Soil Characteristics Constrain the Response of Microbial Communities and Associated Hydrocarbon Degradation Genes during Phytoremediation

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ABSTRACT Rhizodegradation is a promising cleanup technology where microorganisms degrade soil contaminants in the rhizosphere. A symbiotic relationship is expected to occur between plant roots and soil microorganisms in contaminated soils that enhances natural microbial degradation. However, little is known about how different initial microbiotas influence the rhizodegradation outcome. Recent studies have hinted that soil initial diversity has a determining effect on the outcome of contaminant degradation. To test this, we either planted (P) or not (NP) balsam poplars (*Populus balsamifera*) in two soils of contrasting diversity (agricultural and forest) that were contaminated or not with 50 mg kg⁻¹ of phenanthrene (PHE). The DNA from the rhizosphere of the P and the bulk soil of the NP pots was extracted and the bacterial genes encoding the 16S rRNA, the PAH ring-hydroxylating dioxygenase alpha subunits (PAH-RHD α) of Gram-positive and Gram-negative bacteria, and the fungal ITS region were sequenced to characterize the microbial communities. The abundances of the PAH-RHD α genes were quantified by real-time quantitative PCR. Plant presence had a significant effect on PHE degradation only in the forest soil, whereas both NP and P agricultural soils degraded the same amount of PHE. Fungal communities were mainly affected by plant presence, whereas bacterial communities were principally affected by the soil type, and upon contamination the dominant PAH-degrading community was similarly constrained by soil type. Our results highlight the crucial importance of soil microbial and physicochemical characteristics in the outcome of rhizoremediation.

IMPORTANCE Polycyclic aromatic hydrocarbons (PAH) are a group of organic contaminants that pose a risk to ecosystems' health. Phytoremediation is a promising biotechnology with the potential to restore PAH-contaminated soils. However, some limitations prevent it from becoming the remediation technology of reference, despite being environmentally friendlier than mainstream physicochemical alternatives. Recent reports suggest that the original soil microbial diversity is the key to harnessing the potential of phytoremediation. Therefore, this study focused on determining the effect of two different soil types in the fate of phenanthrene (a polycyclic aromatic hydrocarbon) under balsam poplar remediation. Poplar increased the degradation of phenanthrene in forest, but not in agricultural soil. The fungi were affected by poplars, whereas total bacteria and specific PAH-degrading bacteria were constrained by soil type, leading to different degradation patterns between soils. These results highlight the importance of performing preliminary microbiological studies of contaminated soils to determine whether plant presence could improve remediation rates or not.

KEYWORDS phenanthrene, phytoremediation, polycyclic aromatic hydrocarbons, poplar, rhizosphere-inhabiting microbes, soil contamination, soil diversity

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The expansion of the human population coupled with a burst in industrialization has led to a sensible increase in fossil fuel combustion (1). As a result, there has been an increase in all types of pollutant emissions, including polycyclic aromatic hydrocarbons (PAH) (2, 3). PAH are organic compounds formed by a variable number of fused aromatic rings that constitute a priority concern due to their adverse effects on environmental (4) and human health (5). PAH are ubiquitous in the environment and are often found polluting soils (6). This is of special concern, since soil is one of the most important resources upon which many key human economic and social activities depend (7).

To remediate soils, a wide range of cleanup technologies have been developed, ranging from relatively simple but expensive excavation, transportation, washing, and dumping of small affected soil areas (8) to more complex but comparatively less expensive phytoremediation strategies, where the soil is cleaned by the combined action of plants and microorganisms (9). Phytoremediation has proven to be effective to decontaminate soils polluted by PAH (10–13). Among the many types of phytoremediation approaches, rhizodegradation is a genuine plant-microbe coenterprise, with the potential to completely remove PAH from soils through the action of bacterial, fungal, and plant enzymes in the rhizospheric environment (14). Members of the *Salicaceae* family, such as willows and poplars, are often seen as good candidates for phytoremediation in view of their deep rooting system, their extremely rapid growth, their large evapotranspiration rates, their high genetic diversity, their resistance to biotic and abiotic stresses, their ease of clonal propagation through cuttings, and the availability of genetic tools and sequenced genomes. For instance, the model species selected here, *Populus balsamifera*, has a wide distribution in Canada and can thus adapt to various (cold) climates and soil types, making it an ideal candidate for phytoremediation in Canada.

Phenanthrene (PHE) is a low-molecular-weight PAH formed by three fused rings. In Canadian soils, background levels of PHE range between 0.05 and 0.14 mg kg⁻¹ in agricultural soils and are around 0.03 mg kg⁻¹ in rural soils, whereas in urban areas, the median and maximum concentrations measured were 5.7 and 66 mg kg⁻¹, respectively (15). However, the highest PAH concentrations in soils (as mixtures of 16 contaminants) are found near former coking plants, where values as high as 11,473 mg kg⁻¹, before restoration, have been registered (15). PHE is known for its cytotoxic and carcinogenic effects on living organisms (16, 17). Because of this, Canada regulates the amount of phenanthrene that can be found in soils depending on the land use. As such, 50 mg kg⁻¹ of phenanthrene is the maximum amount that can be found in soil for commercial and industrial uses (15). Beyond this threshold, the soil is considered contaminated and no activities can be carried out before the soil is remediated. In this study, in line with this threshold, soils were contaminated with 50 mg kg⁻¹ of phenanthrene.

Fortunately, PHE is relatively easy to degrade by a wide range of microorganisms through aerobic or anaerobic metabolic pathways (18). Most microbial degraders are bacteria or archaea, although PHE degradation has been described among fungi (19, 20). Typically, under aerobic conditions the initial step of the bacterial catabolic pathways starts with the incorporation of molecular oxygen into the aromatic nucleus by a ring hydroxylating dioxygenase (RHD) enzyme system to yield *cis*-dihydrodiol. *Cis*-dihydrodiol is then rearomatized to a diol intermediate that is further transformed by ring-opening dioxygenases into catechols and other intermediates. Finally, these catechols can be converted to tricarboxylic acid (TCA) cycle intermediates (18, 21, 22). RHD genes are often used as markers of PAH degradation, since they catalyze the first step of the degradation process. They are multicomponent enzyme systems composed of a large alpha subunit and a small beta subunit (23). The genes coding for the alpha subunit of PAH-RHD form a monophyletic group (24), and were consequently used to design PCR primers for both Gram-negative and Gram-positive bacteria (25), providing a useful way to track bacterial degraders in PAH-contaminated environments.

Many microorganisms harbor RHD genes, such as members of the genera *Enterobacter* (26–28), *Sphingomonas* (29–31), *Burkholderia* (25, 32–34), *Pseudomonas* (26, 32,

35, 36), *Mycobacterium* (29, 37–39), and *Polaromonas* (30, 40, 41), among others. Several of these microorganisms have been found to thrive in the rhizosphere of many plants (42, 43), including trees, which explains in part the success of phytoremediation approaches.

Unravelling the complex microbe-soil-plant relationships within the context of rhizoremediation of PAH-contaminated soils remains challenging, but has been suggested as key to optimizing phytoremediation to increase its use (14, 44). Many recent studies have demonstrated that the diversity of microbial communities has an important role in the degradation prospects in soils (45, 46) and in the rhizosphere of various *Salicaceae* tree species used for phytoremediation (47, 48). For instance, Bell et al. (45) showed that a soil with a high initial diversity was more efficient at degrading complex hydrocarbon mixtures than a soil inoculated with a consortium of confirmed hydrocarbon degraders. Furthermore, under high levels of hydrocarbon contamination, willows grew better when planted in a diverse bulk soil that was not previously planted than in a less diverse rhizospheric soil of a willow that had previously grown well under the same contamination conditions (47). Taken together, these two studies highlight the importance of a diverse initial microbial community over a less diverse, preselected community for the successful degradation of hydrocarbon in soils. Other studies have shown that the initial community composition also had an importance for the degradation of hydrocarbons in soils. For instance, the initial relative abundance of some of the major families of *Betaproteobacteria* in the soil before contamination were significantly correlated with the amount of diesel degraded across a range of arctic soils (46). In another study, the total amount of zinc taken up by willows after 16 months of growth was strongly linked to the relative dominance of four fungal taxa in the rhizosphere of the plants after 4 months of growth (48). Soil microbiological characteristics appear to have a determining effect on the degradation of hydrocarbons and on the outcome of soil remediation efforts, potentially explaining the variable efficiency of phytoremediation in different soils and, consequently, its low retention as a preferred remediation technique (49).

