Table 1).

A permutation test confirmed that the habitat ($R^2 = 0.58$) explained most of the variance in the fungal community, whereas variations between plots were negligible and not statistically significant (Table S2). The importance of the habitat factor was further corroborated through visualization in a NMDS plot (Fig. 1), and significant dissimilarity between all habitats was confirmed with the ANOSIM test (Table S3). The Bray-Curtis method indicated that the belowground and aboveground communities at the Tavaux site were well separated (Fig. 1a). Considering the global analysis, NMDS plots revealed that root samples exhibited the greatest between-sample variation (Fig. 1a). Furthermore, we showed that stem and leaf samples clustered closely together (Fig. 1a), although the NMDS plot of the belowground (Fig. 1b) or aboveground (Fig. 1c) communities alone showed a net clustering of each sample type in these two communities. The leaf data presented less scattering than the stem data (see sizes of ellipses in Fig. 1c). Overall, these data indicated higher homogeneity of the OTUs distribution in aboveground samples, while the soil and root samples were less homogeneous.

In the Venn diagram analysis, the sums of the total observed fungal OTUs in the four sampled habitats of the Skado plots were 1567, 609, 918 and 948 for the soil, root, stem and leaf samples, respectively (Fig. 2). Overall, 151 OTUs (5.9%) were shared by all habitats. The OTUs that were unique represented 52% and 35% of the belowground and aboveground samples, respectively (Fig. 2). The soil samples exhibited the highest proportion of unique OTUs (36.6%), followed by the leaf (10.2%) and stem (10.4%) samples. Conversely, the root samples shared > 97% of OTUs with another habitat, mostly with the soil habitat (>84.7% of

the root OTUs were detected in soil samples). Our data also revealed that in the poplar phyllosphere, 51% of OTUs were shared by the stem and leaf samples, whereas 31% of OTUs were shared by the soil and root samples.

Symbiotic fungi dominated the belowground habitats, whereas yeast-like fungi dominated the aboveground habitats

The fungal communities across all four habitats were dominated by the phylum *Ascomycota* (54.6% of total relative abundance on average), while *Basidiomycota* represented a smaller portion of the communities (23.5%) (Fig. 3). However, the *Ascomycota*/*Basidiomycota* ratios were significantly higher in the aboveground samples than the belowground samples (Kruskal-Wallis $X^2 = 36.7$; $P < 1.4 \times 10^{-9}$). The largest proportion of *Basidiomycota* was found in the root samples (Kruskal-Wallis $X^2 = 56.1$; $P < 4.0 \times 10^{-12}$). These ratios are very similar to those identified with 454 sequencing technology in fungal communities associated with broadleaf trees [42]. Few members of the known arbuscular mycorrhizal fungi (AMF) phylum *Glomeromycota* were detected in the soil (0.25%, 92 OTUs from the *Entrophospora* and *Rhizophagus* genera) and root (1.30%, 90 OTUs from the *Entrophospora* and *Rhizophagus* genera) samples collected under poplars. OTUs assigned to mycorrhizal species were virtually absent from all aboveground samples. Members of the *Zygomycota* phylum were almost exclusively found in soil samples (5.2%, for 65 OTUs), mostly associated to *Mortierella* species.

Across all samples, we detected a total of 21 distinct fungal classes, which were unequally distributed, suggesting substantial differences between sampled habitats (Fig. 4). The belowground habitats were enriched with *Agaricomycetes* (Kruskal-Wallis $X^2 = 69.8$; $P < 2.2 \times 10^{-16}$), *Pezizomycetes* (Kruskal-Wallis $X^2 = 69.5$; $P < 2.2 \times 10^{-16}$) and *Sordariomycetes* (Kruskal-Wallis $X^2 = 67.9$; $P < \times 2.2\ 10^{-16}$). Moreover, the root samples contained significantly more *Agaricomycetes* (Kruskal-Wallis $X^2 = 16.3$; $P < \times 5.4\ 10^{-5}$), *Pezizomycetes* (Kruskal-Wallis $X^2 =$

