Bacterial diversity associated with poplar trees grown on a Hg-contaminated site: Community characterization and isolation of Hg-resistant plant growth-promoting bacteria

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Elsevier Editorial System(tm) for Science of the Total Environment

Manuscript Draft

Manuscript Number: STOTEN-D-17-06881R1

Title: Bacterial diversity associated with poplar trees grown on a Hg-contaminated site: community characterization and isolation of Hg-resistant plant growth-promoting bacteria

Article Type: VSI: AquaConsoil 2017

Keywords: Phytomanagement, Poplar habitats, Trace elements, Plant growth-promoting bacteria, Mercury-resistant bacteria

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Abstract: Industrial waste dumps are rarely colonized by vegetation after they have been abandoned, indicating biological infertility. Revegetation of industrial tailings dumps is thus necessary to prevent wind erosion, metal leaching and has been shown to restore soil functions and ecosystem services. However, little is known about the microbial colonization and community structure of vegetated tailings following the application of restoration technologies. In this study, we investigated the rhizosphere and phyllosphere bacterial communities of a poplar tree plantation within a phytomanagement-based restoration program of a Hg-contaminated site. We used Illumina-based sequencing combined with culture-dependent approaches to describe plant-associated bacterial communities and to isolate growth-promoting bacteria (PGPB) and Hg-resistant bacteria. The genus Streptomyces was highly specific to the root community, accounting for 24.4 % of the relative abundance but only representing 0.8 % of the soil community, whereas OTUs from the Chloroflexi phylum were essentially detected in the soil community. Aboveground habitats were dominated by bacteria from the Deinococcus-Thermus phylum, which were not detected in belowground habitats. Leaf and stem habitats were characterized by several dominant OTUs, such as those from the phylum Firmicutes in the stems or from the genera Methylobacterium, Kineococcus, Sphingomonas and Hymenobacter in the leaves. Belowground habitats hosted more cultivable Hg-resistant bacteria than aboveground habitats and more Hg-resistant bacteria were found on the episphere than in endospheric habitats. Hg-resistant isolates exhibiting plant growth-promoting (PGP) traits, when used as inoculants of Capsicum annuum, were shown to increase its root dry biomass but not Hg concentration.. The N2-fixing and Hg-resistant species Pseudomonas graminis, observed in the poplar phyllosphere, may be a key microorganism for the restoration of industrial tailings dumps.

Response to Reviewers: See attached file

Dear Editor

We are pleased to submit a revised version of our manuscript: STOTEN-D-17-06881

We hope we have now responded satisfactorily to the comments of the reviewers. The English was initially edited by the "American Journal Expert" author service.

Sincerely yours

M Chalot, Professor

NB: The line numbers provided below correspond to the marked revised version of the pdf.

Reviewer #1:

This study characterizes the bacterial communities associated with poplar trees growing in a mercury-contaminated site. The manuscript presents the results of illumina-based sequencing characterizing bacterial communities associated with the bulk soil and rhizosphere soil, but also of endophytic communities associated with different plant compartments. In addition, the authors used culture-dependent techniques to isolate Hg-resistant bacterial strains with potential application as inoculants in future studies. The overall theme of the study is suitable to the scope of STOTEN, of particular novelty is the characterization of the endophytic bacterial communities using these sequencing approaches. The results are therefore of considerable interest and represent an important advance in this field. Unfortunately, the manuscript is not very well written, and the general quality of English is poor. I would recommend that, at least the introduction, methods and
discussion section, are thoroughly revised. Some changes are proposed below but the article still requires further revision and re-writing of several sections to meet with the standards of STOTEN.

Specific comments:
Abstract:
-Page 2, Line 24-26. This sentence does not make sense, please re-write. e.g. Industrial waste dumps are rarely colonized by vegetation after they have been abandoned, indicating biological infertility.
-Page 2, Lines 26-28. Change to "to prevent wind erosion and to restore soil functions and ecosystem services"
These sentences have now been modified for more clarity lines 24-30.

-Page 2, Line 28-29. This declaration is a bit too vague. For example, studies can now be found on microbial colonization of mine tailings. It would be good if the authors could be a bit more specific about the type of waste dumps to which they are referring.
We agree, this comment has now been rephrased lines 30-31.

-Page 2, Lines 42-43 "were used as inoculants and increased root dry biomass but did not increase Hg concentration in leaves." Specify the plant species you inoculated.
The sentence has been corrected lines 47-49 and plant species have now been specified.

Introduction:
-Page 3, Lines 54-55- The authors give statistics for European agricultural land, is this relevant to waste dumps?

We consider those data are relevant because land allocated to storage of industrial wastes decrease suitability for agricultural activities around contaminated sites. However, we have now added a comment to highlight the link between agricultural lands and waste dumps, lines 61-64. .

-Page 3, Lines 56-57 Phytomanagement-based restoration programs of contaminated soils often use woody plant species which are readily colonized by essential microorganisms. Please provide more details and references regarding this "ready" colonization. Also specify the types of contmainated soils to which you are referring.
We agree, this statement has now been modified for more clarity and reference papers have now been added lines 66-69.

-Page 3, Lines 59-61. Correct to "Among the microorganisms that colonize the plant rhizosphere and phyllosphere, some play important roles in determining plant fitness and are collectively described as plant growth-promoting bacteria (PGPB)."
Done lines 71-75.

-Page 3, Line 63 - indoleacetic acid
Done line 77.

-Page 3, Lines 66-72, this text is a bit confusing
We agree, the sentence has now been modified for more clarity lines 80-86.

-Page 3, Line 71 WHat do you mean here by "Widely"?
The comment has now been toned down line 90-91.

-Page 4, Line 73. "endophyte-infested"?? Do you refer to the transmission of endophytes through seed or plant vegetative parts???
Yes, indeed. Corrections have been made for more clarity lines 92-94.

-Page 4, Line 75 "which are hosted by numerous genera". In several parts of the introduction the authors refer to numerous genera or various bacteria, but they should less vague and could give some examples
We agree, corrections are done and several examples are now listed when needed lines 86-88, lines 95-100, 102-105 and lines 115-117.

-Page 3, Line 76-77 and lines 79-80. Authors should refrain from over-simplification
We agree, correction has been done lines 101-102 with some more details added of the paper by Gal *et al.* lines 106-113.

-Page 3, Lines 80-82. this sentence does not make sense
The whole sentence has now been rephrased lines 120-127.

-Page 3, Lines 84-86. Too vague, the authors should try to highlight the novelty of their own work, and specific situation, and not generalise regarding the advantages of the techniques they used -Page 3, Line 86-88. This phrase needs re-writing
-Page 3, Lines 90-91. Here you should specify that you are investigating the diversity of rhizosphere, endosphere and phyllosphere bacterial communities, so that the author sees the novelty of the work
The whole paragraph has now been rephrased and clarified, lines 133-144.

Methods:
-Page 5 - This section would benefit from further details regarding the site and its

characteristics, and the industrial activities causing the contamination. "industrial dump" is a bit too vague
We agree, some details are now provided lines 149-154.

-Page 5, Line 97. Which major River?
Added line 150.

-Page 5, Line 100. What do you refer to by "control" soil? Page 5, Line 100.
Clarifications have now been added lines 156-157.

-What do you refer to by the "lagoon"?
This term has now been replaced by "dump" line 158.

-Page 5, Line 102-103. Mention that the SRC system was implemented as part of a phytomanagement strategy
Done lines 160.

- Page 5, Line 106-107. Here you state that metabarcoding analysis was carried on samples of bulk soil, root, stem and leaf. It is not always clear what type of samples were used. Later on you mention rhizosphere soil, bulk soil and root endophytic populations. The description of the sampling and type of samples used should be clearer. Also, there is no mention of the plant tissue treatment before DNA extraction and amount of plant tissues used for extraction.
This whole section has now been restructured and detailed lines 164-178.

-Page 7, Lines 161-164. This is confusing
-Page 7, Line 166. What quantity of stem and leaf tissues were used for the isolation process
The sentences have now been modified for more clarity lines 181-190.

-Page 7, Line 167. Root endophytes or rhizosphere soil?
Yes, root endophytes, clarification added lines 177-178.

-Page 7, Did you confirm a successful surface sterilization?
Yes, tests on agar plates confirmed surface sterilization.

-Page 8, Line 175. Please correct here and throughout "cultivabilities" for "densities"
Done: line 260, line 473, line 474, and line 481.

-Page 8, Line 179-180. What do you mean by "further established"? Bacterial strains were maintained in LB broth and 25% glycerol?
Strains were stored in 25 % sterile glycerol containing 75 % brain heart infusion broth.
The sentence has now been modified for more clarity lines 265-268.

-Page 10, Line 245. The authors should explain their choice of plant species for the pot experiment. Do they intend to transfer the results of the inouclation experiment with pepper to the field where they cultivate poplar? Why did they decide to use a crop which can accumulate Hg? Is it of relevance/interest for remediation processes at the contaminated site?
-Page 10, Line 249. Which other crops were tested? why edible crops?
Clarifications have been added to answer all these questions lines 335-343.

-Page 10, Line 250 "liter" not "litter"
Correction done, line 345.

-Page 10, Was the experiment run for 68 days or 46 days. The description is confusing. Were the plants grown in peat and exposed to Hg-atmosphere?

Clarifications have now been added line 343-344.

-Page 10, Line 263 Grown to saturation?
Yes, indeed. We meant grown until maximum Optical Density (OD_{600}) in the culture media. This comment has now been added lines 360-362.

-Page 10, Line 267. Do you refer to the soil inoculum when you talk about the belowground consortium?
The belowground consortium refers to a mixture of the FT79, FT82, FT97 and FT50 bacterial isolates (from the bulk and rhizospheric soils (Table S4). They have been used to inoculate soil in the pot experiment.
Clarifications have been done , lines 365-366.

-Page 12, Line 272. "Hg concentrations" not "amounts"
Correction done, line 371.

-Page 18, Line 432. Units?
Unit is now provided lines 532-533.

Discussion:
-Page 19, Line 440-441. Bacterial communities in four habitats (soil, root, stem and leaf) associated with poplars on a Hg-contaminated sit were characterized using ...
Correction is done, lines 541-545.

-Page 19, Lines 443-445, Lines 445-446. These phrases need re-writing
The sentences have now been modified for more clarity lines 545-554.

-Page 19, Line 447. What do you mean by mid-season?
This term is fully explained in the sentence lines 554-555.

-Page 19, Line 452 "can be problematic due to unwanted chloroplast DNA amplification"
Correction has been done, line 561-562.

-Page 20, Lines 476-486. the authors compare the bacterial communities associated with poplar to the results observed in similar studies using alpine rock cress. The comparison is not directly apparent. The authors should include comparisons with their own previous studies:
We agree, this part of the discussion has now been improved lines 593-598.

Page 20, Line 485. Lopez et al. 2017 worked with natural Ni-rich soils, not contaminated soils. Since the dominance of Chloroflexi is unusual it would be interesting if the authors could comment further on this result.
We agree, this interesting result has now been highlighted lines 600-606.

Page 21, Line 489. this feature has been observed in studies focusing on the cultivable bacterial communities in contaminated soils
Correction is done p22, lines 608-610.

Page 22, Line 518-519. The cultivar should not be given in italics
Corrections are done, line 640 and line 641.

Page 23, Line 554-555. Again comparing densities with clover does not seem a very logical comparison
As stated in the text, the number of studies related to the phyllospheric compartment is scarce. However we agree wit this comment and this statement has now been deleted

Page 23, Line 558-559. What do you mean?
The sentence has now been clarified, line 682-683.