Here, we hypothesize that differences in the microbiological characteristics of soil (namely, microbial diversity) are a major constraint to PAH rhizoremediation, overriding the rhizosphere effect central to this technology. We aimed at determining the response of the microbial communities of two different soils to contamination with phenanthrene and its subsequent rhizoremediation by poplars. To attain this objective, we designed a pot experiment and sequenced the 16S rRNA gene of bacteria and archaea, the ITS1 region of fungi, and the PAH-ring hydroxylating dioxygenase (PAH-RHD α) genes of Gram-negative and Gram-positive bacteria, and determined the residual concentration of phenanthrene after 9 weeks of poplar growth.

RESULTS

Plant morphological response and phenanthrene degradation. All the 48 poplar cuttings survived throughout the experiment and were included in the analysis. Poplar growth was evaluated as the plant aboveground biomass but neither soil type nor contamination had a significant impact on this variable (Fig. 1a).

Phenanthrene degradation was measured as the quantity of remaining phenanthrene after 9 weeks of incubation and was expressed as absolute mg kg⁻¹ retrieved in soil. Differences in soil type ($F = 79.508$, $P < 0.001$), plant presence ($F = 9.442$, $P = 0.004$) and the interaction term ($F = 8.166$, $P = 0.007$) were all significant (Fig. 1b). The interaction term revealed that the effect of soil type depended on plant presence. More precisely, in agricultural soils, both P and NP pots showed similar degradation, with 1.33 and 1.45 mg kg⁻¹ of phenanthrene left in NP and P pots, respectively. In contrast, after 9 weeks the nonplanted forest soils contained significantly more phenanthrene than the planted forest soils (7.97 versus 3.19 mg kg⁻¹ of phenanthrene; Fig. 1b).

FIG 1 Boxplots of poplar biomass and phenanthrene presence. (A) Dried biomass of poplars grown in agricultural and forest soil contaminated with phenanthrene (PHE) or not (CTRL). (B) Quantification of phenanthrene in agricultural and forest soils after 9 weeks of poplar growth in pots (P, for planted) compared to nonplanted pots (NP). Different letters denote significant differences found with the Tukey's HSD *post hoc* test ($n = 12$).

Fungal diversity. Shannon diversity index for fungi ranged from 1.599 to 3.646 (Fig. 2a). The Kruskal-Wallis test showed significant differences among treatments ($\chi^2 = 17.779$, $P = 0.013$). The Dunn's test showed that this difference was due to significantly higher Shannon diversity in the P control (CTRL, uncontaminated) forest soils compared to the NP CTRL and NP PHE agricultural soils and to the NP PHE forest soil (Fig. 2a). The same trends were visible for species richness, but this was not statistically significant (Fig. 2a).

Fungal community structure. Analysis of the beta diversity with the principal-coordinate analysis (PCoA) (Fig. 2b) based on relative abundance showed that the fungal community structure differed between plant presence and soil types. The main factor was plant presence ($R^2 = 49.7\%$, $P = 0.001$), followed by the soil type ($R^2 = 14.5\%$, $P = 0.001$). However, the main plant effect was modulated by the interaction with the soil type factor ($R^2 = 5.0\%$, $P = 0.001$). Contamination, either as a main effect or its interaction with other factors, had no significant influence on the fungal community structure (Table 1).

More specifically, while the fungal communities found in the NP pots diverged significantly from one another depending on the soil type, these communities tended to converge in the P pots. The PERMANOVA analysis confirmed this visual interpretation, with plant presence representing the main source of variation, followed by first order effect of soil type and the interaction of both factors (Table 1).

Fungal community composition. A total of 3,099 fungal operational taxonomic units (OTUs) was retained after the bioinformatic quality filtering pipeline. As to the community structure, the fungal community composition was principally influenced by plant presence. As such, the PHE and CTRL rhizospheres (P) of both forest and agricultural soils were vastly dominated by fungi from the genus *Sphaerospora* (class *Pezizomycetes*; Fig. 2c and Table S2 in the supplemental material). The NP pots of both types of soil showed mixed community composition, strongly distinct from the P pots. The lower diversity of fungi in the agricultural soil could be explained by the prevalence of *Mortierellomycotina* (Fig. 2c and Table S2), which accounted for around 45% and 35% of total OTUs in CTRL and PHE agricultural NP pots, respectively. In contrast, the presence of *Mortierellomycotina* in forest soils was lower than 20%, whereas *Basidiomycota* (*Tremellomycetes* and *Agaricomycetes* classes) were significantly more abundant (Fig. 2c and Table S2). Within the *Ascomycota* phylum, *Leotiomyces* were also significantly more abundant in forest than in agricultural soils (Table S2).

Some significant but weak correlations were found among some fungal OTUs and the plant biomass. The top 10 OTUs had a Pearson's correlation coefficient r ranging from 0.395 to 0.331, and the OTUs belonged mainly to the *Ascomycota* phylum.

FIG 2 Summary of the fungal community diversity, structure, and composition based on OTUs of the ITS region. (A) Boxplots of Shannon diversity index and species richness (as the observed number of OTUs) by treatment. Different letters denote significant differences found with the Tukey's HSD *post hoc* test. (B) Principal-coordinates analysis (PCoA) based on Bray-Curtis dissimilarity of the relative abundance of fungal OTUs showing the effects of contamination, soil type, and plant presence on the fungal community structure. (C) Fungal community composition at the class level. Only taxa with a relative abundance above 0.01 are shown. Values are averaged across treatments ($n = 12$). Legend: NP, nonplanted pots; P, planted pots; CTRL, uncontaminated pots; PHE, pots contaminated with 50 mg kg⁻¹ of phenanthrene.

The significant correlations found between fungi and phenanthrene concentration in soils at the end of the experiment were stronger (Pearson's correlation coefficient r between 0.834 to 0.744). The top 10 OTUs presenting positive correlations belonged to the genera *Aspergillus*, *Acrodontium*, *Imleria*, *Postia*, *Umbelopsis*,

TABLE 1 Summary of permutational multivariate analysis of variance (PERMANOVA) examining differences in microbial composition based on the OTUs of the ITS region for fungi, the 16S rRNA gene for total bacteria, and ASVs for GN and GP PAH-RHD α genes for bacterial degraders^a

Treatment	Df	Sum sqs	Mean sqs	F model	R ²	Pr(>F)
Fungi						
Soil	1	3.938	3.938	44.745	0.145	0.001
Contamination	1	0.109	0.109	1.234	0.004	0.26
Plant	1	13.526	13.526	153.690	0.497	0.001
Soil:contamination	1	0.174	0.174	1.977	0.006	0.101
Soil:plant	1	1.353	1.353	15.377	0.050	0.001
Contamination:plant	1	0.148	0.148	1.678	0.005	0.157
Soil:contamination:plant	1	0.197	0.197	2.241	0.007	0.078
Residuals	88	7.745	0.088	NA	0.285	NA
Total	95	27.189	NA	NA	1.000	NA
Bacteria						
Soil	1	12.348	12.348	115.521	0.512	0.001
Contamination	1	0.530	0.530	4.955	0.022	0.004
Plant	1	0.572	0.572	5.353	0.024	0.003
Soil:contamination	1	0.451	0.451	4.221	0.019	0.009
Soil:plant	1	0.460	0.460	4.301	0.019	0.005
Contamination:plant	1	0.165	0.165	1.542	0.007	0.150
Soil:contamination:plant	1	0.169	0.169	1.583	0.007	0.145
Residuals	88	9.406	0.107	NA	0.390	NA
Total	95	24.101	NA	NA	1.000	NA
PAH-RHDα GN genes						
Soil	1	4.341	4.341	14.912	0.119	0.001
Contamination	1	2.622	2.622	9.007	0.072	0.001
Plant	1	0.283	0.283	0.971	0.008	0.457
Soil:contamination	1	3.091	3.091	10.618	0.085	0.001
Soil:plant	1	0.193	0.193	0.663	0.005	0.811
Contamination:plant	1	0.228	0.228	0.782	0.006	0.659
Soil:contamination:plant	1	0.293	0.293	1.005	0.008	0.401
Residuals	86	25.034	0.291	NA	0.688	NA
Total	94	36.402	NA	NA	1.000	NA
PAH-RHDα GP genes						
Soil	1	4.900	4.900	16.970	0.146	0.001
Contamination	1	1.230	1.230	4.261	0.037	0.001
Plant	1	0.644	0.644	2.232	0.019	0.014
Soil:contamination	1	0.340	0.340	1.176	0.010	0.176
Soil:plant	1	0.417	0.417	1.446	0.012	0.082
Contamination:plant	1	0.441	0.441	1.528	0.013	0.08
Soil:contamination:plant	1	0.670	0.670	2.322	0.020	0.01
Residuals	85	24.54	24.54	NA	0.733	NA
Total	93	36.402	NA	NA	1.000	NA

^aValues in bold indicate significant effects. Df, degrees of freedom; Sum sqs, sum of squares; Mean sqs, mean squares; F, variance ratio; Pr(>F), P value.