25.4; $P < \times 4.6\ 10^{-7}$) and *Glomeromycetes* (Kruskal-Wallis X^2= 17.6; $P < \times 2.8\ 10^{-5}$) but significantly less Sordariomycetes and *Zygomycetes* than the soil samples. The aboveground habitats were enriched in *Dothideomycetes* (Kruskal-Wallis X^2= 65.7; $P < 5.4 \times 10^{-16}$) and *Taphrinomycetes* (Kruskal-Wallis X^2= 69.8; $P < 2.2 \times 10^{-16}$) from the *Ascomycota* phylum. While some *Dothideomycetes* members were detected in root and soil samples, *Taphrinomycetes* were virtually absent from all belowground samples. Although stem and leaf sample habitats contained members of *Basidiomycota* classes, belonging to *Agaricostillbomycetes* (Kruskal-Wallis X^2= 69.8; $P < 2.2 \times 10^{-16}$), *Exobasidiomycetes* (Kruskal-Wallis X^2= 69.8; P < 2.2×10^{-16}), *Microbotryomycetes* (Kruskal-Wallis X^2= 69.7; P < 2.2×10^{-16}) and *Tremellomycetes* (Kruskal-Wallis X^2= 69.0; $P < 2.2 \times 10^{-16}$), these classes were virtually absent from all belowground samples. The high proportion of classes of unassigned fungi in the stem habitat (63.5%) highlights the need for additional investigations of the diversity of the fungi living in this particular habitat.

The assignment tools revealed that root and soil habitats were dominated by OTUs identified as *Hymenogaster griseus, Thelephoraceae* and *Hebeloma hiemale*, all of which belong to *Agaricomycetes* from the *Basidiomycota* phylum (Figs. 3 and 5). However, *Hebeloma* (Kruskal-Wallis X^2= 8.7; $P < \times 10^{-10}$0.003) and *Thelephoraceae* (Kruskal-Wallis X^2= 48.5; $P < 1.7 \times 10^{-10}$) OTUs dominated the root samples (Figs. 3 and 5), whereas *Hymenogaster* OTUs (Kruskal-Wallis X^2= 4.3; $P < 0.04^{-10}$) were the most abundant in soil samples (Fig. 3 and Fig. 5). By contrast, the aboveground samples were dominated by *Ascomycota* OTUs, mostly belonging to 9 genera (*Alternaria, Aureobasidium, Bensigtonia, Lalaria, Davidiella, Sphaerulina, Rhodotorula, Cryptococcus, Taphrina)*. The *Lalaria* (Kruskal-Wallis X^2= 29; $P < 1.7 \times 10^{-8}$) and *Davidiella* (Kruskal-Wallis X^2= 33; $P < 9.2 \times 10^{-9}$) genera were most abundantly found in leaves while a Pleosporale OTU was the most abundant in stems (Kruskal-Wallis

X^2=3.5; $P < 0.05$) (Fig. 3). The *Basidiomycota* OTUs in aboveground samples were mostly assigned to the species *Bensigtonia yuccicola* (Figs. 3 and 5).

Each fungal OTU was further assigned to functional or morphological groups of fungi using FUNguild (http://www.stbates.org/guilds/app.php) [43] (Fig. 6). For every assignment, the FUNguild tool provides a confidence ranking, while referring to previously peer reviewed data (Table S4). To examine the distribution of OTUs within the functional categories, the abundance of the various OTU groups was set to 100%, and the OTUs were classified into guilds (Fig. 6A) and morphological categories (Fig. 6B). The investigation of trophic status in the belowground habitats revealed dominance of symbiotrophs in the root habitat (70.5%), while the soil community was composed of saprotrophs (45%), symbiotrophs (40%) and biotrophs (14%). In the aboveground habitats, saprotrophic fungi appeared to be dominant (stem, 53%; leaf, 65%) (Fig. 6a). The symbiotrophic fungi identified in the aboveground habitats belong mostly to the lichenized genus *Sphaerulina*, whereas symbiotrophs from the belowground habitats were identified as ectomycorrhizal fungi from the *Hymenogaster* genus. Another dichotomy was clearly revealed between the belowground and aboveground habitats through the analysis of growth form morphology (Fig. 6b). Indeed, as the soil and root habitats were dominated by gasteroid (soil: 67%; root: 48%) and agaricoid (soil: 21%; root: 45%) fungi, the fungal communities from the stem and leaf habitats were essentially dominated by yeasts (stem: 4%; leaf: 11%), dimorphic yeasts (stem: 62%; leaf: 38%), thallus fungi (stem: 27%; leaf: 1%) or rot fungi (stem: 11%; leaf: 37%). The presence of basidiomycetous or ascomycetous yeast in the phyllosphere has previously been observed in plants from temperate, tropical and Mediterranean climates [44, 45], in agreement with our results.