Reviewer #2

This paper investigates the effects of poplar trees on mercury contaminated soil, with a focus on soil and plant associated bacterial communities. The authors sequence partitions (soil, root, stem and leave) from six trees on three different mercury contaminated sites at one time point. Number of replicates sample per plant are not detailed i.e. do soil, leave, stem numbers = 1 per tree? If so how do the authors know these are representative of the entire sample?
This is now detailed lines 164-179.

Subsequently they isolated mercury resistant bacteria from each of the habitats, and tested them for plant growth promoting traits (PGT). Using a consortium of these bacteria a growth experiment was conducted whereby the microorganisms are added to soil leaves etc. of mercury contaminated soils plus and minus plants. The rational and specific hypothesis for this experiment are not indicated until the discussion (L582-584) this should be brought forward. Indeed, the final paragraph of introduction,focuses only on the sequencing. The overall hypothesis for this is that poplar tree plantation "will significantly and differently ship the bacterial communities from different soil and poplar habitats" yet they do not have a control site without poplar trees, nor poplar trees grown on non-contaminated sites. Thus, they do not address this hypothesis in the paper. This should be re-phrased to reflect this. Further, the isolation and pot experiments rational/hypothesis are not reflected in the introduction.
We agree. The introduction has now been rephrased lines 135-144.

Minor comments
Section 2.2 Metabarcoding
- Experimental design - only soils with trees planted on them were examined. No control soil, or control poplar plants from non-contaminated sites were included.
We have previously compared microbial communities from native Salicaceous (inc. poplar) trees grown on the dump with microbial communities from native salicaceous growing outside the dump (Zappelini et al., 2015). The two communities shared ca. 65% of their OTUs. We have also compared vegetated soil with non-vegetated soil. In the present paper, as now highlighted in the introduction, we mostly aimed at comparing the different poplar habitats, as the literature is lacking this kind of information. We did not intend to compare with unplanted soil as there is a large plant diversity that may bring some further unnecessary complexity.

- How many soil, root, stem, leaf replicates were collected per tree? It appears to be only 1?
For each habitat, 4 samples were collected for each of the six random trees that were selected on three replicated plots (24 samples per habitat). This is now detailed lines 164-179.

- Why were different extraction methods used for soil and leaves, and importantly what effect did/could this have on the data and comparison of soil to root/stem/leaf?
Using a single extraction method for the various matrices is barely done in papers where different matrices are studied. A key feature when using metabarcoding approaches is indeed that comparable amounts of DNA should be used for further processing, which is not reached when using a single extraction protocol. In a first trial, we found that DNA extraction from soil samples using procedures or commercial kits used for plants

resulted in very low DNA amounts of poor quality, unsuitable for Illumina sequencing. DNA extraction from soil sample is frequently done using specific DNA extraction mixture (kits) that counteracts the inhibitory effect of e.g. polyphenols.
For instance, the use of different extraction protocols for various matrices is frequently reported in the literature:
- Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. Knief et al. 2012
- Diazotrophic potential among bacterial communities associated with wild and cultivated Agave species. Desgarennes et al. 2014
- Biogeography and cultivation affect microbiome composition in the drought-adapted plant Subgenus Agave. Coleman-Derr et al. 2016
-Beckers 2017

L116 and throughout: use 16S rRNA, not just 16S
Corrected throughout the manuscript

L118: Details of PCR amplification and library preparation are missing.
The details have now been added lines 198-204.

L155: Why 25% of habitat samples, this seems low, especially if overall sample number per compartment are low (see comment above on number of replicates per plant).
As detailed now in the text (lines 164-179), there were 24 replicates per habitat (4 replicates collected on each of the 6 trees); we considered that at least an OTU should be present in 6 samples over the 24.

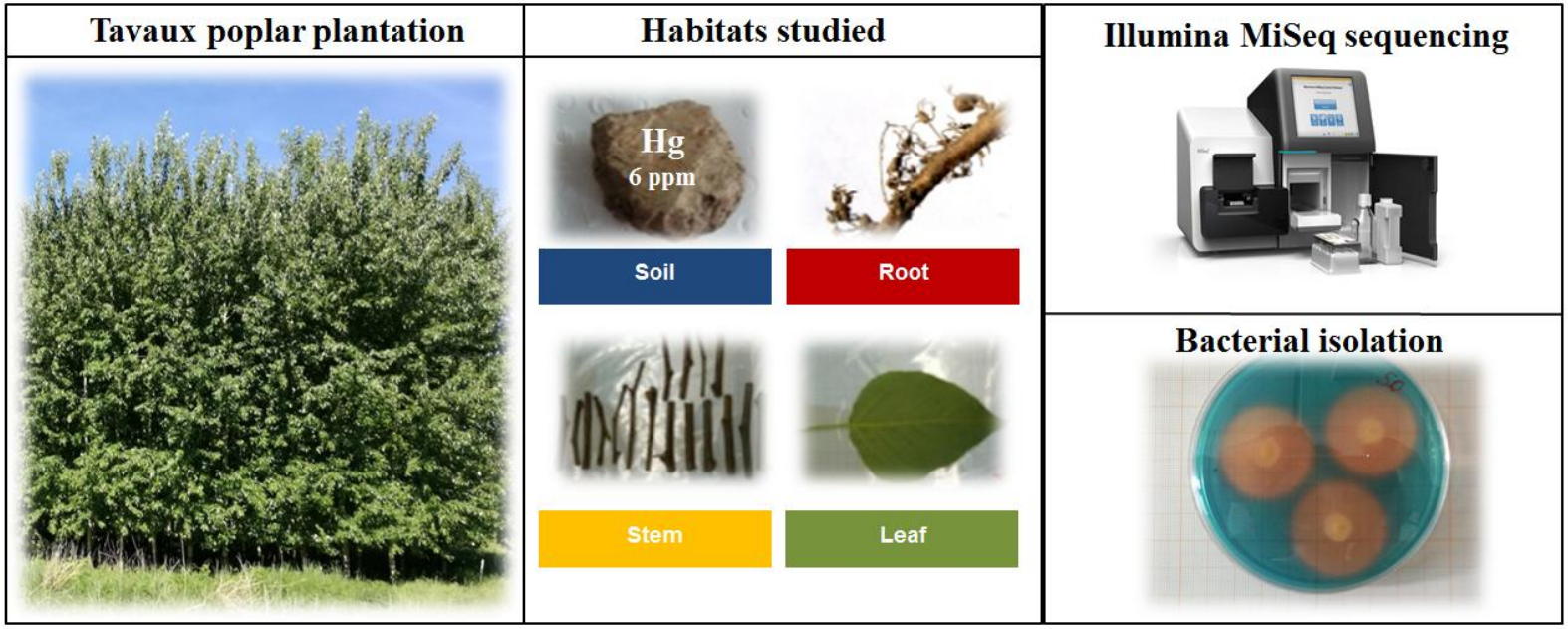
L189: Still the 16S rRNA gene even if you targeted DNA
Corrected throughout the manuscript

L245: pepper plants?
Now explained lines 334-344.

Pot experiment: was there any measure of the viability of the microorganisms added to the soil/leaves over time? How can you know they survived? Results indicated that the inoculum did not affect the rate of Hg accumulation, but did increase root growth. How do the authors know that the inoculated cells survived within the soil/on leaves etc.?
Rational for the pot experiments needs to be outlined
Unfortunately, we have not measured the colonization ability nor the viability of the inoculum. However, as we have seen an effect in root growth we believed that at least part of the inoculum was able to successfully establish at the soil and/or plant level. On the other side, it would be very interesting to know if all the strains were successfully established or the positive response was due to one specific strain. Moreover, due to the short-term duration of the experiment, we believe that a positive response in root growth is significant since this would be the first part of the plant influenced by the microorganisms / the main part that the plant uses for its establishment in a new environment. We are aware of the importance of these types of measurements for a correct interpretation of inoculation protocols. However, unfortunately, the protocols to carry out these measurements in bacteria are still not fully developed or available, besides being expensive in time and money.

*Graphical Abstract

GA V3



Highlights

- Poplar habitats host Hg-resistant PGPB in a Hg-contaminated site
- Bacterial communities were assessed through Illumina Miseq sequencing
- Belowground habitats host higher diversity than aboveground habitats
- *Chloroflexi* phylum was especially abundant in the Hg-enriched soil
- Bulk soils host more cultivable Hg-resistant bacteria than other habitats

Bacterial diversity associated with poplar trees grown on a Hg-contaminated site: community characterization and isolation of Hg-resistant plant growth-promoting bacteria

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Abstract

~~Large dumps used to store industrial tailings are unable to generate significant vegetated surfaces after they have been abandoned, suggesting that these surfaces become biologically infertile.~~ Industrial waste dumps are rarely colonized by vegetation after they have been abandoned, indicating biological infertility. Revegetation of industrial tailings dumps ~~produced by various industrial activities~~ is thus necessary to prevent wind erosion, metal leaching and has been shown to restore soil functions and ecosystem services~~to prevent dust storms and erosion and to restore soil ecosystem services~~. However, little is known about the microbial colonization and community structure of ~~re~~vegetated tailings following the application of ~~site~~ restoration technologies~~ of Hg contaminated site. s.~~ In this study, we investigated the rhizosphere and phyllosphere bacterial communities of a poplar tree plantation within a phytomanagement-based restoration program ~~at~~ of a Hg-contaminated site . We used Illumina-based sequencing combined with culture-dependent approaches to ~~isolate and characterize plant~~describe plant-associated bacterial communities and to isolate growth-promoting bacteria (PGPB) and Hg-resistant bacteria. The genus *Streptomyces* was highly specific to the root community, accounting for 24.4 % of the relative abundance but only representing 0.8 % of the soil community, whereas OTUs from the *Chloroflexi* phylum were essentially detected in the soil community. Aboveground habitats were dominated by bacteria from the *Deinococcus-Thermus* phylum, which were not detected in belowground habitats. Leaf and stem habitats were characterized by several dominant OTUs, such as those from the phylum *Firmicutes* in the stems or from the genera *Methylobacterium*, *Kineococcus*, *Sphingomonas* and *Hymenobacter* in the leaves. Belowground habitats hosted more cultivable Hg-resistant bacteria than aboveground habitats and more Hg-resistant bacteria were found on the episphere than in endospheric habitats. Hg-resistant isolates exhibiting plant growth-promoting (PGP) traits, when ~~were~~ used as inoculants of *Capsicum annuum*, ~~and~~were shown

to increased its root dry biomass but did not increase Hg concentration in leaves.were incubated and increased root dry biomass but didn't increased Hg concentration in leaves. The N_2-fixing and Hg-resistant species *Pseudomonas graminis*, observed in the poplar phyllosphere, may be a key microorganism for the restoration of industrial tailings dumps.

Keywords: Phytomanagement, Poplar habitats, Trace elements, Plant growth-promoting bacteria, Mercury-resistant bacteria

1. Introduction

Soil contamination by metal(loids) and trace elements (TEs) such as Ag, As, Cd, Cr, Cu, Hg, Ni, Pb, Se, or Zn is a threat to ecosystem stability and a risk to human health. In 2011, 127,000 sites in 33 European Union countries were confirmed to have contaminated soils, and approximately 45 % of these sites (58,000) were under remediation project management to reduce associated risk (remediation) (Panagos et al., 2013). Data published in 2016 showed that 6.24 % (137,000 km^2) of European agricultural lands were unsafe for food production, requiring local assessment and eventual remediation (Toth et al., 2016). Due to the frequent lack of a plant cover at industrial dumps, processes such as wind erosion or leaching can led to the contamination of adjacent agriculture lands leading to the decrease in soil productivity.