Acrophialophora, *Wilcoxina*, an unclassified *Basidiomycota*, and *Leptodontidium* (Table S3).

Bacterial diversity. A total of 45 archaeal OTUs were found in the soil samples representing a combined relative abundance of less than 0.1%, therefore they were not retained for the analyses.

In terms of bacterial diversity, there were no significant differences between treatments, although slightly higher values were found in NP PHE soils for the Shannon index (Fig. 3a). For the species richness, the forest soils seemed to present higher number of OTUs in PHE pots while the agricultural soils showed higher OTU numbers in CTRL soils (Fig. 3a), but again this difference was not significant.

Bacterial community structure. The PCoA based on relative abundance (Fig. 3b) along with the PERMANOVA analysis (Table 1) showed that the soil type was the main factor shaping the bacterial community ($R^2 = 51.2\%$, $P = 0.001$), separating agricultural

FIG 3 Summary of the bacterial community diversity, structure, and composition based on OTUs of the 16S rRNA gene. (A) Boxplots of Shannon diversity index and species richness (as the observed number of OTUs) by treatment. Different letters denote significant differences found with the Tukey's HSD *post hoc* test. (B) Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity of the relative abundance of bacterial OTUs showing the effects of contamination, soil type, and plant presence on the bacterial community structure. (C) Bacterial community composition at the class level. Only taxa with a relative abundance above 0.01 are shown. Values are averaged across treatments ($n = 12$). Legend: NP, nonplanted pots; P, planted pots; CTRL, uncontaminated pots; PHE, pots contaminated with 50 mg kg^{-1} of phenanthrene.

from forest soil samples along the first axis, in sharp contrast to what was observed in the fungal community structure. Moreover, plant presence ($R^2 = 2.4\%$, $P = 0.003$) and the contamination status ($R^2 = 2.2\%$, $P = 0.004$) had minor but significant main effects and also modulated the effect of soil type through significant interaction terms ($R^2 =$

1.9%, $P = 0.005$ and $P = 0.009$, respectively; Table 1). These interaction effects are visible in Fig. 3b, as the bacterial communities found in the agricultural soils all clustered together, whereas the bacterial communities of the forest soil were also clustering loosely based on soil contamination and plant presence on the second axis of the ordination.

Bacterial community composition. A total of 5,716 OTUs were retained from the 16S rRNA gene sequences. From those, the vast majority belonged to *Bacteria*, and only 45 OTUs were *Archaea*.

The 5,671 bacterial OTUs were classified in 35 phyla, although the majority of OTUs belonged to *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Gemmatimonadetes*, *Planctomycetes*, and *Verrucomicrobia*. Despite that agricultural and forest soils presented very different community structures (Fig. 3b), the composition at the phylum level was relatively homogeneous among treatments (Fig. 3c), with a few significant differences in terms of the overall relative abundance of phyla (Table S4).

In contrast, major differences were observed in the relative abundance at the genus level (Table 2). Compared to forest soils, agricultural soils had significantly higher relative abundance of the following genera: unclassified uncultured *Acidobacteriaceae* subgroup 1 (*Acidobacteria*, mean of 14.84% versus 2.52%), HSB OG53-F07FA (*Chloroflexi*, 8.07% versus 0.12%), DA101 soil group FA (*Verrucomicrobia*, 5.06% versus 0.36%), Candidatus *Solibacter* (*Acidobacteria*, 2.40% versus 0.94%) *Sphingomonas* (*Alphaproteobacteria*, 3.51% versus 2.50%), and *Gemmatimonas* (*Gemmatimonadetes*, 2.40% versus 1.27%), the latest being mostly absent in the forest soils. In contrast, the forest soils had significantly higher relative abundances of the genera *Ramlibacter* (*Betaproteobacteria*, 1.11% versus 4.66%), *Geothrix* (*Acidobacteria*, 0.02% versus 5.61%), and *Variibacter* (*Alphaproteobacteria*, 2.88% versus 4.6%).

Also, the forest soils presented some particularities linked to the effects of the contamination and plant presence that were absent in agricultural soils, and that explained the different community structure captured in the PCoA (Fig. 3b). For instance, regarding the effect of plant presence, *Polaromonas* (*Betaproteobacteria*), *Aquicola* (*Betaproteobacteria*), and *Acidothrmus* (*Actinobacteria*) were nearly absent from the P pots, while the *Acidobacteria* subgroup 2CL was significantly more abundant in the planted forest soils compared to the NP soils (Table 2). Moreover, the significant effect of the interaction between plant presence and contamination was largely explained by the higher mean relative abundance of bacteria from the genus *Polaromonas* (*Betaproteobacteria*) in NP PHE forest soils (10.95%) compared to the P PHE of forest soils (1.46%), while being nearly absent in CTRL pots (Table 2).

As it was for fungi, the correlations between bacterial OTU relative abundances and the biomass of the plant were weak. The top 10 significant positive correlations had a Pearson's correlation coefficient r ranging from 0.476 to 0.414. These OTUs were diversely classified as *Alphaproteobacteria*, *Chloroflexi*, *Acidobacteria*, *Armatimonadetes*, *Actinobacteria*, and *Gammaproteobacteria*. The strongest positive correlation involved an OTU from the genus *Sphingomonas*.

The OTU with the strongest positive correlation with the amount of phenanthrene in soil was a member of the *Polaromonas* genus (Pearson's correlation coefficient $r = 0.923$), which was one of the more abundant genera found in NP PHE forest soils. Moreover, the top 10 highly correlated OTUs all presented very high correlation coefficients (Pearson's correlation coefficient r ranging from 0.880 to 0.824) and were mostly present in the NP pots (Table S3).

Alternatively, the strongest negative correlations between OTUs and phenanthrene concentrations in soil were mostly for OTUs well represented in the agricultural soil (Table S3).

PAH-RHDα Gram-negative (GN) gene diversity. Three phyla were found in the PAH-RHDα GN gene data set: *Bacteroidetes* (aggregated with other phyla under "Other

TABLE 2 Summary of the three-way analysis of the variance (ANOVA), with White's correction for heteroscedasticity on the relative abundance of the 16S rRNA gene OTUs identified at the genus level of bacteria^a