Interactions with Hg

The analysis of Hg in the various matrices revealed that the belowground habitats contained 100 times more Hg compared with the aboveground habitats (Fig. S2). In detail, the average values of Hg were 42.5 ng/g DM in poplar leaves and 3.6 ng/g DM in poplar stems, which are within the range of previously published data [21]. The root samples exhibited Hg concentrations of approximately 2.4 µg/g of DM. The soils exhibited an average Hg concentration of 5.6 µg/g DM, in agreement with our previous data [13], but ranged from 2.92 to 9.08 µg/g DM within the various harvested soil samples. Given the large variations in Hg concentrations in each habitat, we analyzed the correlations with Hg concentrations in the various matrices and found that only soil samples showed significant correlations between the Hg concentration and the diversity or richness indices. Specifically, we found a significant negative correlation between the soil Hg content and fungal richness indices (Observed richness: Spearman correlation coefficient of r^2 = -0.68 and $p < 0.001$; Chao1 index: Spearman correlation coefficient of r^2 = -0.42, and $p < 0.05$). The abundance of the two fungal classes, *Eurotiomycetes* (r^2 = 0.63, and $p < 0.001$) and *Sordariomycetes* (r^2 = 0.41, $p < 0.05$), were correlated with soil Hg concentrations, as well as the abundance of the two following OTUs, corresponding to a *Thelephoraceae* (r^2 = 0.44, and $p < 0.05$) and a *Trichoderma* (r^2 = 0.48, and $p < 0.05$) species. Conversely, the abundance of an OTU identified as *Hymenogaster griseus* was significantly negatively correlated with Hg (r^2 = -0.46, and $p < 0.05$). None of the diversity, richness or abundance indices were significantly correlated in root, leaf or stem with the Hg concentrations (data not shown).

The number of yeast cells isolated from the phyllosphere was $2.5.10^7$ UFC/ml without Hg but decreased to $1.9.10^7$ UFC/ml, $1.2.10^6$ UFC/ml, 1.10^6 UFC/ml and 2.10^5 UFC/ml on media enriched with 2, 5, 10 or 20 µM of $HgCl_2$, respectively. At 10 µM $HgCl_2$, only 2 species were isolated, namely *Nakazawaea populi* formely known as *Candida populi*, and *Aureobasidium pullulans*, which was one of the most abundant OTU (2.5 % of detected

sequences) of the metabarcoding dataset obtained from the leaf habitat. The MIC values for Hg of the isolated strains were 32 µM for *Nakazawaea populi* and 16 µM for *Aureobasidium pullulans* (Table 2).

The co-occurrence network revealed rare fungal OTUs with a high level of interaction in the community

We built co-occurrence networks to further assess the links within the fungal communities of the four habitats (Fig. 8 and Table 3). After network calculations, some topological properties that are commonly used in network analysis were completed to reveal complex patterns [46]. The root and soil habitats harbored the highest network connectivity, as exemplified by the highest number of edges and nodes (Fig. 8, Table 3). The co-presence and mutual exclusion of OTUs in the whole dataset were equally well distributed in the leaf, stem and root, habitats, whereas soil showing the highest mutual exclusion percentage (Table 3).

The network indices allowed us to define the 10 dominant keystone OTUs for each habitat (Table S5), which were defined as being important to maintain the function and structure of the microbial community and were arbitrarily identified here based on the number of connections established with the rest of the network [47]. Some taxa can be less abundant but highly connected with other taxa (as shown by the number of degrees within the node). These keystone OTUs can be divided in two groups: those generating positive connections (co-presence) and those generating negative connections (mutual exclusion). Both (+ and -) groups were evident in this subset of keystone OTUs in the leaf, stem and root habitats, whereas the soil contained mostly OTUs exhibiting negative connections, as observed for the whole soil dataset. The tendency of OTUs to cluster is revealed by the clustering coefficient, which was two-fold higher for the root habitat. In the leaf habitat, OTUs from the genus *Myrothecium* and from the class *Dothideomycetes* (unassigned genus) produced the highest number of negative