Phytomanagement-based restoration programs for contaminated soils may use woody plant species that are readily colonized by essential microorganisms (Bell et al., 2014; Partida-Martínez and Heil, 2011; Yergeau et al., 2012), especially in highly stressed and nutrient-poor environments, such as heavy metalsTE- contaminated sites (Durand et al., 2017; Foulon et al., 2016a, 2016b) (Partida-Martínez and Heil, 2011). However, knowledge of these associated microbial communities remains scarce, particularly with respect to aboveground tissues. Among the microorganisms that colonize the plant rhizosphere and phyllosphere, some are

known to play important roles in determining plant fitness and are collectively described as plant growth-promoting bacteria (PGPB) ~~Among the microorganisms that colonize the rhizosphere and the phyllosphere of a plant, some play important roles in plant fitness and are collectively described as plant growth-promoting bacteria (PGPB)~~ (Glick, 2014; Lindow and Brandl, 2003; Pieterse et al., 2016). PGPB directly benefit host plants via hormone production (*e.g.*, ~~Correction has been done~~~~indole acetic acid~~indoleacetic acid, cytokinin, and zeatin) and improved nutrition and indirectly by acting as biocontrol agents, inducing systemic resistance and ethylene stress resistance (Bringel and Couée, 2015; Glick, 2005; Lindow and Brandl, 2003; Maignien et al., 2014; Trouvelot et al., 2014; Vorholt, 2012). Belowground and aboveground plant ~~, plant~~ tissues provide ~~various~~ habitats, which can be easily colonized by specific PGPB ~~for bacteria growth/colonization and may host specific PGPB in or on those tissues~~ ~~Belowground, various bacteria live in, on, or around plant tissues and may stimulate plant growth, nutrition and health~~ (Vessey, 2003), ~~while aboveground, leaves and stems provide a broad habitat for bacteria and may host specific PGPB in or on tissues~~ (Vorholt, 2012). In the soil, free-living bacteria of various genera ~~exert beneficial effects on plants~~ such as *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Curtobacterium*, *Klebsiella*, *Enterobacter*, *Pseudomonas*, and *Serratia* exert beneficial effects on plants (Kloepper *et al.*, 1989 ; Glick *et al.*, 1999 ; Benizri *et al.*, 2001) and are classified as rhizospheric PGPB. ~~Widely, p~~Plant-associated bacteria also include endophytic bacteria, which colonize internal plant tissues ~~of plants~~. They are originating from the epiphytic bacterial communities of the rhizosphere and phyllosphere ~~as well as from~~but may also arise from transmission through seeds or plant vegetative parts ~~endophyte-infested seeds or planting materials~~ (Hallmann et al., 1997). ~~These bacterial communities may host endophytic PGPB which are hosted by numerous genera such as~~Members of the genera ~~such as~~ *Acinetobacter*, ~~—~~ *Aminobacter*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Curtobacterium*,

Devosia, *Enterobacter*, *Gluconacetobacter*, *Herbaspirillum*, *Methylobacterium*, *Microbacterium*, *Ochrobactrum*, *Paenibacillus*, *Pseudomonas*, *Phyllobacterium*, *Rahnella*, *Shinella*, *Sphingomonas* and *Staphylococcus* have been described as plant endophytes (Compant et al., 2009; De Meyer et al., 2015; Doty et al., 2009). Other bacterial endophytes that are only localized in specific structures, such as members of the genera *Azorhizobium*, *Bradyrhizobium*, *Devosia*, *Ensifer*, *Frankia*, *Mesorhizobium*, *Microvirga*, *Ochrobactrum*, *Phyllobacterium and Rhizobium* (Glick 2014, Kloepper et al. 1989; Wang et al. 2012; De Meyer et al. 2015). Aboveground, phyllospheric PGPB are subjected to fitness stresses (*e.g.* direct solar radiation, drought), that are very different than those found belowground, suggesting a specific selection pressure (Lindow and Brandl, 2003). It has been demonstrated that the survival capacity of *Pseudomonas fluorescens* SBW25 in the phyllosphere is dependent on the expression of habitat specific genes that are significantly more correlated to the fitness of the bacteria in the phyllosphere that in the soil or the rhizosphere (Gal et al., 2003). Albeit, a full understanding of these bacterial communities has not been reached (Vacher et al., 2016), several phyllospheric PGPB have been already previously described such as members of *Arthrobacter*, *Azotobacter*, *Beijerinckia*, *Klebsiella*, *Methylobacterium*, *Nostoc*, *Pantoea*, *Pseudomonas*, *Scytonema*, *Sphingomonas and Stigonema* (Lindow and Brandl, 2003; Vacher et al., 2016). However, gaining an understanding of the structure and composition of microbial communities *in situ* is difficult due to the diversity and complexity of these communities. Metabarcoding using high-throughput sequencing technologies offers a solution to describe the bacterial communities associated with environmental habitats with high

resolution, as exemplified recently in our previous work (Durand et al., 2017; Foulon et al., 2016a, 2016b). We demonstrated for instance that *Alphaproteobacteria* and *Actinobacteria* dominated root bacterial communities, whereas soil samples were dominated by *Alphaproteobacteria* and *Acidobacteria* (Foulon et al., 2016b). These innovative approaches still need complementary culture-dependent approaches to allow for the functional characterization and selection of the best-performing PGPB.

~~Metabarcoding using high-throughput sequencing technologies offers a solution to describe the bacterial communities associated with environmental habitats with high resolution. However, culture-dependent approaches are far from obsolete. Indeed, functional characterization must be performed to select the best PGPB, and bacterial isolation therefore remains unavoidable.~~

In this study, we combined a metabarcoding approach that employs a high-throughput Illumina MiSeq platform (Durand et al., 2017; Foulon et al., 2016a, 2016b; Schmidt et al., 2013; Wu et al., 2015) and a culture-dependent approach~~es~~ to investigate the composition and assembly of ~~characterize~~ bacterial communities ~~investigate bacterial diversity~~ and isolate Hg-resistant PGPB in the rhizosphere, endosphere and phyllosphere of ~~a polar~~poplars ~~plantation~~ at a Hg-contaminated site. ~~We hypothesized that the presence of a poplar tree plantation on an industrial dump would significantly and differentially shape the bacterial communities from the different soils and poplar habitats.~~ We expected that we would observe clear differences in the relative abundance and composition of bacterial communities across poplar habitats that may improve our understanding of the microbial ecology of these environments. We also aimed at better understanding the role of isolated bacterial strains in Hg transfer from the atmosphere to plant tissues using PGPB inoculation of the model plant *Capsicum annum*.

2. Materials and Methods

2.1. Site description

The chlor-alkali ~~study~~ site is located in Tavaux, France, in Western Europe along ~~a major~~the Saône river ~~(Saône)~~ and is surrounded by several urban aggregates. ~~The study site is a dump/This~~The ~~study was carried out at an~~ industrial dump ~~which~~ was exploited as a sediment storage area from the 1950s to 2003. These sediments were originated from the effluents produced during the chlor-alkali Hg-cell process used ~~during~~for the electrolysis of NaCl~~tic processes associated with a Hg cell chlor-alkali process~~. ~~–~~A detailed map of the industrial site ~~is~~ was provided by Maillard et al. (2016). ICP-AES analyses revealed that total Hg and total As were significantly increased in the dump compared to the levels in ~~control~~ soil from an adjacent undisturbed forest~~nearby~~,. Further details ~~of soil analyses~~on soil characteristics~~-~~ of the ~~lagoon~~ dump are provided in Zappelini et al. (2015). The poplar cultivar Skado (*P. trichocarpa* x *P. maximowiczii*) was planted in 2011 as a short rotation coppice (2200 stems ha^{-1}) as part of a phytomanagement strategy, fully described in Durand et al. (2017).

2.2. Sampling design~~Metabarcoding analysis~~

~~Samples of soil (bulk), root, stem and leaf (named from now on as habitats) were collected in August 2014.~~ Four~~Four~~ replicates were sampled in August 2014 ~~for each habitat~~ at ~~two~~six random trees ~~selected from three different field plots~~, making a total ~~(a total~~ of 24 samples per habitat~~)~~.(bulk soil, root, stem and leaf). ~~–~~Soil samples composed of bulk soils

from under the canopy of the poplars were sieved to <4 mm. In more details, the roots were collected after litter removal from the upper 20 cm layer of soil from under the canopy of the trees. They were separated from the soil via 2 distilled water baths, and the smallest roots were selected and separated from larger roots by cutting them with a scalpel. Woody samples were collected from poplar branches of the axe 2 (0.8-1.2 cm in diameter) at a *ca*. 5 m height, corresponding to the half-crown of the poplar. Leaf samples were composed of 3 leaves collected from the branches collected as described above. 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. ~~Detailed information about sampling procedure is described in Durand et al 2017~~

2.3. Metabarcoding analysis

~~Sampling for metabarcoding analysis was carried out in August 2014 on the 4 different habitats: bulk soil, root, stem and leaf. For each habitat, 4 samplesSamples were collected from each of the six random trees that were selected on three replicated plots (2 trees per plot, 24 samples per habitat), with DNA extracted as previously described in Durand et al. (2017) . Briefly, environmental~~ DNA was extracted from biological samples within 2 months after sampling. Plant material (root, stem and leaf samples), was thoroughly washed with distillated water ~~?~~and DNA was extracted from ~~XX~~20 mg of fresh material using an adapted cetyl trimethylammonium bromide/chloroform/isoamyl alcohol protocol (Lefort and Douglas 1999; Healey et al. 2014) ~~for, root, stem and leaf samples, whereas environmenta~~. ~~Soil~~ DNA ~~from soil samples~~ was extracted from 100 mg of sieved fresh ~~material~~soil, using a PowerSoil DNA Isolation Kit following the manufacturer's instructions (MoBio Laboratories, Inc., Carlsbad, CA USA). Extracted DNA was purified using a Power Clean® Pro DNA

193 Clean-Up Kit (MoBio Laboratories, Inc., Carlsbad, CA USA). Equimolar DNA pools were
194 adjusted to 10 ng μl^{-1}, and PCR conditions sequencing of the bacterial ~~16S~~ 16S rRNA region
195 was performed on an Illumina MiSeq platform (Microsynth AG, Switzerland). PCR
196 amplification of the 16S rRNA gene was performed~~-~~ using the bacterial primers 799f (5'-
197 AAC MGG ATT AGA TAC CCK G -3') and 1115r (5'- AGG GTT GCG CTC GTT G -3'),
198 which amplify the V5-V6 region (Kembel et al., 2014). These primers target a section of the
199 bacterial 16S rRNA region and generate a small amplicon (~300 bp) that is appropriate for
200 Illumina sequencing. Reactions were performed in triplicate for each sample with the
201 following conditions: 30 s initial denaturation at 98 °C, followed by 20 cycles of 10 s at 98
202 °C, 30 s at 64 °C, and 30 s at 72 °C, with a final 10-min elongation at 72 °C. ~~. These primers~~
203 ~~target a section of the bacterial 16S region and generate a small amplicon (~300 bp) that is~~
204 ~~appropriate for Illumina sequencing.~~

205 Reads were assigned to each sample according to a unique barcode, and contigs were
206 then assigned using the MOTHUR pipeline (Schloss et al., 2009). Raw reads were filtered by
207 length and quality. ~~16S~~ 16S rRNA reads were pre-clustered using sumaclust (Mercier et al.,
208 2013) at 0.99 identity. Only sequences with at least 8 reads were retained (76 % of initial
209 reads). The retained ~~16S~~ 16S rRNA sequences were aligned with those present in the Silva
210 database to remove non-~~16S~~ 16S rRNA sequences, and plant DNA contamination was
211 removed by suppressing reads identified as k__Bacteria (100), p__Cyanobacteria (100),
212 c__Chloroplast (100), o__Streptophyta (100), and unclassified (100). Taxonomic assignments
213 were made using a Bayesian approach (Wang et al., 2007) with the Greengenes database
214 (DeSantis et al., 2006). Finally, OTUs were derived using the Needleman distance and
215 average neighbor clustering at a distance of 0.03.