Treatment	Df	F	Pr(>F)
<i>Acidobacteria</i> subgroup 2CL			
Soil	1.000	6.402	0.013
Contamination	1.000	1.200	0.276
Plant	1.000	0.333	0.565
Soil:contamination	1.000	0.232	0.632
Soil:plant	1.000	18.052	<0.001
Contamination:plant	1.000	2.681	0.105
Soil:contamination:plant	1.000	0.236	0.629
Residuals	88.000		
<i>Actinobacteria Acidothermus</i>			
Soil	1.000	202.047	<0.001
Contamination	1.000	2.195	0.142
Plant	1.000	54.144	<0.001
Soil:contamination	1.000	0.606	0.438
Soil:plant	1.000	8.664	0.004
Contamination:plant	1.000	0.034	0.855
Soil:contamination:plant	1.000	2.662	0.106
Residuals	88.000		
<i>Gammaproteobacteria Aquincola</i>			
Soil	1.000	1.083	0.301
Contamination	1.000	29.161	<0.001
Plant	1.000	2.822	0.097
Soil:contamination	1.000	2.972	0.088
Soil:plant	1.000	18.062	<0.001
Contamination:plant	1.000	0.634	0.428
Soil:contamination:plant	1.000	0.034	0.853
Residuals	88.000		
<i>Actinobacteria Pseudarthrobacter</i>			
Soil	1.000	23.154	<0.001
Contamination	1.000	0.165	0.685
Plant	1.000	6.909	0.010
Soil:contamination	1.000	6.797	0.011
Soil:plant	1.000	3.357	0.070
Contamination:plant	1.000	2.648	0.107
Soil:contamination:plant	1.000	0.531	0.468
Residuals	88.000		
<i>Acidobacteria Geothrix</i>			
Soil	1.000	46.073	<0.001
Contamination	1.000	1.607	0.208
Plant	1.000	1.799	0.183
Soil:contamination	1.000	4.526	0.036
Soil:plant	1.000	1.005	0.319
Contamination:plant	1.000	0.004	0.947
Soil:contamination:plant	1.000	0.710	0.402
Residuals	88.000		
<i>Betaproteobacteria Massilia</i>			
Soil	1.000	0.038	0.847
Contamination	1.000	6.642	0.012
Plant	1.000	1.497	0.224
Soil:contamination	1.000	1.138	0.289
Soil:plant	1.000	3.685	0.058
Contamination:plant	1.000	0.491	0.486
Soil:contamination:plant	1.000	2.378	0.127
Residuals	88.000		
<i>Betaproteobacteria Polaromonas</i>			
Soil	1.000	71.821	<0.001
Contamination	1.000	0.612	0.436
Plant	1.000	0.173	0.679

(Continued on next page)

TABLE 2 (Continued)

Treatment	Df	F	Pr(>F)
Soil:contamination	1.000	4.101	0.046
Soil:plant	1.000	10.727	0.002
Contamination:plant	1.000	1.798	0.183
Soil:contamination:plant	1.000	10.349	0.002
Residuals	88.000		
<i>Alphaproteobacteria Variibacter</i>			
Soil	1.000	67.316	<0.001
Contamination	1.000	0.057	0.811
Plant	1.000	9.031	0.003
Soil:contamination	1.000	1.088	0.300
Soil:plant	1.000	8.056	0.006
Contamination:plant	1.000	0.200	0.656
Soil:contamination:plant	1.000	2.349	0.129
Residuals	88.000		
<i>Alphaproteobacteria Sphingomonas</i>			
Soil	1.000	8.023	0.006
Contamination	1.000	5.332	0.023
Plant	1.000	12.001	<0.001
Soil:contamination	1.000	5.730	0.019
Soil:plant	1.000	1.643	0.203
Contamination:plant	1.000	0.407	0.525
Soil:contamination:plant	1.000	1.358	0.247
Residuals	88.000		
<i>Betaproteobacteria Ramlibacter</i>			
Soil	1.000	133.801	<0.001
Contamination	1.000	29.482	<0.001
Plant	1.000	0.486	0.488
Soil:contamination	1.000	4.124	0.045
Soil:plant	1.000	4.620	0.034
Contamination:plant	1.000	3.097	0.082
Soil:contamination:plant	1.000	0.673	0.414
Residuals	88.000		
<i>Gemmatimonadetes Gemmatimonas</i>			
Soil	1.000	57.249	<0.001
Contamination	1.000	9.794	0.002
Plant	1.000	1.408	0.239
Soil:contamination	1.000	0.023	0.879
Soil:plant	1.000	1.705	0.195
Contamination:plant	1.000	0.266	0.608
Soil:contamination:plant	1.000	2.930	0.090
Residuals	88.000		
<i>Acidobacteria Candidatus Solibacter</i>			
Soil	1.000	299.754	<0.001
Contamination	1.000	1.219	0.273
Plant	1.000	28.450	<0.001
Soil:contamination	1.000	0.019	0.892
Soil:plant	1.000	10.756	0.001
Contamination:plant	1.000	0.290	0.591
Soil:contamination:plant	1.000	0.392	0.533
Residuals	88.000		
<i>Verrucomicrobia DA101 soil groupFA</i>			
Soil	1.000	603.476	<0.001
Contamination	1.000	3.051	0.084
Plant	1.000	0.018	0.894
Soil:contamination	1.000	0.228	0.634
Soil:plant	1.000	19.820	<0.001
Contamination:plant	1.000	0.037	0.849
Soil:contamination:plant	1.000	0.643	0.425
Residuals	88.000		

(Continued on next page)

TABLE 2 (Continued)

Treatment	Df	F	Pr(>F)
<i>Chloroflexi</i> HSB OF53 F07FA			
Soil	1.000	580.036	<0.001
Contamination	1.000	0.273	0.603
Plant	1.000	20.951	<0.001
Soil:contamination	1.000	0.170	0.681
Soil:plant	1.000	1.232	0.270
Contamination:plant	1.000	2.152	0.146
Soil:contamination:plant	1.000	0.000	0.993
Residuals	88.000		
<i>Acidobacteria</i> uncultured <i>Acidobacteriaceae</i> subgroup 1			
Soil	1.000	605.363	<0.001
Contamination	1.000	0.201	0.655
Plant	1.000	11.038	0.001
Soil:contamination	1.000	1.004	0.319
Soil:plant	1.000	11.469	0.001
Contamination:plant	1.000	3.830	0.054
Soil:contamination:plant	1.000	0.019	0.891
Residuals	88.000		

^aValues in bold indicate significant effects. Df, degrees of freedom; F, variance ratio; Pr(>F), P value.

Bacteria" in Fig. 4c), *Firmicutes* ("Bacilli, *Lysinibacillus*" in Fig. 4c) and *Proteobacteria*. When looking at the Shannon diversity index, the mean values ranged from 2.442 to 3.801, with significant differences among treatments (Fig. 4a). For P and NP agricultural soils, PAH-RHD α GN Shannon diversity was significantly higher in the PHE soils compared to their corresponding CTRL (Fig. 4a). This pattern was similar for forest soils, but not statistically significant.

Similar trends were observed for species richness (Fig. 4a), with the number of amplicon sequence variants (ASVs) significantly higher in the PHE agricultural soil compared to the CTRLs, for both the P and the NP pots.

PAH-RHD α GN gene community structure. The PCoA of the PAH-RHD α GN gene data set showed that in CTRL soils all the samples clustered more or less together, but that in the presence of contaminant the PAH-RHD α GN communities of the two soil types clustered separately, at the two opposite ends of the first axis (Fig. 4b). PERMANOVA analysis corroborated the significant effects of soil type ($R^2 = 11.9\%$, $P = 0.001$), contamination ($R^2 = 7.2\%$, $P = 0.001$) and the interaction of soil type and contamination ($R^2 = 8.5\%$, $P = 0.001$), and a lack of effect for the presence of plants and the related interactions (Table 1).

PAH-RHD α GN gene community composition. The community composition of PAH-RHD α GN genes reflected well the results of the PCoA and PERMANOVA, with similar communities across all treatments for the CTRL treatment, and largely different communities between the two soil types under PHE conditions (Fig. 4c). The PHE agricultural soils were dominated by the *Betaproteobacteria* genera *Comamonas* and *Ralstonia*, whereas in the PHE forest soils, the *Betaproteobacteria* genus *Delftia* replaced *Ralstonia* (Fig. 4c and Table 3).

For phenanthrene concentrations, we found significant positive correlations with the relative abundance of various *Delftia* spp., (Pearson's correlation coefficient r ranging from 0.537 to 0.515), and significant negative correlations with the relative abundance of two *Ralstonia* spp. (Pearson's correlation coefficient r -0.421 and -0.426) and various *Comamonas* spp. (Pearson's correlation coefficient r ranging from -0.410 to -0.398) (Table S3).

PAH-RHD α GN gene abundance. The abundance of PAH-RHD α GN genes was significantly affected by soil type ($F = 74.64$, $P < 0.001$), contamination ($F = 37.968$, $P < 0.001$), and the interaction between these two factors ($F = 11.142$, $P < 0.001$). Both NP and P PHE forest soils harbored significantly more PAH-RHD α GN gene copies than did agricultural samples and CTRL forest samples (Fig. 4d).