connections. The genus *Myrothecium*, previously detected in mulberry, has been identified as a foliar pathogen producing mycotoxins [48]. Our work revealed that OTUs from this genus found on poplar leaves had an overall negative impact on other microbes from the leaf community. In the stem habitat, an OTU from the *Exobasidiomycetes* class showed only negative connections with all other fungal OTUs. The other keystone OTUs from the stem belonged to the *Dothideomycetes* class and exhibited mostly negative connections. Similarly, in the root habitat, two keystone OTUs belonging to *Glomeromycetes* exhibited mostly negative connections with other OTUs. In contrast to the leaf and stem habitats, other keystone OTUs presented mostly positive connections, constituting a cluster highlighted in Figure 8. *Rhodotorula* and *Lalaria* OTUs from this cluster were rather rare in the root habitat but were frequently encountered in the leaf habitat (Fig. 3). The soil habitat was characterized by keystone OTUs exhibiting mostly negative connections. The *Peziza* OTU (ITS-75-68665) displayed the greatest number of connections among all keystone OTUs by far (Table S5).

Discussion

We used the Illumina MiSeq sequencing platform to characterize fungal communities from a poplar plantation at a Hg-contaminated site. It is important to bear in mind that we were unable to distinguish between endophytes and epiphytes in each of the three plant habitats (root, stem and leaf) and instead considered the fungal communities in these habitats in their entirety. Although we did not set out to study seasonal dynamics of the belowground and aboveground fungal communities, we should bear in mind that differing seasonal patterns between belowground [49] and aboveground [50, 51] fungal taxa have been described previously. It was concluded that the variation of foliar chemistry across growing seasons should not be considered a major driver of the observed fungal dynamics.

Rarefaction analyses and richness estimators indicate that much of the total diversity detectable with the Illumina-based sequencing was obtained. The finding of higher richness and diversity in aboveground habitats compared with the root habitat was poorly predictable, as there are considerably fewer available studies on aboveground communities, compared with belowground communities. A previous study revealed a low percentage of fungal OTUs shared by leaf and root samples in *Fagus sylvatica* trees [42], while another study showed that a majority of aboveground OTUs were also present in the belowground compartment of agave plants [3]. Our dataset unequivocally revealed that i) less than 6% of the OTUs were detected in all four habitats, and ii) the aboveground fungal communities from poplar leaves were extremely diverse, although they were represented by only a few abundant taxa and numerous rare taxa [15, 52]. Overall, our results strongly indicate that belowground habitats host fungal communities almost completely isolated from from the aboveground habitats communities in terms of taxonomy, growth morphology, and relationship with trees or microbial interactions. Considering previous studies, the finding of lower richness and diversity in the root compared with the soil habitat was expected [3, 11, 53]. Clear separation of microbiomes has been reported for soil and root samples from mature poplars [54] and 2-year-old poplars [11]. In our study, we showed that 87% of the detectable OTUs of the roots habitat were also found in the soil habitat but few taxa were strongly associated to root. Thus, the root fungal communities also displayed lower homogeneity of the species distribution compared with soil communities.

We demonstrated that the fungal communities associated with *Populus* roots mostly consisted of ectomycorrhizal fungi, which are known to develop mutually beneficial interactions with their hosts. These plant-microorganism interactions in the root compartment are probably one of the factors explaining the reasonable adaptation of *Populus skado* to this particular soil. The *Thelephorales* OTUs in the root samples accounted for the main contribution to the dominance of symbiotrophs in roots, as most members of this order are

known to be ectomycorrhizal and to live in symbiosis with various host plants across Northern America and Europe. *Thelephoraceae* are indeed abundant colonizers of *Salix caprea* or *Populus tremula* roots in TE-contaminated soils [55–57]. Additionally, OTUs corresponding to the *Hebeloma*, *Cortinarius* and *Geopora* genera were also detected in our root and soil samples, in agreement with previous studies [55, 56]. Members of the *Hebeloma* mycorrhizal genus (notably *H. mesophaeum*) are frequently found within unvegetated soils [55, 56] and have been shown to promote the growth of host trees in soils contaminated with metals [58]. At the family level, both this and a previous study by our group [13] identified *Agaricomycetes* and *Pezizomycetes* as the most frequent fungal families in the belowground compartment. Similarly, five of the 6 most common genera (*Hebeloma*, *Mortierella*, *Tuber*, *Geopora* and *Cortinarius*, but not *Hymenogaster*) identified in this study were among the top five detected previously.