216 Diversity parameters were calculated based on a dataset in which numbers of reads per
217 sample were rarefied to 3,000 reads per sample. We calculated rarefaction curves at a 97 %

similarity level to verify whether the amount of sequencing reflected the diversity of the original microbial communities. Rarefaction curves were created using the "rarefaction" function and the Vegan package in R (Work et al., 2010). A 2-dimensional non-metric multi-dimensional scaling (NMDS) plot was calculated using the Bray Curtis method (k = 3) based on the standardized (Wisconsin double) and square root transformation of OTU abundance using the "metaMDS" function in the Vegan package. We used ANalysis Of SIMilarities (ANOSIM) to obtain P (i.e., significance levels) and R (i.e., the strength of the factors on the samples) values. These results were paired with a heatmap of Spearman's correlations between the 20 most abundant OTUs in each habitat (a total 59 OTUs) created with "heatmap.2" from the "gplots" package. In addition, in the same pool of OTUs, we use the "multipatt" function of the "indicspecies" package to evaluate the best bioindicator OTUs (De Cáceres et al., 2012). OTU-based analysis of alpha diversity was performed with the following functions using Mothur calculators: "sobs", "chao", "shannoneven", "shannon", "invsimpson", "coverage" and "nseqs" (Schloss et al. 2009). These estimates included observed OTU richness, Chao estimation (Chao, 1949), Shannon diversity index, inverse of Simpson diversity index, and measure of evenness based on the Shannon index and coverage. The coverage calculator returns Good's coverage for an OTU definition (Good, 1953). Coverage was calculated using the following equation: C=[1-(*n*/*N*)]*100 (%), where *n* is the number of OTUs and *N* is the number of sequences. The numbers of OTUs that were shared between habitats were visualized using Venn diagrams that were generated in Mothur with the function "venn". We considered an OTU to be present in a compartment if it was present in at least 25 % of the habitat samples.

2.43. Isolation ~~and characterization~~ of Hg-resistant bacterial strains

Bacterial strains were isolated from bulk and rhizospheric soils and from stem and leaf phyllospheric habitats of poplar trees. Soil properties and Hg concentrations were detailed in

our previous study (Durand et al., 2017). Bulk soil samples (3.5 g) were taken far from the root system under the canopy and placed in a flask containing 35 ml of phosphate buffer (PBS: 8 g of NaCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 per l, pH 7.2). Roots with adhering soil (defined as the rRhizospheric soil) Rhizospheric soil, defined as root-adhering soil, was separated from roots after were washed in a 30 mina PBS bath in a rotatory shaker (30 min, 150 rpm). Roots were separated from the soil suspension andthen rRhizospheric soil solutions werewas recovered after centrifugationed at (14,000 g for 10 min), and 1 g of the rhizospheric precipitated soil was then rediluted suspended by shaking in 35 ml of PBS (shaking?). Phyllospheric epiphytes, defined as the microbes living on the surfaces of aboveground habitats, were isolated from leaves and stems.– Three g of leaves or 2020 g of stems or 3g leaves and addedwere thoroughly washed? with distilled water and suspended in to 150 ml of PBS extraction solution, then placed on a rotatory shaker (150 rpm) for 1 h. Isolation of endophytes from root, stem and leaf habitats was carried out using the same tissues sampled for epiphytic isolation after surface sterilization with 2 % sodium hypochlorite for 2 min. Samples were thoroughly rinsed with sterile distilled water and then were crushed in 35 ml of PBS and macerated for 1 h on a rotatory shaker (150 rpm). Soil and plant suspensions were diluted in 10-fold series and plated onto 1/10 rich medium (869) as well as onto environmental medium (284) (Eevers et al. 2015). Cultivabilities Densities of Hg- resistant bacteria were determined using the same media supplemented with 0, 2 or 10 µM $HgCl_2$ (Francois et al., 2012). The agar plates were then incubated in the dark at 27°C for 6 days. The number of bacteria was expressed as log(log (CFU) g^{-1} dry weight (DW) of sample. Hg-resistant colonies were isolated and purified by subculture on Hg-enriched 1/10 869 or 284 medium. A bacterial collection was further established by growing each previous isolated strains in 6 ml of Luria Bertani (LB) broth (Sigma-Aldrich, Steinheim, Germany) for

15 h on a rotatory shaker (150 rpm) at 30°C and stored in 25 % sterile glycerol containing 75 % brain heart infusion broth (Roth, Karlsruhe, Germany) at -80°C for further analyses.

2.5. Taxonomic identification

For taxonomic identification, ~~E~~each Hg-resistant isolate was cultured in 15 ml of LB medium for 24 h on a rotatory shaker (120 rpm) at 27°C. After centrifugation, DNA was extracted from the pellets using an EZNA bacterial DNA isolation kit (OMEGA Bio-tek, Inc, Norcross, Georgia, USA) according to the manufacturer's instructions. The BOX-PCR fingerprinting method was used to group genotypic profiles at a similarity level of 97 % as previously described (Becerra-Castro et al., 2011). One strain was selected for each BOX-group and R~~16S~~ rDNA genes were amplified from 5 µl of bacterial DNA in a final reaction volume of 50 µl containing 25 µl of 2x PCR master mix (Thermo Fisher Scientific, Carlsbad, California, USA) and each universal primer at 0.5 µM (Eurofins Genomics, Paris, France), specifically 27f (5′- AGA GTT TGA TCM TGG CTC AG -3') and 1492r (5′- GGT TAC CTT GTT ACG ACT T -3′) (Mark Ibekwe et al., 2007). DNA amplification was carried out in a thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) under the following conditions: 4 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, with an additional 15-min cycle at 72°C (Durand et al., 2015). All 16S rR~~D~~NA PCR products were sequenced by Sanger sequencing (Genewiz Beckman Coulter Genomics, United Kingdom). DNA sequences were edited with BioEdit software v7.2.6. (Hall, 1999) screened against the GenBank database using the BLASTn tool at the NCBI website (http://www.ncbi.nlm.nih.gov/) and then aligned with reference sequences of bacteria (LN551925, KR922297, HQ256858, KU587965, KU523561, JX827229, HQ256858, HQ224627, KT825724, NR_028626, LN774179, JF496259, KR476438, JQ956529, FJ380956, and KC790245) using Clustal W (Thompson et al., 1994) as implemented in Bioedit. Neighbourg Joining (NJ) tree reconstructions were produced using MEGA 5 (Tamura

et al., 2011). The most appropriate model of ~~16S~~ 16S rRNA sequence evolution was K2P model (Kimura, 1980).

2.6. Functional characterization

Each isolate of the bacterial collection was screened for the following plant growth-promoting traits: siderophore production, IAA synthesis and phosphate solubilization. The siderophore production of each bacterial strain was determined using chrome azurol sulfonate (CAS) agar medium (Alexander and Zuberer, 1991; Schwyn and Neilands, 1987). After 5 days of incubation on CAS medium at 30°C, a red-orange halo around colonies indicated the production of siderophores. For each strain, the ratio between the diameter of the halo and the diameter of colony was recorded. The IAA synthesis of each bacterial strain was determined using Salkowski's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35 % $HClO_4$) as described by (Barillot, 2012). The phosphate-solubilizing capacity of bacterial strains was determined on Pikovskaya medium (Mehta and Nautiyal, 2001). After 5 days of incubation at 30°C, a discolored halo around colonies demonstrated phosphate solubilization. For each strain, the ratio between the halo diameter and the diameter of colony was recorded. Additionally, MICs (minimal inhibitory concentrations) of Hg were determined for each isolated strain. Briefly, microtitration plates were prepared using 2-fold dilutions of Hg in LB liquid broth, from 0 to 256 µM Hg. The strain *Pseudomonas aeruginosa* PA14, which is resistant to Hg above 256 µM, was used as a positive control. Each well was inoculated with 2 µl of a bacterial suspension, and plates were incubated for 48 h at 27 °C on a rotatory shaker table (180 rpm). After incubation, the bacterial growth was evaluated by measuring the optical density at 600 nm (OD_{600}). Nitrogen fixation ability was evaluated using N-free semisolid malate medium (NFb) (Baldani and Döbereiner, 1980) inoculated with the assayed strains. After 10 days, strains that successfully grew were transferred to fresh NFb medium, and after 7 days of incubation, the characteristic pellicles of diazotrophic bacteria were observed.

Using the previous extracted bacterial DNA, the presence of the genes *merA* and *nifH* in the genome of each strain was assayed. PCR amplification of the *merA* gene was performed using 3 µl of bacterial DNA in a final volume of 20 µl that also contained 10 µl of 2x PCR master mix (Thermo Fisher Scientific, Carlsbad, California, USA) and each primer at 0.5 µM, specifically merA2F (5'- CCT GCG TCA ACG TCG GCT G -3') and merA2R (5'- GCG ATC AGG CAG CGG TCG AA -3') (Poulain et al., 2015). DNA amplification was carried out in a thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) using the following conditions: 10 min at 95°C, followed by 35 cycles of 30 sec at 95 °C, 15 sec at 63 °C, and 30 sec at 72°C, with an additional 5-min incubation at 72 °C. PCR amplification of the *nifH* gene was performed in a final volume of 25 µl containing 5 µl of bacterial DNA, 12.5 µl of 2x PCR master mix, and each primer at 0.5 µM including nifHF (5'- TAC GGN AAR GGS GGN ATC GGC AA -3') and nifHI (5'- AGC ATG TCY TCS AGY TCN TCC A -3') (Laguerre et al., 2001). DNA amplification was carried out in a thermocycler under the following conditions: 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 53.5 °C, and 2 min at 72 °C, with an additional 15-min incubation at 72 °C.