FIG 4 Summary of the diversity, structure, and composition of PAH-degrading bacterial communities based on ASVs of the PAH-RHD α GN genes. (A) Boxplots of Shannon diversity index and species richness (as the observed number of ASVs) by treatment. Letters denote significant differences (Continued on next page)

TABLE 3 Summary of the three-way analysis of the variance (ANOVA), with White's correction for heteroscedasticity on the relative abundance of PAH-RHD α amplicon ASVs identified at the genus level of Gram-negative and Gram-positive bacteria^a

Treatment	Df	F	P value
PAH-RHD α Gram negative			
<i>Betaproteobacteria Comamonas</i>			
Soil	1	0.9105	0.34314
Contamination	1	6.7655	0.01124
Plant	1	0.1857	0.66782
Soil:contamination	1	1.3443	0.25006
Soil:plant	1	0.3172	0.57499
Contamination:plant	1	0.8946	0.34735
Soil:contamination:plant	1	1.5475	0.21749
Residuals	73		
<i>Betaproteobacteria Ralstonia</i>			
Soil	1	0.9909	0.32281
Contamination	1	7.163	0.009184
Plant	1	2.0175	0.159747
Soil:contamination	1	25.5137	<0.001
Soil:plant	1	0.2757	0.601121
Contamination:plant	1	0.2601	0.611605
Soil:contamination:plant	1	1.4116	0.238641
Residuals	73		
<i>Betaproteobacteria Delftia</i>			
Soil	1	30.7704	<0.001
Contamination	1	1.6399	0.204396
Plant	1	0.016	0.899766
Soil:contamination	1	8.4392	0.004857
Soil:plant	1	0.0143	0.90525
Contamination:plant	1	0.4815	0.489946
Soil:contamination:plant	1	0.0542	0.816595
Residuals	73		
PAH-RHD α Gram positive			
<i>Actinobacteria Microbacterium</i>			
Soil	1	17.863	<0.001
Contamination	1	0.968	0.329
Plant	1	1.155	0.287
Soil:contamination	1	4.273	0.043
Soil:plant	1	15.902	<0.001
Contamination:plant	1	2.338	0.132
Soil:contamination:plant	1	7.418	0.009
Residuals	58		
<i>Actinobacteria Mycobacterium</i>			
Soil	1	1.478	0.229
Contamination	1	43.171	<0.001
Plant	1	7.798	0.007
Soil:contamination	1	4.302	0.043
Soil:plant	1	3.534	0.065
Contamination:plant	1	0.193	0.662
Soil:contamination:plant	1	8.304	0.006
Residuals	58		

^aValues in bold indicate significant effects. Df, degrees of freedom; F, variance ratio.

PAH-RHD α Gram-positive (GP) gene diversity. The Shannon diversity of the PAH-RHD α GP genes was generally higher in the forest soils compared to the agricultural soils (Fig. 5a). Kruskal-Wallis tests showed that the differences among treatments were significant ($\chi^2 = 65.466$, $P < 0.001$), and the Dunn's test revealed that the P and NP CTRL agricultural soils had a significantly lower diversity than all the other treat-

FIG 4 Legend (Continued)

found with the Tukey's HSD *post hoc* test. (B) Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity of the relative abundance of the PAH-RHD α GN ASVs showing the effects of contamination, soil type, and plant presence on the PAH-degrading Gram-negative bacterial community structure. (C) PAH-degrading Gram-negative bacterial community composition at the class level. Only taxa with a relative abundance above 0.01 are shown. Values are averaged across treatments ($n = 12$). (D) PAH-RHD α GN gene copy numbers determined by real-time PCR quantification on DNA. Legend: NP, nonplanted pots; P, planted pots; CTRL, uncontaminated pots; PHE, pots contaminated with 50 mg kg⁻¹ of phenanthrene.

FIG 5 Summary of the diversity, structure, and composition of PAH-degrading bacterial communities based on ASVs of the PAH-RHDα GP genes. (A) Boxplots of Shannon diversity index and species richness (as the observed number of ASVs) by treatment. Letters denote significant (Continued on next page)

ments, except for the P PHE agricultural soils (Fig. 5a). The trends for the abundance of ASVs observed were similar, with the P and NP CTRL agricultural soils showing a significantly lower abundance of PAH-RHD α GP ASVs compared to all the forest soil treatments.

PAH-RHD α GP gene community structure. The PCoA ordination of the PAH-RHD α GP gene data set showed a strong differentiation between the community structure of the two soil types ($R^2 = 14.6\%$, $P = 0.001$). The main effects of contamination ($R^2 = 3.7\%$, $P = 0.001$) and plant presence ($R^2 = 1.9\%$, $P = 0.019$) were also significant, as well as the interaction between the three factors ($R^2 = 2.0\%$, $P = 0.01$; Table 1). For the forest treatment, there was a clear separation between the communities in NP and P pots, but not so much between the PHE and CTRL communities. In contrast, for the agricultural treatment, the PHE and CTRL communities were distinct from one another, but we also observed a separation of the NP and P communities on the first (CTRL) or second (PHE) axis of the ordination (Fig. 5b).

PAH-RHD α GP gene community composition. All the treatments, regardless of soil type, plant presence or contamination status were dominated by *Microbacterium* spp. ASVs (*Actinobacteria*) that accounted for more than 50% of the total amplicon sequences recovered for the PAH-RHD α GP genes (Fig. 5c). The other 50% of ASVs were divided among the phyla *Actinobacteria*, *Proteobacteria*, and other unclassified *Bacteria*. The difference between the PHE agricultural P and NP pots observed in the PCoA could be due to the higher relative abundance of *Mycobacterium* (*Actinobacteria*) in the P PHE agricultural soils (Fig. 5c and Table 3). Also in accordance with the trends observed in the PCoA, the two different soil types showed different PAH-RHD α GP community compositions (Fig. 5c).

The top 10 most positive significant correlations with phenanthrene concentrations were for some *Microbacterium* and undefined *Actinobacteria* showing Pearson's correlation coefficient r ranging from 0.505 to 0.599 (Table S3). In this case, no significant negative correlations were found.

PAH-RHD α GP gene abundance. There was a highly significant main effect of soil type ($F = 72.674$, $P < 0.001$) and of plant presence ($F = 10.523$, $P = 0.002$) but not of contamination on the number of PAH-RHD α GP gene copies (Fig. 5d). Forest soils showed higher numbers of PAH-RHD α GP genes than the agricultural soils, whereas the P pots harbored generally higher abundance of GP gene copies compared to the NP pots (Fig. 5d).

DISCUSSION

Recent studies have suggested that the initial soil diversity was linked to the outcome of soil remediation and phytoremediation (45–48). Similarly, the initial soil diversity has been identified as responsible for the variable efficiency of phytoremediation in practice (49). Based on those studies, we had hypothesized that differences in the microbiological characteristics of soil are a major constraint to PAH rhizoremediation, overriding the rhizosphere effect central to this technology. Although it is difficult to completely disentangle the effects of soil physicochemical and microbiological characteristics with our experimental design, our results confirmed that soil types had a major influence on the microbial communities and degradation functions, which resulted in different remediation patterns for the PAH phenanthrene. Indeed, soil type constrained the response of the general bacterial community to the rhizosphere effect and to contamination, with shifts mostly visible for the forest soil. Similarly, for both hydrocarbon-degradation functional genes targeted, the response of the com-

FIG 5 Legend (Continued)

differences found with the Tukey's HSD *post hoc* test. (B) Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity of the relative abundance of the PAH-RHD α GP ASVs showing the effects of contamination, soil type, and plant presence on the PAH-degrading Gram-positive bacterial community structure. (C) PAH-degrading Gram-positive bacterial community composition at the class level. Only taxa with a relative abundance above 0.01 are shown. Values are averaged across treatments ($n = 12$). (D) PAH-RHD α GP gene copy numbers determined by real-time PCR quantification on DNA. Legend: NP, nonplanted pots; P, planted pots; CTRL, uncontaminated pots; PHE, pots contaminated with 50 mg kg $^{-1}$ of phenanthrene.

munity to contamination was constrained by soil type. These microbiological differences were mirrored in the phenanthrene degradation, which was significantly higher in the agricultural soil, with no rhizosphere effect, and lower in the forest soil, where the presence of plants significantly decreased contaminant concentrations. In contrast, fungal communities responded strongly to the rhizosphere effect, regardless of soil type and contamination, and a soil type effect was only visible in the absence of a plant.