The analysis of growth morphology clearly resulted in clustering of the belowground and aboveground habitats. The soil and root habitats were dominated by agaricoid and gasteroid fungi, as highlighted by the presence of *Hebeloma* and *Hymenogaster* species, respectively. The fungi from stem and leaf communities were essentially identified as yeasts or facultative yeast morphotypes (Fig. 6b), as exemplified by *Lalaria* OTUs [59]. Abundance of *Lalaria* OTUs in the phyllosphere has previously been reported on the leaves of *Fagus sylvatica* [60] and in the *Quercus* phyllosphere [15]. *Davidiella tassiana*, also known as *Mycosphaerella tassiana* or *Cladosporium herbarum*, is a leaf pathogenic fungus from the Helotiales order that is commonly encountered in the phyllosphere of trees [61]. Reader should keep in mind that many fungi have several names, which can lead to mistakes and thus have always to be taken in consideration when using dated data. Standardization at a global scale of fungal names should only profit to fungal ecology. The stem tissues were enriched in *Pleosporales sp. and Sphaerulina pseudovirgaureae* OTUs. Previous studies have shown that phyllosphere

endophytic fungi can play an important role in enhancing plant health [20], acting as biocontrol agents against other plants, insects and pathogens.

Hg is known to be a toxic element, but only few studies have explored the impact of Hg on fungal communities in field trials. Müller (2001) showed that the soil fungal biomass was not affected by the Hg along a Hg gradient ranging from 7-522 mg THg/kg of soil. Over a narrow gradient in terms of the Hg concentration, only a negative correlation between arbuscular mycorrhizal fungi and Hg was observed in the literature, while no correlation was found between ectomycorrhizal (ECM) fungi and Hg [63]. Therefore, this study is the first to describe a significant negative effect of Hg on soil fungal richness and diversity under long-term, natural Hg exposure. In contrast, Hg exposure was not a major driver of the root, stem and leaf communities, probably due to the limited variations and the limited impact these variations may have on cellular processes. Nevertheless, we were able to isolate some Hg resistant yeast strains from the leaf habitat. Resistance here refer to the fact that these strains were isolated on Hg-enriched growth media, and to the MIC measured for these strains, which are comparable to previously published data [64]. As most of the Hg detected in poplar leaves entered through the atmospheric pathway [21], we indeed focused on the isolation of Hg resistant fungi from this habitat. We thus isolated *Aureobasidium pullulans* Hg resistant strains, also highly represented in the leaf metabarcoding dataset (Fig. 3). This species is recognized as an active phylloplane colonizer [65], which showed some capacity to bind metals to the cell surfaces [66]. Other, authors previously revealed that melanized fungi such as *Aureobasidium pullulans*, *Cladosporium spp.* and *Alternaria alternate* have been isolated from soil samples treated with toxic industrial wastes containing high concentrations of copper and mercury and may also be dominant members of the mycobiota of metal-contaminated phylloplanes [67]. We also isolated *Nakazawaea populi* Hg resistant strains, that were not detected in our metabarcoding dataset, probably due to an uncomplete

assignment, and that may be part of the unassigned *Ascomycota* cluster (Fig. 3). This strain has been recently assigned to the *Nakazawaea* genus, and was previously known as a member of the *Candida* genus [68]. *C. populi* was indeed isolated from poplar sap exudate [69]. These strains will be further used in inoculation experiments, to better understand the role of leaf yeast communities on the overall Hg cycle between soil, atmospheric and leaf compartments.

This study is the first to explore the organization of the fungal communities of soil, roots, stems and leaves using a co-occurrence approach at the scale of a clonal tree stand. Indeed, previous studies have focused on species abundance and diversity, but not on the interactions among species, which could be more important to ecosystem functioning [70]. Co-occurrence networks represent individual microbes (operational taxonomic units (OTUs)) as nodes and feature–feature pairs as edges, where an edge may imply a biologically or biochemically meaningful relationship between features, and are based on correlations [34]. For instance, one may expect that mutualistic microbes, or those that benefit each other, will co-occur across samples. In contrast, antagonistic relationships between microbes, such as competition for the same niche, result in a mutual exclusion. It has been observed that phylogenetically related microbes have a tendency to positively co-occur [71]. In practice, microbes may exhibit positive or negative correlations for indirect reasons, based on their environmental preferences. The overall dataset revealed that non-abundant OTUs might play a significant role in the network of interactions. Co-occurrence network analysis of the fungal communities from the four habitats established a clear dichotomy between soil and the three other habitats, where the soil community was dominated by negative edges, known as mutual exclusion. It should be noted that sequencing depth impacts the percentage of positive edges in the network, with a low depth resulting in spurious positive correlations [37]. Thus, the large number of negative correlations found in our study can be correlated with our extremely high sequencing depth. This dominance of negative degrees found in the soil (80%), but not in the