2.~~7~~4. Pot experiment with Hg-resistant bacteria and pepper

Capsicum annuum seeds (Yolo Wonder Pepper) were planted in peat (Brill, Germany) and grown for 68 days. As part of a bioindicator programme implemented at the chlor-alkali site, we found out that –*Capsicum annuum* was the highest Hg-accumulator among other edible (*Solanaceae* or *Brassicaceae*) or woody (poplar, willow) species, well reflecting the actual Hg atmospheric content (Assad, unpublished data). These edible species were chosen, as they are currently cultivated in the vicinity of the chlor-alkali site in private gardens. ~~In a preliminary experiment, this plant species was found to be the highest Hg-accumulator among~~

~~the other edible or woody crops tested and thus may be the most sensitive to any changes of Hg accumulation. This plant was considered as an adequate candidate for their use (in the adjacent areas of industrial sites).as bioindicator of atmospheric Hg pollution~~ ~~Next~~The 68-day-~~s~~ old plants~~,~~ were used to start a 46-day pot experiment ~~was performed~~. Each mesocosm was defined as a pepper plant growing in a 0.5 lit~~t~~er pot in a growth chamber (photoperiod, 16 h; temperature, 18 °C (night) and 22 °C (day); relative humidity, 60-80 %; photosynthetic photon flux density, 250 mmol m^{-2} s^{-1}). In total, 16 *Capsicum annuum* plants were used for each of the following treatments: non-~~un~~inoculated and Hg-unexposed (ni-); inoculated and Hg-unexposed Hg (i-); uninoculated and Hg-exposed (ni+); and inoculated and Hg-exposed (i+). Hg-unexposed plants (ni- and i-) were grown in the same growth chamber, while Hg-exposed plants (ni+ and i+) were grown in a separate growth chamber. Hg-enriched soil (6.38 mg kg^{-1} Hg) from the industrial tailings described above was added to the Hg-contaminated growth chamber to create an Hg-enriched atmosphere as previously described (Assad et al., 2016). The soil was not in direct contact with the plants in any way but provided an atmospheric enrichment with Hg, similar to that observed in the natural environment. Atmospheric Hg in the contaminated and uncontaminated growth chambers was measured at 21.0 and 3.48 ng m^{-3}, respectively. Using the previously isolated bacteria stored at -80 °C, two bacterial consortia were produced to inoculate both the belowground and aboveground compartments of *Capsicum annuum*.

~~-~~The bacteria were grown at 30 °C with shaking at 200 rpm ~~to~~ until maximum optical density (OD_{600}) in ~~saturation in~~the liquid 869 media ~~for~~ was reached~~24 h at 30 °C with shaking at 200 rpm~~. To generate the final phyllospheric consortium (PC), four *Pseudomonas graminis* strains (AT3, AT7, AT8 and AT9), isolated from the phyllosphere were added in equal proportions and adjusted to a final OD_{600} of 1 in ~~0.5 l of~~ PBS. To generate the final belowground consortium (BC), two *Pseudomonas* sp. strains (FT79, FT82) isolated from the

rhizospheric soil and two *Bacillus subtilis* strains isolated from the bulk soil (FT97 and FT50) were used in equal proportions and adjusted to a final OD_{600} of 1 in ~~0.5 l of~~ PBS. For each plant, 4.8 -ml of PC was sprayed onto the leaves, and 10 ml of BC was added near the base of the stems. The same amounts of PBS were added using the same methods with uninoculated plants. In addition, unplanted control pots were prepared.

Stem lengths and ~~the amounts of~~ Hg concentrations in older leaves were measured at 0, 15, 32 and 46 days. In addition, at harvest (46 days), dry biomass of belowground and aboveground tissues ~~plants were~~was measured as well as the total Hg content in younger leaves, fruits, stem and roots. All samples were dried at room temperature and ground into a homogenous powder in a Mixer Mill for 3 min at 30 Hz (model MM400; Retsch Inc., Newtown, Pennsylvania, USA). We used Biolog Ecoplate™ microplates (Biolog Inc., Hayward, CA.) following the manufacturer's recommendations to determine the metabolic capacity of soil microbial communities under the different treatments.

2.~~8~~5. Hg analysis in the substrate and biomass

Hg was measured in the soil and ~~pepper~~ plant samples using an AMA-254 (Advanced Mercury Analyzer) cold vapor atomic absorption (CV-AAS) Hg analyzer (Altec Co., Czech Republic) under the standard conditions recommended by the manufacturer (120 sec drying, 150 sec heating, and 45 sec cooling). The validity of the analytical method was assessed using certified reference material (CRM), specifically Oriental Basma Tobacco Leaves (INCT-OBTL-5) with a certified Hg content of 20.9 ± 1.3 ng g^{-1} DM (Samczyński et al., 2012). Quality controls were regularly performed as described elsewhere (Maillard et al., 2016).

2.~~9~~6. General statistical analysis

The Shapiro test and the Bartlett test were used to check the normality and homoscedasticity of the data, respectively. Our data verifying normality and homoscedasticity were assessed using analysis of variance (ANOVA). In other cases the data were examined using the Kruskal-Wallis test. Statistical analyses were performed with R software, version 3.3.2 (Team R, 2014).

3. Results

3.1. Contrasting microbial communities in poplar tree habitats revealed by metabarcoding

Illumina MiSeq sequencing of bacterial 16S rRNA genes from environmental DNA isolated from four habitats (soil, root, stem and leaf of poplar trees) yielded 4,437,316 sequences. After filtering ~~artifactual~~artiefactual, chimeric and unaligned sequences, 76 % (3,222,285) of the initial sequences were retained, distributed into 2,699 OTUs. After the removal of non-bacterial OTUs, 2,661,227 bacterial sequences remained and composed the operational dataset. Subsampling of the dataset was adjusted to 3,000 reads clustered in 2,414 OTUs (Table S1). Our results showed large variations in total OTU numbers in aboveground *versus* belowground samples.

Rarefaction curve analysis, which assesses species richness ~~from the results of sampling~~, showed that all curves from each habitat were almost parallel with the x-axis, indicating that overall bacterial diversity was well represented (Fig. S1). Visualization of a global analysis of the four habitats using NMDS plots was difficult due to the gap between the aboveground and belowground habitats; therefore, they were represented separately. The NMDS plot of the belowground habitats (root and soil habitats) showed clear clustering of the two microbial communities (Fig. 1a), while the bacterial communities of aboveground ~~habitats~~ tissues (stem and leaf habitat) clustered more closely (Fig. 1b). ANOSIM analyses

confirmed a clear separation between the root and soil bacterial communities (R = 1; P value = 0.001) while a less marked dissimilarity between the stem and leaf communities was observed (R = 0.61; P value = 0.001). Roots (Fig. 1a) and stems (Fig. 1b) data exhibited the greatest between-sample variations, evidenced by their higher scattering.

Diversity and richness indices were calculated for each habitat (Table 1). Most indices occurred in the order soil > root > leaf ≥ stem, revealing that aboveground habitats were significantly less rich than belowground habitats. Comparing within belowground habitats, soil appeared to be significantly more diverse and rich than the root habitat, while that when comparing the aboveground compartments stem habitat was as rich or richer than the leaf habitat but less diverse. Indeed, the Chao1 and Shannon indices indicated that the highest richness and diversity of the microbial community were present in soil samples, followed by the root ~~microbiome~~. Soil reads were evenly spread among diverse OTUs, as revealed by high Simpson indices and high evenness.

The Venn diagram highlights shared and unique OTUs for each habitat (Fig. S2). The sums of the total observed bacterial OTUs in the four sample types in the poplar tree plots were 475, 337, 94 and 77 for soil, root, stem and leaf samples, respectively (Fig. S2). Overall, 9 OTUs (1.4 %) were shared by all compartments, and only 4.2 % of OTUs were found in both belowground and aboveground habitats. Moreover, aboveground habitats shared 50.8 % of their OTUs, while belowground habitats shared 43.9 % of their OTUs. Soil samples contained the highest proportions of unique OTUs (47.3 %), followed by leaf (31.2 %), root (25.2 %) and stem (18.1 %) samples.

Through environmental 16S rRD~~D~~NA sequencing, bacterial communities from the four habitats (soil, root, stem and leaf) were categorized into 30 phyla and 80 classes, the most abundant of which are shown in Fig. 2. ~~The entire dataset was dominated by~~ *Actinobacteria*

(28 %) and *Alphaproteobacteria* (26 %) dominated the entire dataset. The largest proportion of *Actinobacteria* was found in the root habitat (41.7 %) compared to the other compartments (Kruskal-Wallis $X^2 = 45.8$; $P < 1.3 \times 10^{-11}$), and the aboveground samples contained more *Alphaproteobacteria* (35.7 %) compared to belowground samples (Kruskal-Wallis $X^2 = 59.7$; $P < 1.1 \times 10^{-14}$). Altogether, belowground habitats were enriched for *Acidobacteria* (Kruskal-Wallis $X^2 = 68.9$; $P < 2.2 \times 10^{-16}$), *Chloroflexi* (Kruskal-Wallis $X^2 = 66.9$; $P < 2.9 \times 10^{-16}$), *Betaproteobacteria* (Kruskal-Wallis $X^2 = 42.7$; $P < 6.3 \times 10^{-11}$), *Deltaproteobacteria* (Kruskal-Wallis $X^2 = 66.7$; $P < 3.2 \times 10^{-16}$) and *Gammaproteobacteria* (Kruskal-Wallis $X^2 = 66.6$; $P < 3.2 \times 10^{-16}$), whereas aboveground habitats were enriched for *Deinococcus-Thermus* (Kruskal-Wallis $X^2 = 68.0$; $P < 2.2 \times 10^{-16}$) and *Bacteroidetes* (Kruskal-Wallis $X^2 = 59.1$; $P < 1.5 \times 10^{-14}$). Although soil and root habitats were closely related to each other compared to aboveground habitats, both root and soil samples demonstrated also specificity in terms of the composition of their bacterial communities. The soil habitat was distinguished from the root habitat by a higher proportion of *Chloroflexi* (Kruskal-Wallis $X^2 = 66.6$; $P < 3.2 \times 10^{-16}$) and *Gemmatimonadetes* (Kruskal-Wallis $X^2 = 34.6$; $P < 4.1 \times 10^{-9}$) and lower *Actinobacteria* (Kruskal-Wallis $X^2 = 33.1$; $P < 8.6 \times 10^{-9}$). Additionally, soil was the only habitat containing bacteria from the phylum *Nitrospirae*. Bacterial communities inhabiting aboveground habitats were similar in both stem and leaf samples, although *Firmicutes* was only found in stems (8.5 %). A heatmap constructed with the most abundant OTUs in each of the 4 habitats (Fig. 3, 59 OTUs in total) indicated a net clustering between bacterial communities in belowground and aboveground habitats (Fig. 3). Moreover, the "multipatt" function of the "indicspecies" package identified the most specific OTUs for soil, root and phyllosphere habitats (Table S3). Among these 59 OTUs, only 4 were shared by all four habitats, exemplified by OTU00025 assigned to *Pseudomonas* and OTU00023 assigned to *Comamonadaceae*, with both OTUs comprising between 0.5 and 1.5 % of the relative abundance of each habitat. A total of 34

OTUs were specific to belowground samples, and 16 were specific to aboveground samples. Five OTUs, all from the phylum *Firmicutes*, were essentially found in the stem habitat, exemplified by OTU00083 assigned to *Paenibacillus amyloticus*. Among the 16 OTUs shared by stem and leaf communities, the most abundant were species from the genera *Hymenobacter*, *Deinococcus*, *Sphingomonas*, *Kineococcus* and *Methylobacterium*. Among the 34 OTUs shared in belowground habitats, several were more specific to the root habitat, such as *Streptomyces* (OTU00003) and *Micromonosporaceae* (OTU00005 and OTU00014). Indeed, *Streptomyces* was most specific to the root community, accounting for 24.4 % of the relative abundance, but represented only 0.8 % of the soil community. In contrast, OTUs affiliated to the *Chloroflexi* phylum were the most abundant in the soil community.