In terms of phenanthrene degradation, the agricultural soil outperformed the forest soil. However, there were no significant differences in phenanthrene degradation in planted and unplanted pots for the agricultural soil. Both treatments efficiently degraded nearly 98% of the spiked phenanthrene at the end of the 9th week, suggesting that the mere fact of distributing the soil in pots and watering them stimulated the microbial degradation enough that the poplar trees had no effect in agricultural soils. On the contrary, significant differences in the degradation of phenanthrene were observed between forest planted and unplanted pots. In the unplanted pots, we observed a mean total degradation around 80% compared to 90 to 95% in the planted pots. This means the effectiveness of rhizoremediation is strongly influenced by the soil type and its initial microbial diversity. Although we did not measure the initial soil microbial communities, the strong differences in fungal and bacterial communities observed in the control unplanted pots at the end of the experiment suggest that the soils initially harbored very different microbial communities. Some soils appear to effectively degrade phenanthrene and potentially other PAH through the action of the microbial communities alone, without the need to introduce a plant to shift the microbial communities, whereas other soils with a different bacterial diversity and physicochemical characteristics may need stimulation from a plant in order to more effectively degrade phenanthrene. Other studies had already shown the divergences that occur during remediation of organic compounds (50), with communities of microbial degraders being significantly different depending on the soil type and the original microbial diversity. When it comes specifically to PAH, the response of different soil microbial communities was also divergent, with groups of different microbial degraders arising when exposed to PAH alone or in cocontamination (51). These contrasting observations may result from the initial soil microbial diversity, which might explain the variable efficiency of phytoremediation across different sites.

Ideally for rhizoremediation to work across sites with different soil types, the rhizosphere effect should be stronger than the soil effect. Maybe poplars do not offer this level of rhizosphere effect for PAH decontamination. Nevertheless, it is important to notice that we used a single pure contaminant in this study, which is rarely the case in nonexperimental conditions. Other studies with this and other plant species have shown the overriding effect of soil characteristics on bacterial communities in a variety of studies, compared to plant characteristics (52–55). Alternatively, as fungi were shown here and elsewhere to be generally more affected by plant characteristics (47) and identity (56), rhizoremediation based on fungi could be more successful across various soil types. However, metatranscriptomic studies have shown that hydrocarbon degradation genes are mainly expressed by bacteria during rhizoremediation using willows (27, 57, 58).

Even though the community composition of PAH Gram-negative degraders was similar in control soils for both soil types, the communities widely differed when soils were contaminated, with *Ralstonia* and *Comamonas* dominating in the agricultural soils versus *Comamonas* and *Delftia* dominating in the forest soils. The divergence we observed in our results, depending on the soil type, is in agreement with the findings of other studies (50, 51). This is a good example of functional redundancy that is one of the trademark aspects of soil diversity (59) and can be defined as the coexistence of multiple distinct taxa or genomes capable of performing the same biochemical function (60). The relationship between biological diversity and ecological processes is far from being completely understood at a mechanistic level (60). Our study has shown that even in the context of high-functional redundancy among PAH degraders, the outcome at the process level, namely the degradation of phenanthrene, varies widely

among different soils. Similar results were previously reported, where two different soils showed specific degradation patterns for phenanthrene (30). Conversely, in the unplanted pots, PAH-degrader communities were similar for both soil types, but resulted in different degradation rates of phenanthrene, pointing toward an interaction between degrader communities and soil physicochemical characteristics or other microbial communities. Taken together, these results suggest that not all degraders are equal, and that depending on the pool of degraders in the original soil, of the soil physicochemical and microbiological characteristics, and on the level of influence of the plant on the degraders, the outcome of the remediation will vary for similar levels of degrader diversity. Interestingly, *Lysinibacillus* (*Firmicutes*), a well-known Gram-positive bacteria, was amplified in control soils using the PAH-RHD α Gram-negative primer set. The genus *Lysinibacillus* contains some species capable of degrading hydrocarbons and phenanthrene (61, 62). However, to our knowledge, this is the first time *Lysinibacillus* has been amplified with the PAH-RHD α Gram-negative primer set. It could well be that the sequence of the PAH-RHD α of *Lysinibacillus* presents enough homology to the primer sequence to allow for annealing with the Gram-negative primer set.

The bacterial community was mainly shaped by the soil type, but the contamination and the presence of a plant also influenced the community structure and composition. These effects were not evident at the phylum level but were clearly visible at finer taxonomical levels. For instance, *Polaromonas* and *Sphingomonas* were relatively more abundant in the PHE soils, in line with previous reports (30). *Polaromonas* is a genus containing known hydrocarbon-degrading species such as *Polaromonas naphthalenivorans* (30, 41, 63). It was accordingly correlated positively with the phenanthrene concentrations in soils, as it was found mainly in nonplanted forest soils, where the phenanthrene degradation was probably still ongoing. Based on our qPCR quantification of PAH-RHD α Gram-positive genes, PAH degraders were significantly more abundant in planted pots compared to unplanted pots, regardless of the contamination status of the soil. This agrees with previous studies that reported higher abundance of hydrocarbon degradation genes and transcripts in the rhizosphere of plants, even in the absence of contaminant (63), which was suggested to be linked with the structural similarity between several plant secondary metabolites present in root exudates and PAH (64). In contrast, the rhizosphere effect on PAH-RHD α Gram-negative genes was stronger in the contaminated soils, an effect that had been previously reported (57). Moreover, the soil contamination has also been found to override the willow rhizosphere effect on transcript abundance patterns in field studies (58).

In contrast to bacterial and to PAH-degrader communities, fungal communities were very strongly influenced by the presence of poplar and not so much by contamination. These results suggest that the fungal community is relatively stable when it comes to responding to stress caused by phenanthrene contamination, both in the rhizosphere of poplars and in the absence of plants. Most importantly, the convergence from strikingly different fungal communities in the absence of plants toward the more homogeneous communities found in rhizosphere indicate a strong plant effect. The main reason behind this trend was the complete dominance of the ectomycorrhizal fungus *Sphaerosporella* in the rhizosphere of poplars across all treatments. This fungus has been found to dominate the rhizosphere of various *Salicaceae* tree species in a range of pot and field experiments involving remediation of different contaminants. For instance, Dagher, Pitre, and Hijri (65) found that willow (*Salix miyabeana* clone SX67) inoculation with *Sphaerosporella brunnea* alone was capable of significantly improving biomass production and Ba, Zn, and Cd phytoextraction with bioaccumulation in the shoot plant biomass, and an additional decrease of Cu, Pb, and Sn concentrations in soil. However, Bell et al. (48) reported that early colonization by *S. brunnea* negatively impacted the Zn accumulation efficiency of willows. In another study, the relative abundance of *S. brunnea* was positively correlated with the shoot biomass of willows (47). In Tardif et al. (66), *S. brunnea* showed an increase in its relative abundance in the rhizosphere of willows with increasing petroleum hydrocarbon concentrations. Simi-

larly, willows were shown to associate more strongly with *Sphaerospora* when growing in highly contaminated fields of Canada, but this trend was only visible for North American willows and not for Asian or European genotypes (54). Therefore, it appears that *Sphaerospora* plays a growth-promoting or stress-relieving role for *Salicaceae* growing in contaminated soils, supporting the idea of a dual effect of the microbial community in rhizoremediation processes, where some elements would play a degradative role while others would stimulate the plant growth via direct plant growth promotion, or by relieving stress (14). Despite that this is not directly related to the degradation of contaminants, it is still of the utmost importance for rhizoremediation, as this technology is only effective where roots penetrate the soil and an impaired growth is likely to reduce efficiency. In the present study, since *Sphaerospora* colonized all the rhizospheres regardless of the soil type or the contamination status, it is difficult to conclude any positive effects on poplar growth and/or tolerance to contamination stress. One indication is that poplars did not present any visible stress response to phenanthrene exposition and produced equal amounts of biomass in the contaminated and noncontaminated pots. Alternatively, it could also be that the concentration of phenanthrene used was not high enough to trigger a strong stress response that would hinder plant development, as was observed in previous studies (67–69). Yet again, other microbes in the rhizosphere could also have contributed by degrading enough phenanthrene in the proximity of the roots to allow for plant roots to grow without contaminant stress (14). Finally, the lack of an apparent response of fungi to phenanthrene contamination could well be due to the presence of relic DNA in the soil, which could have obscured this and other results. However, previous studies arrived at similar conclusions using methods that were not affected by relic DNA, such as metatranscriptomics, which suggests that the trend observed here is most likely due to live organisms and not to the sequencing of relic DNA.