roots nor in the aboveground habitats, could reflect a high degree of competition between fungi in the soil due to a lack of nutrient availability in the absence of tree exudates. It is also possible that the soil microorganisms under the canopy were in competition with the plants for nutrients such as nitrogen, exacerbating the nutrient competition between microorganisms [72]. The network obtained from the fungal sequencing data also revealed that the root compartment present the highest number of interactions between fungi and the highest clustering coefficient, with predominance of *Glomeromycetes*, showing a great number of interactions with other fungi. Arbuscular mycorrhizas (AM) formed by *Glomeromycetes* are widespread in living plants, supporting the ancestral origin of the plant–*Glomeromycetes* symbiosis, as fully supported by the literature [73]. We noted that the class *Glomeromycetes* produced many degrees of mutual exclusion with other classes and between the most interactive *Glomeromycetes* themselves. The hub in the root compartment network typical of the leaf compartment could correspond to the transfer of microorganisms during leaf fall in the root area, but the real explanation is still unclear.

We may conclude that each habitat that we studied represents a unique niche for the fungal communities in a monoclonal plantation of the cultivar Skado (*P. trichocarpa* x *P. maximowiczii*) implemented in 2011 as a short-rotation coppice (SRC, 2200 stems/ha). Aboveground and belowground poplar habitats host completely different fungal communities, as highlighted by the core microbiome of the four habitats that represent only reduced to 5.9% of the total OTUs. We will further explore the role of fungal organisms in the Hg cycle, which deserves attention. We believe that our findings will be instructive for the design of future ecological restoration practices.

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Figure legends

Figure 1. Non-parametric multidimensional scaling (NMDS) plot of fungal communities associated with the four poplar habitats, using the Bray-Curtis dissimilarity measure. Each point represents the fungal community of a given sample. Each color represents one of the 6 trees sampled. Confidence area of ellipses = 0.95. (a) All habitats, (b) belowground habitats, and (c) aboveground habitats.

Figure 2. Venn diagram showing the overlap of the fungal communities from the four poplar habitats, based on OTUs. OTU delineation was based on a threshold of < 97% sequence similarity

Figure 3. Proportion and taxonomic assignment of abundant and rare (< 0.5% relative abundance) operational taxonomic units (OTUs) from the various poplar habitats. The assignments are given at the lowest taxonomic level possible, with relative proportions presented in parentheses. The abundance of the major phyla and the total number of reads are provided on the left side of each graph, color coded as follows: *Ascomycota* (red), *Basidiomycota* (blue), *Zygomycota* (yellow), *Chytridiomycota* (brown), *Glomeromycota* (green) and unassigned fungi (grey).

Figure 4. Composition of the fungal communities from the various poplar habitats at the class level. The data were derived from MiSeq sequencing of the ITS1 region.

Figure 5. Heat map and hierarchical cluster analysis of the relative abundance of fungal OTUs from the various poplar habitats. Letters indicate significantly different abundances at $p < 0.05$ (Kruskal-Wallis comparison test), n=23 (root and leaf) or n=24 (soil and stem). The dendrogram represents linkage clustering using Euclidean distance measures. OTU delineation was based on a threshold of < 97% sequence similarity. The number associated with the OTU corresponds to the relative abundance rank of that OTU in the total dataset. Assignments between brackets show the lowest taxonomic level associated with the OTU using the UNITE database, k: kingdom, p: phylum, o: order, c: class, f: family, s: genus_species.

Figure 6. Relative proportions of fungal sequences from the various poplar habitats assigned to major fungal guilds (a) and morphological groups (b).

Figure 7. Box plots of the Hg concentration (ng/mg DM) in (a) belowground habitats, and (b) aboveground habitats. Letters indicate significant differences between habitats (p-value < 0.05).

Figure 8. Co-occurrence network of microbial taxa detected in the four habitats via a high-throughput DNA sequencing (Illumina MiSeq). Nodes represent fungal OTUs, whereas edges represent significant positive correlations between pairs of OTUs. The node size corresponds to the number of connections, and taxa with many correlations are within densely connected areas of the network. Green edges between nodes represent co-presence, while red edges represent mutual exclusion.