3.2. Mercury resistance and functional traits of bacteria isolated from belowground and aboveground habitats

Table 2 shows ~~the cultivability density~~ densities of culturable bacteria from the four habitats and the densities~~y~~~~cultivability~~ of Hg-resistant bacteria. In the media without Hg, a higher number of CFUs were isolated in all habitats when using 1/10 869 medium compared to 284 medium, and followed this gradient: rhizospheric soil > bul~~c~~k soil > root endosphere > stem endosphere > leaf episphere > stem episphere > leaf endosphere. The sizes of the cultivable microbial communities were compared to the sizes of communities exposed to 10 µM $HgCl_2$ for all habitats. A decrease in all cultivable bacterial communities was observed after exposure to 10 µM $HgCl_2$. The bacteria living in the phyllosphere demonstrated the lower ~~–~~density~~cultivability~~ when exposed to 10 µM $HgCl_2$ compared to bacteria from the belowground habitats. Hg-resistant bacteria were isolated from bulk soil, rhizospheric soil, epiphytic stem and epiphytic leaf samples, whereas no Hg-resistant endophytic bacteria were found between the isolated strains. A few root endophytic bacteria strains were isolated

485 initially but were not confirmed in subsequent steps. Among all habitats, the bulk soil hosted
486 the highest densities of Hg-resistant bacteria (at 10 µM $HgCl_2$).

487 The list of isolated Hg-resistant species in belowground and aboveground habitats is
488 shown in Fig. 2. Phenotypic and genotypic characterizations were performed for the 56
489 identified Hg-resistant strains (34 isolated from aboveground habitats and 22 from
490 belowground habitats) (Fig. 4 and Table S4). All strains were identified after BLAST analysis
491 of 16S rRNA sequences, and NJ-phylogenetic trees were subsequently established. For
492 example, in the phyllosphere we identified strains from the genera *Pseudomonas*,
493 *Sphingomonas*, *Frigoribacterium*, *Curobacterium*, *Micrococcus*, *Paenibacillus* and
494 *Staphylococcus* (Fig. 4). For those 56 identified strains, the Hg resistance of bacteria from
495 belowground habitats was higher than that of bacteria from aboveground habitats (Table S4).
496 Indeed, the strains found in aboveground habitats presented MIC values between 4 and 64 µM
497 $HgCl_2$ (Fig. 4), whereas the strains from belowground habitats exhibited MIC values between
498 16 and 254 µM $HgCl_2$. In bacteria from various habitats, the presence of the *merA* gene did
499 not consistently correlate with their Hg MICs (Table S4).

500 Regarding the study of PGP traits, ~~S~~surprisingly, the *nifH* gene was observed in all 13
501 aboveground *Pseudomonas graminis* strains (Fig. 4 and Table S4), and an experiment in
502 semi-solid nitrogen-free medium confirmed the ability of these strains to fix N_2. In both
503 aboveground and belowground habitats, all strains demonstrated IAA production, with
504 variations in the amount of IAA produced (mg l^{-1}) between strains. The lowest IAA producers
505 were AT38 (*Paenibacillus amylolyticus*) in the phyllosphere (with 0.9 mg l^{-1} IAA) and FT43
506 (*Pseudomonas fluorescens*) in the belowground samples (with 2.1 mg l^{-1} IAA), while the best
507 IAA producers in the phyllosphere were AT8 (*Pseudomonas graminis*), with 12 mg l^{-1} IAA,
508 and FT97 (*Bacillus subtilis*), with 12.5 mg l^{-1} IAA (Table S4). In belowground habitats, all
509 strains with the exception of ST81 (*Bacillus licheniformis*) showed siderophore activity,

reported as the ratio of the diameter of the orange-colored zone compared to the diameter of the colony; ratios ranged from 1.8 to 4.8. For the belowground bacterial consortium, we selected strains with ratios higher than 3.0. In aboveground samples, 28 of the 34 colonies demonstrated siderophore production, with ratios ranged from 1.1 to 3.0. Phosphate solubilization ability (ratio > 1) was more frequently detected in aboveground bacteria (31 of 34 colonies) than in belowground bacteria (6 of 22 colonies) (Table S4). Finally, the strains with the best profiles in terms of Hg resistance and PGP potential were selected for inoculation (IAA > 4 mg l^{-1} and siderophore ratio > 2.4, plus *nifH* detection, high Hg resistance, phosphate solubilization potential). The strains AT3, AT7, AT8 and AT9 were part of the consortium to inoculate the phyllosphere, and the strains FT79, FT82, FT97 and FT50 were part of the consortium to inoculate belowground.

3.3. Influence of the Hg-resistant bacteria PGPB consortia on plant growth and Hg accumulation in pepper

The Hg-contaminated atmosphere significantly lowered the stem length in both inoculated and uninoculated pepper plants after 32 days compared to the non-contaminated atmosphere, but no differences were observed after 46 days of growth (data not shown) and at harvest, Hg had no impact on plant dry biomass. However, inoculation significantly increased the dry root biomass of Hg-unexposed plants (increase of 24%), and the same trend appeared for Hg-exposed plants (increase of 8%) (Fig. 5a). We did not observe significant differences between various treatments in terms of shoot and total dry biomasses (Fig. 5b and c). Plants from Hg-enriched atmosphere chamber had significantly higher Hg concentrations in leaves at harvest (4 fold higher) than Hg-unexposed plants (Fig. 6). However, inoculation with the selected bacterial consortia had no impact on Hg accumulation. Hg amounts in root samples (expressed in ng mg^{-1} DW) were below quantification limit (0.01 ng Hg). The metabolic activities estimated using Biolog Ecoplate™ microplates revealed that planted soils had

higher metabolic activities than unplanted soils and no differences between Hg-exposed or Hg-unexposed pots were observed (Fig. S3). Among the 31 substrates tested, we observed significant differences for L-phenylalanine, which was metabolized to a greater degree by soil microorganisms of the inoculated plants compared to uninoculated plants.

4. Discussion

The b~~B~~acterial communities ~~in~~of the ~~four~~4 habitats (soil, root, stem and leaf) associated with poplars growing at~~on~~ ~~a~~an Hg-contaminated site were characterized using ~~The experimental design provided us with the opportunity to characterize bacterial communities in 4 habitats (soil, root, stem and leaf) at a poplar tree plantation on a Hg-contaminated site using~~ metabarcoding tools and traditional isolation-based techniques. In ~~this~~the present study, analyses of metabarcoding data made no distinction between epiphytices~~s~~ and endophytices~~s~~ microbial communities of the plant habitats ~~and considered the total community~~. ~~In other~~~~There are other~~Previous studies ~~which~~ showed that~~y~~ only a ~~small~~limited ~~part~~proportion of the microbiome ~~were is~~was ~~found to be~~ specific of the epiphytic or endophytic habitats ~~suggesting a continuum of the microbiome between outside and inside the plant~~ ~~Regarding the metabarcoding approach, at each habitat, the community was considered in its entirety, and no distinction was made between endophytes and epiphytes for the three plant habitats. In other studies were both compartments were analyzed separately a major overlap between the two bacterial communities was observed~~ (Coleman-Derr et al., 2015). We sampled communities under trees during the growing season (from leaf emergence to leaf fall). Notably, sampling was carried out in mid-season (July-August), and we did not attempt to study the seasonal dynamics of our habitats. According to previous studies, the mid-season community in the phyllospheric habitat is more diverse and contains more unique species than the early (May-Jun) or late-season (September-October) communities (Redford and Fierer, 2009).

Illumina-based sequencing of bacterial communities from plant habitats, such as roots, stems and ~~leaves,~~leaves can be problematic due to unwanted chloroplast DNA amplification~~was difficult due to unsuitable chloroplast DNA amplification~~. A similar study investigating oak phyllospheric bacterial communities in oak trees using 16S rRNA amplification of the V6 region with Illumina technologies, revealed higher proportions of oak chloroplast sequences (up to 92.1 %- from the total sequences) than bacterial sequences ~~, with the total sequence pool reaching up to 92.1 % chloroplast sequences~~ (Jakuschkin et al., 2016) when using a previously described amplification protocol (Gloor et al., 2010). Using a different amplification~~a different~~ protocol ~~(~~described by ~~(~~Kembel et al.~~,~~ ~~(~~2014) with specific primers (799F-1115R, V5-V6 region) and optimized hybridization conditions (64°C), we still observed chloroplast DNA amplification for the stem and leaf habitats. However, the majority of amplified sequences were of bacterial origin, and the total pool of sequences (2,661,227 effective sequences) was consistent with high Good's coverage and satisfactory taxonomical assignment. In our study, Illumina MiSeq sequencing allowed us to obtain a greater number of reads per sample, and the alpha diversity indices were comparable to those found in a previous study (Beckers et al., 2017).

Analysis of our datasets revealed that bacterial communities were habitat-specific. Moreover, aboveground and belowground habitats were clearly different between them, as shown in Fig. 3. Similar results were ~~also~~ found by Knief et al. (2012)~~ in rice~~, who ~~. These authors found~~demonstrated that the rhizosphere of rice~~ rhizosphere~~ hosts a bacterial community that is distinct to that in the phyllosphere regarding composition and complexity ~~(Knief et al., 2012)~~. The aboveground and belowground communities shared few OTUs, and the aboveground communities were less enriched and diverse than the belowground communities. Similarly, a recent study carried out on ~~seven~~ 7 tree species revealed lower alpha diversity in aboveground habitats compared to belowground habitats, which is

consistent with our results (Lambais et al., 2014). On the other side, this results seems to be plant species-specific since Coleman-Derr et al., (2015) ~~have~~ found that the phyllosphere of the agave plant hosts a diversity of prokaryotes, comparable to the rhizosphere. ~~(Coleman-Derr et al., 2015).~~

The soil and root habitats shared approximately half of their OTUs, which was lower than the proportions previously observed for *Arabis alpina* (> 64 % for the soil, rhizosphere and root communities) (Dombrowski et al., 2016). However, evidence of a lower alpha diversity in the roots compared to the soil was highlighted in our results. The dataset obtained for root and soil samples in ~~this~~ the present ~~study~~ work corroborates our previous studies~~y~~, which was carried out at the same site on native tree species (poplar and willow), revealing the dominance of *Proteobacteria* and *Actinobacteria* (Zappelini et al., 2015). *Proteobacteria* and *Actinobacteria* also dominate in root bacterial communities from other polluted (Foulon et al., 2016b) or non polluted (Beckers et al., 2016) soils, although ~~-~~these authors used 454 pyrosequencing in their study. The dominance of the phylum *Chloroflexi* in soil samples from the present study may be explained by the adaptability of members of this phylum to oligotrophic environments (Barton et al., 2014). Moreover, this phylum ~~and~~ ~~have~~has been found to be~~is~~ dominant within the *Alyssum~~.~~ murale* rhizosphere growing ~~in~~on ultramafic soils (Lopez et al., 2017) and also geothermal soils, low-temperature meadow soils, sea and lake sediments, and hydrothermally active sediments (Yamada and Sekiguchi, 2009) ~~in TE-contaminated soil, suggesting that this phylum is abundant in extreme conditions (Lopez et al., 2017)~~. These findings suggest that this phylum may be particularly abundant in extreme environments. ~~/conditions.~~

The root habitat was significantly enriched for spore-forming bacteria, such as those from the genus *Streptomyces*, compared with the other three habitats, this feature has been observed in studies focusing on the cultivable bacterial communities in contaminated soils~~a~~

feature that was observed in several studies performed on contaminated soils (Kuffner et al., 2008; Sas-Nowosielska et al., 2008; Schmidt et al., 2009). The predominance of these spore-forming bacteria on or inside the roots could be explained by the fact that the abundance of r-strategists (bacteria capable of rapid growth and utilization of resources) decreases in the root area in disturbed soils (such as metal-contaminated soils), while k-strategists (bacteria with reduced growth and metabolic activity) such as *Streptomyces* may be privileged (Kunito et al., 2001). In addition, the *Streptomyces* genus contains many strains that are highly resistant to several metals (Álvarez et al., 2013) and thus may be more competitive than other bacteria in TE-enriched environments such as the tailings dump of Tavauxinvestigated in the present study.