In conclusion, rhizoremediation using poplars significantly improved the degradation of phenanthrene in forest soil, but not in agricultural soil. The fungal communities were the only ones strongly affected by the presence of poplar, whereas the effects of poplar on the diversity, composition, and abundance of the total bacterial and PAH-degrader communities were strongly constrained by soil type, probably leading to this different degradation patterns between soils. This has important implications for rhizoremediation, since these results highlight the importance of preliminary microbiological or physicochemical studies of contaminated soils in order to determine whether plant presence could improve remediation rates. Further studies could improve the predictive power of such preliminary analyses, leading to a wider adoption of this green remediation technology.

MATERIALS AND METHODS

Experimental design. The experimental design consisted of a full factorial experiment involving three factors: (i) soil contamination, (ii) presence of the plant, and (iii) soil type. The contamination factor consisted of two levels: soil contaminated with 50 mg kg⁻¹ dry soil of phenanthrene (PHE) and uncontaminated soils (CTRL). Plant presence consisted of either a pot without a plant (NP) or a planted pot (P). Finally, the soil type factor consisted of one agricultural and one forest soil. These three factors with two levels each produced 8 treatment combinations. The 12 total replicates per treatment were arranged in four blocks. Pots were randomly distributed inside blocks. A total of 96 pots were placed outside at the Laurentian Forestry Center (Natural Resources Canada, Sainte-Foy, QC, Canada). After planting, the pots were covered with coconut fiber to reduce phenanthrene evaporation and photooxidation. The plants were subsequently connected to an automated irrigation system and received approximately 400 ml of water every day at 8 a.m. Phenanthrene leakage into the running water was evaluated and judged negligible for both soils in a preliminary experiment (data not shown). Therefore, no water collection system was implemented.

Soil and biological material. In this experiment, two types of soils were selected: an agricultural soil and a forest soil. The agricultural soil was collected from 15 cm of the upper soil layer from two sites closely situated within a family farm located in Ste-Famille de l'Île d'Orléans, QC, Canada (GPS coordinates site one: 46.941037, -70.974966 and site two: 46.937038, -70.968887). The farm had been alternating crops for more than 30 years, with rotations including corn, soy, oat, hay, and with fallow every 3 years. The forest soil used in this experiment was a mix of two different soils. Two thirds (vol/vol) of the forest soil mixture corresponded to soil collected from the upper layer of two closely situated forested plots of the Valcartier forest research station located in Saint-Gabriel-de-Valcartier, Quebec Canada (GPS coordi-

nates for site one: 46.950110, -71.497922 and for site two: 46.951730, -71.497097). Due to the high content of organic matter and low mineral proportion of this forest soil, 1/3 of garden topsoil was incorporated to the forest soil. This was done in order to better compare the two soil type matrices in terms of physical characteristics. The garden topsoil was acquired from a local company (Les développements Robko Inc., Quebec City, QC, Canada). All the soils used were thoroughly homogenized prior to sieving (4 mm mesh) and before setting the experiment. Physicochemical analyses of the experimental potting mixes were performed at AgroEnviroLab, La Pocatière, QC, Canada (Table S1).

Half of each soil was spiked with 50 mg kg^{-1} dry soil of phenanthrene according to the following protocol. One-tenth of the soil was spiked while leaving the rest untreated. Five hundred milligrams of phenanthrene (Sigma-Aldrich) diluted in 100 ml of acetone was applied to 1-kg batches of dried 2-mm-sieved soil. The soil spiked this way was left outside protected from direct sunlight for 24 h until the acetone was completely evaporated. Then, the spiked batches were thoroughly incorporated into 9 kg of uncontaminated soil in a cement mixer until the potting mix reached homogeneity.

The plants used for the experiments were cuttings harvested from the same adult poplar tree. Cuttings were propagated following a previously described method (70). Briefly, cuttings were kept at 4°C until rooting. Dormancy was disrupted by soaking the cuttings in a rooting mixture for a few weeks, then planting in 500 g of potting medium. Subsequently, between 19 July and 21 July 2017, a total of 48 trees were planted into 6-liter pots containing approximately 5 kg of either agricultural or forest mix soil, PHE or CTRL. Forty-eight extra pots containing only soil were used for the nonplanted treatments (NP).

Sampling and plant trait measurements. The experiment was sampled between 18 and 20 September 2017, 9 weeks after planting. Aboveground biomass was clipped, collected, and kept at 4°C until biomass measurements. For the planted pots, the soil adhering to the roots after a vigorous shaking of the root system was considered “rhizosphere” (P soil). The nonplanted soil was collected at a depth of approximately 10 cm (NP soil). Soil samples were kept at 4°C until transportation to the lab (around 2 h), where they were placed at -20°C . Soil samples were used for phenanthrene and microbial community analyses. Harvested plants were weighed fresh and again after 24 h at 60°C to obtain dry biomass. The remaining contaminated soils were left outdoors for photooxidation and complete biodegradation of phenanthrene for 14 months. Following an analytical confirmation of the complete disappearance of the phenanthrene, the soil was disposed of as regular soil.

Phenanthrene quantification. Soil samples were analyzed for phenanthrene content at the end of the experiment for both NP and P pots. Two phenanthrene measurements were taken per biological replicate. The phenanthrene extraction protocol consisted of the following in-house method developed in the lab.

For sample preparation, approximately 4 g of frozen soil was placed in a screw cap glass tube. Ethyl acetate ($900 \mu\text{l}$) was added to each sample together with 3 ml of distilled water, and 10 ppm of phenanthrene- d_{10} in $100 \mu\text{l}$ of ethyl acetate was added as the internal standard. Glass tubes containing samples were shaken to thoroughly mix the soil with the organic solvent. Next, the tubes were placed in an ultrasonic bath at 60 kHz for 15 min to detach the remaining phenanthrene from the soil organic matter. Then, the tubes were placed horizontally in a shaker at 300 rpm and left overnight at room temperature. The next day, the tubes were centrifuged at $270 \times g$ for 10 min. Afterward, the ethyl acetate phase was recovered and further centrifuged at $5,000 \times g$ for 1 min. Lastly, the ethyl acetate containing the phenanthrene was recovered and placed in a GC vial for gas chromatography mass spectrometry (GC-MS) analysis.

Extracts were analyzed for phenanthrene content with a Trace GC Ultra system (Thermo Scientific) equipped with a $30 \times 0.25 \text{ mm}$ ($0.25 \mu\text{m}$ thickness) DB-5 MS capillary column (Agilent J & W capillary GC) and coupled to a Polaris Q benchtop ion trap mass spectrometer. The injector and analyzer temperatures were at 250°C and 350°C , respectively. The GC-MS temperature program consisted of a 2-min hold at 70°C , increasing temperature to 310°C at $30^{\circ}\text{C min}^{-1}$, followed by a 6-min hold at 310°C . Helium was used as carrier gas at a flow rate of 0.3 ml/min . The injection volume was $3 \mu\text{l}$ and the MS scan range was from 70 to 600 m/z .

Calibration curves for the samples were prepared using phenanthrene as a marker. The phenanthrene was weighed and dissolved in ethyl acetate at concentrations ranging from 1 ppm to 100 ppm. Standard calibration curves were calculated by plotting the peak areas against the corresponding concentration of reference.