In the leaf and stem habitats, OTUs from the phylum *Deinococcus-Thermus*, such as those from the genus *Deinococcus* (Figs. 2 & 3), were among the most abundant OTUs and were not detected in either the soil or root habitats. This group of bacteria consists of poly-extremophile bacteria that are resistant to very high doses of radiation and long periods of desiccation (Mattimore and Battista, 1996). The genus *Methylobacterium* was also identified in the phyllosphere of the plantation; this genus constitutes a considerable and generally stable fraction of the phyllospheric bacterial communities of terrestrial plants under varying environmental conditions (Knief et al., 2010). The other taxa that were most more characteristic of the phyllosphere habitats belonged to the genera *Hymenobacter* and *Sphingomonas*. Members of these bacterial genera were previously identified as more characteristic of the urban ivy (*Hedera helix*) phyllosphere than that of non-urban ivy (Smets et al., 2016). Atmospheric contamination may promote the growth of these specific taxa to a greater degree than other taxa.

Our study is one of the first to compare bacterial communities of leaf and stem habitats in trees using Illumina-based MiSeq sequencing; thus, little data is available in the

literature for comparison. Both habitats clustered closely together (Table S2), but the stem appeared to be significantly less diverse than the leaf. The stem appeared to host a number of specific bacteria that were not detected in the leaf habitat. Indeed, *Firmicutes* were essentially only detected in the stem samples~~, not the leaf samples~~, and the genus *Paenibacillus* was the dominant member of this phylum. An abundance of this genus in the stem was previously observed for endophytes isolated from *Populus* cv. Hazendans ~~*cv. Hazendans*~~ but not for endophytes isolated from *Populus* cv. Hoogvorst ~~*cv. Hoogvorst*~~ (Moore et al., 2006). Several dominant bacterial OTUs in the stem group were assigned to *Paenibacillus amylolyticus* (Table S3). Some environmental or physiological specific factors associated with the stem are likely responsible for the presence of this bacterium. This species was previously identified in the bract phyllosphere of ~~the plant~~ *Phoenix dactylifera*, which dries out and remains on the plant (Rivas et al., 2005). Other studies investigating the rhizosphere (Hosoda et al., 2003) or the phyllosphere (Rivas et al., 2005) revealed that most of the plant polysaccharide-degrading bacteria isolated ~~from plant habitats~~ belonged to the *Paenibacillus* genus. These bacteria readily digest polysaccharides, such as cellulose, pectin, starch, esculin and xylan. Moreover, we tested our phyllospheric-isolated strains for pectinase and cellulase activities (according ~~using~~ previously described protocols (Verma et al., 2001)~~)~~ and we observed that the isolated *Paenibacillus amylolyticus* strain was positive for pectinase activity and negative for cellulase activity (data not shown). Based on these results, it seems that tissues of terrestrial plants represent ~~various~~ different habitats that host specific microbial communities. It may be interesting to decipher the structure and composition of these communities using both DNA sequencing and culture-dependent methods, each of which ~~have~~ has constraints and advantages.

We further assessed densities of bacteria isolated from various habitats of the poplar tree plantation when Hg was added as a stressor. The bacterial communities from the

phyllospheric habitats were altogether less resistant than the communities hosted in the belowground habitats, potentially due to the direct contact between soil bacterial communities and ~~high loads~~higher amounts of Hg ~~within~~ in the soil. The rhizospheric soil hosted a bacterial community that was richer than that of the bulk soil, which was expected. Indeed, previous works have shown that planted soils host significantly richer bacterial communities compared to unplanted soils (Durand et al., 2015). This is explained by the presence of plant exudates in the soil surrounding the roots, which play a role in bacterial growth (Baudoin et al., 2003). However, bacteria isolated from the bulk soil were more resistant to Hg than bacteria isolated from the rhizospheric soil. We isolated Hg-resistant strains harboring PGP traits, such as isolates of *Bacillus subtilis* and *Pseudomonas* sp. from each habitat. The species *Bacillus subtilis* is known for its antifungal properties, siderophores, IAA production and biocontrol effects on *Fusarium wilt* (Zaidi et al. 2006; Chebotar' et al. 2009). This species was previously tested as a bioinoculant to improve Ni bioaccumulation by *Brassica juncea* and promoted the growth of pepper plants (Yu et al., 2011). Many plant-associated *Pseudomonas* species directly and indirectly promote plant growth, a phenomenon that has previously been reviewed (Preston, 2004). These species compete with and suppress pathogenic microorganisms to promote plant growth. ~~(cite).~~

~~In endophytic habitats, our results of culturable bacterial densities regarding root and stems habitats were comparable with previous data from *Trifolium pretense* (Sturz et al., 1997); however, density of leaf endophytic cultivable bacteria was lower in our study.~~ The ~~epispheric habitat of the~~ stem ep~~h~~isphere exhibited a lower cultivable bacterial community than the stem ~~endospheric habitat~~endosphere, ~~whereas but contrary~~and in contrast, the leaf episphere hosted more cultivable bacteria than its endosphere. The structures of these plant organs might play a role in the development of these communities/~~ay~~ be correlated with these results. In ~~this~~ our study, several *Pseudomonas sp.* (including *P. graminis*) were isolated from

the phyllosphere of poplar in the Hg-enriched growth medium. The presence of this genus was also observed using the metabarcoding technique essentially with the OUTU00025 (Fig. 3 and Table S3), which were mostly detected in the leaf (0.96 %), the root (0.66 %) and soil (0.73 %) habitats. *Pseudomonas* are ubiquitous bacteria that survive on plant surfaces and inside plant tissues in both aboveground and belowground habitats (Schreiber et al., 2004). Specifically, *Pseudomonas graminis* is found in phyllospheric habitats (Behrendt et al., 1999) and was previously isolated from poplar (Doty et al., 2009). HoweverMoreover, this strain was also previously defined as an endophyte and wasbut unable to grow in N-free media (Hutner, 1972). However, Wwe observed the presence of the *nifH* gene in all *Pseudomonas graminis* strains, and we confirmed that these strains were able to reduce atmospheric N_2 in nitrogen-free medium. A previous study showed that a *Pseudomonas graminis* strain isolated from poplar and re-inoculated on sterile *Populus trichocarpa* as part of a microbial consortium promoted root production, improved total biomass production and increased leaf area (Knoth et al., 2014). In addition, inoculation resulted in a 25 % increase in the total nitrogen content. The presence of high Hg resistance and the potential to fix atmospheric N_2 such as the *Pseudomonas graminis* strains identified in this study might be valuable traits to further investigate in a future study and may therefore be key microorganisms for the restoration of disturbed environments. We were unable to isolate endophytic Hg resistant bacteria from any of the three habitats tested,studied; suggesting the internal plant tissues may not host resistant bacteria.

As previously reviewed, the microbial communities of the phyllosphere have a central role in plant function and atmospheric trace gas dynamics (Bringel and Couée, 2015). Thus, using PGPB inoculation in both the rhizosphere and phyllosphere, we sought to improve the uptake of Hg by aan Hg-accumulating plant, specifically, pepper. However, our results revealed that inoculation did not affect the rate of Hg accumulation in pepper leaves, although

it ~~did~~ increased root growth. This growth may be related to a significant increase in the metabolism of L-phenylalanine by microbial communities of the inoculated plants. Indeed, the increased activity of phenylalanine ammonia-lyase in *Bacillus subtilis* leads to the release of ammonia into the soil, which then acts as a fertilizer (Podile and Laxmi, 1998).

In conclusion, the application of a metabarcoding approach to the study of bacterial communities associated to the poplar cultivar Skado (*P. trichocarpa* x *P. maximowiczii*) planted at an industrial phytomanaged site revealed contrasting microbial communities in different poplar habitats. In parallel, a more conventional approach allowed us to isolate strains with functional traits of interest. We believe that the present findings will be instructive for the design of future restoration practices for industrial dumps.

Acknowledgments

This work was supported by the French Environment and Energy Management Agency [PROLIPHYT 1172C0053], the Région Franche-Comté [Environnement-Homme-Territoire 2014-069], the Pays de Montbéliard Agglomération [13/070-203-2015], and the French national programme EC2CO/MicrobiEen FREIDI-Hg. A.D. received a PhD grant from the Région Franche-Comté. V.A.L. received a post-doc grant from the Région Franche-Comté.

Figure legends

Figure 1. NMDS plot of bacterial communities associated with the leaf, stem, root and soil habitats using the Bray-Curtis dissimilarity measure and 1,000 iterations. Each point represents the bacterial community of a given sample, a: belowground habitats; b: aboveground habitats.

Figure 2. A barplot showing the bacterial community composition at the phylum or class level for each habitat (leaf, stem, root and soil) based on 16S rRDNA sequencing of samples using the metabarcoding approach. The species of Hg-resistant strains isolated from belowground and aboveground habitats are indicated.

Figure 3. A heatmap comparing the abundance of bacterial OTUs among the 20 most abundant OTUs in at least one of the four poplar habitats. The dendrogram represents linkage clustering using Euclidean distance measures. OTU delineation was based on a cutoff of < 97 % sequence similarity. Assignments between brackets indicate the lower taxonomic level associated with the OTU using the Greengenes database, k: kingdom, p: phylum, o: order, c: class, f: family, s: genus and species.

Figure 4: Phylogeny of the Hg-resistant bacteria isolated from the phyllosphere in 1/10 869 medium containing 2 µM $HgCl_2$. The phylogenic tree was constructed using a K2P Neighbor-joining model based on aligned DNA sequences (697 bp) with 1,000 iterations. The parentage of branches is given in the tree. Phenotypic and genotypic characterizations for each strain are also shown.

Figure 5: Violin plot of the root, shoot and total dry biomasses at harvest (46-day mesocosms) for uninoculated and Hg-unexposed (ni-), uninoculated and Hg-exposed (ni+), inoculated and Hg-unexposed (i-) and inoculated and Hg-exposed (i+) plants. Different letters indicate significant differences, P value < 0.01.

Figure 6: Hg concentration (ng mg DM^{-1}) in the dry biomass of older leaves of peppers grown for 46 days with the following treatments: uninoculated and Hg-unexposed (ni-), uninoculated and Hg-exposed (ni+), inoculated and Hg-unexposed (i-) and inoculated and Hg-exposed (i+). Different letters indicate significant differences, P value < 0.01.

Tables

Table 1. Alpha diversity estimations of bacterial communities from the four poplar habitats. All diversity statistics were calculated using an OTU threshold of ≥ 97 % sequence similarity on randomly subsampled data at the lower sample size (3,000 reads). Mean values followed by different letters are significantly different at P < 0.05 (Kruskal-Wallis comparison test). Numbers between brackets indicate the standard error (SE).

Table 2: Cultivable colony forming units per gram dry weight soil (CFUs g^{-1}) on solid media and percentage (%) of Hg-resistant cultivable bacteria to 2 and 10 µM $HgCl_2$ in the different habitats.