DNA isolation and amplicon library preparation. DNA was extracted from up to 250 mg of soil using the DNeasy Powersoil DNA isolation kit (Qiagen) following the protocol provided. The bead-beating step was carried out on a FastPrep-24 (MP Biomedicals) at 6 m/s for 45 s, twice. Bacterial and archaeal V4 regions of the 16S rRNA gene were amplified using the primer set 515F ($5'-\text{GTGCCAGCMG CCGCGGTAA-3'}$) and 806R ($5'-\text{GGACTACHVGGGTWTCTAAT-3'}$) (71). The fungal internal transcribed spacer region 1 (ITS1) was amplified using the primers ITS1F ($5'-\text{CTTGGTCATTTAGAGGAAGTAA-3'}$) and 582R ($5'-\text{CTGCGTTCTTCATCGAT-3'}$) (72). The PAH-RHD α gene cluster corresponding to Gram-negative (GN) bacteria (*nahAc*, *nahA3*, *nagAc*, *ndoB*, *ndoC2*, *pahAc*, *pahA3*, *phnAc*, *phnA1*, *bphAc*, *bphA1*, *dntAc*, and *arhA1*) was amplified using the primer set 610F ($5'-\text{GAG ATG CATACC ACG TKG GTT GGA-3'}$) and 916R ($5'-\text{AGC TGT TGT TCG GGA AGAYWG TGC MGT T-3'}$). The gene cluster corresponding to Gram-positive (GP) bacteria (*narAa*, *phdA/pdoA2*, *nidA/pdoA1*, and *nidA3/fadA1*) was amplified using the primer set 641F ($5'-\text{CGG CGC CGA CAA YTT YGT NGG-3'}$) and 933R ($5'-\text{GGG GAA CAC GGTGCC RTG DAT RAA-3'}$) developed elsewhere (25). All microbial regions were amplified in $25\text{-}\mu\text{l}$ volumes using $19.125 \mu\text{l}$ of sterile water, 50 nM bovine serum albumin (BSA), 20 mM *Taq* reaction buffer (20 mM Tris HCl [pH 8.4]), 400 nM ($0.5 \mu\text{l}$ at $20 \mu\text{M}$) of each primer, 500 nM MgCl_2 , 200 nM dNTP, 0.25 U *Taq* DNA polymerase, and $1 \mu\text{l}$ of

DNA template (5 to 10 ng/ μ l) on a T100 thermal cycler (Bio-Rad). Thermal cycling conditions for *Bacteria* and *Archaea* were as follows: initial denaturation at 95°C for 5 min; 35 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 1 min; and a final elongation phase at 72°C for 5 min. For fungi, a similar protocol was used except that the annealing temperature was at 59°C and 30 cycles were performed. PAH-RHD α genes also had different annealing temperatures set at 57°C and 54°C for GN and GP primer sets, respectively, for a total of 30 cycles. Following amplification, PCR products were cleaned using 16 μ l of magnetic beads (Agencourt AMPure XP, Beckman Coulter Life Science) following the Illumina protocol 16S metagenomic sequencing library preparation guide (part number 15044223 Rev. B). Then, 400 nM each of Nextera XT unique index primers (2 μ l at 5 μ M) was added to 5 μ l of our PCR product, in a PCR mix containing 50 nM BSA, 20 mM *Taq* reaction buffer (20 mM Tris HCl [pH 8.4]), 500 nM MgCl₂, 200 nM dNTP, 0.25 U *Taq* DNA polymerase, and 12.175 μ l of sterile water. The PCR product was tagged under the following thermal cycling conditions: 95°C initial denaturation phase for 5 min, followed by 8 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 68°C for 30 s, and a final elongation phase at 68°C for 5 min. The indexed amplicons were purified using magnetic beads as described above, and the DNA was quantified using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen). The libraries were combined in an equimolar ratio and sent for 2 $\times$ 250 bp paired-end sequencing on an Illumina Miseq at the Genome Quebec Service Center (Montréal, QC, Canada).

Quantification of PAH-degrading bacteria. The real-time quantitative PCR (qPCR) was conducted on a Stratagene Mx3005P qPCR system (Agilent Technologies), associated with the corresponding software MxPro Mx3005P (v4.10; Agilent). The qPCR analyses were performed using the primers designed by Cébron et al. (25) in 20 μ l total volume containing 1 $\times$ iTaq universal SYBR Green reaction mix supplemented with 300 μ M of each primer (Integrated DNA Technologies) and 5 μ l of DNA template at a concentration around 5 to 10 ng μ l⁻¹ or distilled sterile water (i.e., negative no-template control).

The amplifications were carried out following the protocol provided in Cébron et al. (25) with some modifications. Briefly, the first step consisted of denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing for 35 s at either 57°C (GN) or 54°C (GP), and an elongation step at 72°C for 75 s, after which the SYBR green signal intensities were measured. At the end of the run, a melting curve analysis was performed where signal intensities were measured at 0.5°C temperature increments every 5 s from 51 to 95°C. Standards for each gene were made from 10-fold dilutions of linearized plasmid containing the gene fragment of interest, cloned from amplified soil DNA (52).

At the end of the qPCR run, the threshold line was automatically defined within the logarithmic increase phase of the acquired fluorescence data. The threshold cycle (C_T) values were assessed for all the samples and the gene copy numbers were deducted from the standard curve based on the C_T value. Efficacy of the qPCRs were 89.3% (standard curve $R^2 = 0.998$) and 92% ($R^2 = 0.995$) for PAH-RHD α GN genes and 90.6% ($R^2 = 0.995$) and 85.8% ($R^2 = 0.999$) for PAH-RHD α GP genes.

Bioinformatic analyses. Sequences were analyzed by AmpliconTagger (73). Briefly, raw reads were scanned for sequencing adapters and PhiX spike-in sequences. Remaining reads were filtered based on quality (Phred) score and remaining sequences were dereplicated/clustered at 97% identity (16S rRNA and ITS genes) and then processed for generating operational taxonomic units (OTUs) (DADA2 v1.12.1) (74). Chimeras were removed with the DADA2 internal removeBimeraDeNovo (method = “consensus”) method followed by UCHIME reference (75). OTUs for which abundance across all samples was lower than 5 were discarded. A global read count summary throughout the pipeline steps is provided in Table S5 for all data sets. OTUs were assigned a taxonomic lineage with the RDP classifier (76) using an in-house training set containing the complete Silva release 138 database (77) supplemented with eukaryotic sequences from the Silva database and a customized set of mitochondria, plasmid, and bacterial 16S sequences. For ITS OTUs, a training set containing the Unite DB was used (sh_general_release_s_04.02.2020 version). The RDP classifier assigns a score (0 to 1) to each taxonomic depth of each OTU. Each taxonomic depth having a score of ≥ 0.5 was kept to reconstruct the final lineage. Taxonomic lineages were combined with the cluster abundance matrix obtained above to generate a raw OTU table, from which a bacterial organism OTU table was generated.

Custom RDP classifier training sets were generated for both PAH-RHD GP and GN amplicon data types. Raw reads were processed as described above up to the quality filtering step. Then, remaining sequences were dereplicated/clustered at 100%, thus generating amplicon sequence variants (ASVs) (DADA2 v1.12.1) (74).

Each ASV was subject to a BLAST search (BLASTn) against the NCBI nucleotide (nt) database (downloaded as of 21 February 2020) with `-max_target_seqs` set to 20. BLAST output was filtered to keep hits that had an E value of $\leq 1e-20$, alignment length of at least 100 bp, and alignment percentage of at least 60%. Taxonomic lineages of each filtered BLAST hit were fetched from the NCBI taxonomy database (version downloaded December 4 2019). RDP training sets were generated as described (<https://github.com/jtremblay/RDP-training-sets>).

Statistical analyses. Statistical analyses were performed in R (v.3.5.0) (78), with basic analysis performed with the stats package (78). Normality and homoscedasticity of data were calculated with Shapiro-Wilk and Bartlett tests, respectively. When distributions did not attain normality, variables were log transformed or converted using a Box-Cox transformation using the MASS package (79). The differences in plant trait results and phenanthrene concentration were assessed through univariate analysis of variance using ANOVA followed by *post hoc* Tukey's honestly significant difference (HSD) tests. When transformations did not yield linearity, Kruskal-Wallis analysis of the variance was performed instead, with the Dunn's test performed for multiple comparisons. Univariate analysis of relative abundance of genera was performed with ANOVA test with White's correction for heteroscedastic data using the car package (80). Differences were considered significant at the 0.05 level.

Shannon diversity index and species richness were calculated with the *otuSummary* package (81).

Principal-coordinate analyses (PCoA) were performed to assess differences in community composition and between treatments using normalized OTU or ASV tables with the Bray-Curtis dissimilarity index calculated with the *vegan* package (82). PERMANOVA analyses tested the interaction between treatments in the microbial communities through 999 permutations using the *Adonis* function from *vegan*. All graphs were created with the *ggplot2* package (83). Finally, correlation analyses were performed with the *stats* package.

Data availability. The raw sequence data were deposited in the short read archive (SRA) of the National Center for Biotechnology Information (NCBI) under the BioProject accession [PRJNA666553](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA666553).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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We declare no competing interests.

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