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1023

Bacterial diversity associated with poplar trees grown on a Hg-contaminated site: community characterization and isolation of Hg-resistant plant growth-promoting bacteria

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Abstract

Industrial waste dumps are rarely colonized by vegetation after they have been abandoned, indicating biological infertility. Revegetation of industrial tailings dumps is thus necessary to prevent wind erosion, metal leaching and has been shown to restore soil functions and ecosystem services. However, little is known about the microbial colonization and community structure of vegetated tailings following the application of restoration technologies. In this study, we investigated the rhizosphere and phyllosphere bacterial communities of a poplar tree plantation within a phytomanagement-based restoration program of a Hg-contaminated site. We used Illumina-based sequencing combined with culture-dependent approaches to describe plant-associated bacterial communities and to isolate growth-promoting bacteria (PGPB) and Hg-resistant bacteria. The genus *Streptomyces* was highly specific to the root community, accounting for 24.4 % of the relative abundance but only representing 0.8 % of the soil community, whereas OTUs from the *Chloroflexi* phylum were essentially detected in the soil community. Aboveground habitats were dominated by bacteria from the *Deinococcus-Thermus* phylum, which were not detected in belowground habitats. Leaf and stem habitats were characterized by several dominant OTUs, such as those from the phylum *Firmicutes* in the stems or from the genera *Methylobacterium, Kineococcus, Sphingomonas* and *Hymenobacter* in the leaves. Belowground habitats hosted more cultivable Hg-resistant bacteria than aboveground habitats and more Hg-resistant bacteria were found on the episphere than in endospheric habitats. Hg-resistant isolates exhibiting plant growth-promoting (PGP) traits, when used as inoculants of *Capsicum annuum*, were shown to increase its root dry biomass but not Hg concentration.. The N_2-fixing and Hg-resistant species *Pseudomonas graminis*, observed in the poplar phyllosphere, may be a key microorganism for the restoration of industrial tailings dumps.

Keywords: Phytomanagement, Poplar habitats, Trace elements, Plant growth-promoting bacteria, Mercury-resistant bacteria

1. Introduction

Soil contamination by metal(loids) and trace elements (TEs) such as Ag, As, Cd, Cr, Cu, Hg, Ni, Pb, Se, or Zn is a threat to ecosystem stability and a risk to human health. In 2011, 127,000 sites in 33 European Union countries were confirmed to have contaminated soils, and approximately 45 % of these sites (58,000) were under remediation project management to reduce associated risk (Panagos et al., 2013). Data published in 2016 showed that 6.24 % (137,000 km^2) of European agricultural lands were unsafe for food production, requiring local assessment and eventual remediation (Toth et al., 2016). Due to the frequent lack of a plant cover at industrial dumps, processes such as wind erosion or leaching can led to the contamination of adjacent agriculture lands leading to the decrease in soil productivity.

Phytomanagement-based restoration programs for contaminated soils may use woody plant species that are colonized by essential microorganisms (Bell et al., 2014; Partida-Martínez and Heil, 2011; Yergeau et al., 2012), especially in highly stressed and nutrient-poor environments, such as TE- contaminated sites (Durand et al., 2017; Foulon et al., 2016a, 2016b). However, knowledge of these associated microbial communities remains scarce, particularly with respect to aboveground tissues. Among the microorganisms that colonize the plant rhizosphere and phyllosphere, some are known to play important roles in determining plant fitness and are collectively described as plant growth-promoting bacteria (PGPB) (Glick, 2014; Lindow and Brandl, 2003; Pieterse et al., 2016). PGPB directly benefit host plants via hormone production (*e.g.*, indoleacetic acid, cytokinin, and zeatin) and improved nutrition and indirectly by acting as biocontrol agents, inducing systemic resistance and ethylene stress resistance (Bringel and Couée, 2015; Glick, 2005; Lindow and Brandl, 2003;

Maignien et al., 2014; Trouvelot et al., 2014; Vorholt, 2012). Belowground and aboveground plant tissues provide habitats, which can be easily colonized by specific PGPB (Vessey, 2003), (Vorholt, 2012). In the soil, free-living bacteria of various genera such as *Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Curtobacterium, Klebsiella, Enterobacter, Pseudomonas,* and *Serratia* exert beneficial effects on plants (Kloepper *et al.,* 1989 ; Glick *et al.,* 1999 ; Benizri *et al.,* 2001) and are classified as rhizospheric PGPB. Plant-associated bacteria also include endophytic bacteria, which colonize internal plant tissues. They are originating from the epiphytic bacterial communities of the rhizosphere and phyllosphere but may also arise from transmission through seeds or plant vegetative parts (Hallmann et al., 1997). Members of the genera *Acinetobacter, Aminobacter, Arthrobacter, Bacillus, Burkholderia, Curtobacterium, Devosia, Enterobacter, Gluconacetobacter, Herbaspirillum, Methylobacterium, Microbacterium, Ochrobactrum, Paenibacillus, Pseudomonas, Phyllobacterium, Rahnella, Shinella, Sphingomonas* and *Staphylococcus* have been described as plant endophytes (Compant et al., 2009; De Meyer et al., 2015; Doty et al., 2009). Other bacterial endophytes that are only localized in specific structures are referred to as symbiotic PGPB such as members of the genera *Azorhizobium, Bradyrhizobium, Devosia, Ensifer, Frankia, Mesorhizobium, Microvirga, Ochrobactrum, Phyllobacterium and Rhizobium* (Glick 2014, Kloepper et al. 1989; Wang et al. 2012; De Meyer et al. 2015). Aboveground, phyllospheric PGPB are subjected to fitness stresses (*e.g.* direct solar radiation, drought), that are very different than those found belowground, suggesting a specific selection pressure (Lindow and Brandl, 2003). It has been demonstrated that the survival capacity of *Pseudomonas fluorescens* SBW25 in the phyllosphere is dependent on the expression of habitat specific genes that are significantly more correlated to the fitness of the bacteria in the phyllosphere that in the soil or the rhizosphere (Gal et al., 2003). Albeit, a full understanding of these bacterial communities has not been reached (Vacher et al., 2016), several

phyllospheric PGPB have been already previously described such as members of *Arthrobacter, Azotobacter, Beijerinckia, Klebsiella, Methylobacterium, Nostoc, Pantoea, Pseudomonas, Scytonema, Sphingomonas and Stigonema* (Lindow and Brandl, 2003; Vacher et al., 2016). However, gaining an understanding of the structure and composition of microbial communities *in situ* is difficult due to the diversity and complexity of these communities. Metabarcoding using high-throughput sequencing technologies offers a solution to describe the bacterial communities associated with environmental habitats with high resolution, as exemplified recently in our previous work (Durand et al., 2017; Foulon et al., 2016a, 2016b). We demonstrated for instance that *Alphaproteobacteria* and *Actinobacteria* dominated root bacterial communities, whereas soil samples were dominated by *Alphaproteobacteria* and *Acidobacteria* (Foulon et al., 2016b). These innovative approaches still need complementary culture-dependent approaches to allow for the functional characterization and selection of the best-performing PGPB.

In this study, we combined a metabarcoding approach that employs a high-throughput Illumina MiSeq platform (Durand et al., 2017; Foulon et al., 2016a, 2016b; Schmidt et al., 2013; Wu et al., 2015) and a culture-dependent approach to investigate the composition and assembly of bacterial communities and isolate Hg-resistant PGPB in the rhizosphere, endosphere and phyllosphere of poplars at a Hg-contaminated site. We expected that we would observe clear differences in the relative abundance and composition of bacterial communities across poplar habitats that may improve our understanding of the microbial ecology of these environments. We also aimed at better understanding the role of isolated bacterial strains in Hg transfer from the atmosphere to plant tissues using PGPB inoculation of the model plant *Capsicum annum*.

2. Materials and Methods

120 *2.1. Site description*

121 The chlor-alkali site is located in Tavaux, France, in Western Europe along the Saône
122 river and is surrounded by several urban aggregates. The industrial dump was exploited as a
123 sediment storage area from the 1950s to 2003. These sediments were originated from the
124 effluents produced during the chlor-alkali Hg-cell process used for the electrolysis of NaCl. A
125 detailed map of the industrial site was provided by Maillard et al. (2016). ICP-AES analyses
126 revealed that total Hg and total As were significantly increased in the dump compared to the
127 levels in soil from an adjacent undisturbed forest. Further details on soil characteristics of the
128 dump are provided in Zappelini et al. (2015). The poplar cultivar Skado (*P. trichocarpa* x *P.*
129 *maximowiczii*) was planted in 2011 as a short rotation coppice (2200 stems ha^{-1}) as part of a
130 phytomanagement strategy, fully described in Durand et al. (2017).

131 *2.2. Sampling design*

132 Four replicates were sampled in August 2014 at six random trees, making a total of 24
133 samples per habitat.(bulk soil, root, stem and leaf). Soil samples composed of bulk soils from
134 under the canopy of the poplars were sieved to <4 mm. In more details, the roots were
135 collected after litter removal from the upper 20 cm layer of soil from under the canopy of the
136 trees. They were separated from the soil via 2 distilled water baths, and the smallest roots
137 were selected and separated from larger roots by cutting them with a scalpel. Woody samples
138 were collected from poplar branches of the axe 2 (0.8-1.2 cm in diameter) at a *ca*. 5 m height,
139 corresponding to the half-crown of the poplar. Leaf samples were composed of 3 leaves
140 collected from the branches collected as described above. All samples were obtained over a
141 one-day period to reduce any heterogeneity imparted by climatic conditions. The samples
142 were either freeze-dried and stored at -20°C for molecular analysis or dried at ambient
143 temperature (24°C ± 1) for physico-chemical analyses. Thus, we considered the belowground
144 and aboveground habitats to include both endophytic and epiphytic fungi.

2.3. Metabarcoding analysis

DNA was extracted from biological samples within 2 months after sampling. Plant material (root, stem and leaf samples), was thoroughly washed with distillated water and DNA was extracted from 20 mg of fresh material using an adapted cetyl trimethylammonium bromide/chloroform/isoamyl alcohol protocol (Lefort and Douglas 1999; Healey et al. 2014). DNA was extracted from 100 mg of sieved fresh soil, using a PowerSoil DNA Isolation Kit following the manufacturer's instructions (MoBio Laboratories, Inc., Carlsbad, CA USA). Extracted DNA was purified using a Power Clean® Pro DNA Clean-Up Kit (MoBio Laboratories, Inc., Carlsbad, CA USA). Equimolar DNA pools were adjusted to 10 ng μl^{-1}, and PCR conditions sequencing of the bacterial 16S rRNA region was performed on an Illumina MiSeq platform (Microsynth AG, Switzerland). PCR amplification of the 16S rRNA gene was performed using the bacterial primers 799f (5'- AAC MGG ATT AGA TAC CCK G -3') and 1115r (5'- AGG GTT GCG CTC GTT G -3'), which amplify the V5-V6 region (Kembel et al., 2014). These primers target a section of the bacterial 16S rRNA region and generate a small amplicon (~300 bp) that is appropriate for Illumina sequencing. Reactions were performed in triplicate for each sample with the following conditions: 30 s initial denaturation at 98 °C, followed by 20 cycles of 10 s at 98 °C, 30 s at 64 °C, and 30 s at 72 °C, with a final 10-min elongation at 72 °C.

Reads were assigned to each sample according to a unique barcode, and contigs were then assigned using the MOTHUR pipeline (Schloss et al., 2009). Raw reads were filtered by length and quality. 16S rRNA reads were pre-clustered using sumaclust (Mercier et al., 2013) at 0.99 identity. Only sequences with at least 8 reads were retained (76 % of initial reads). The retained 16S rRNA sequences were aligned with those present in the Silva database to remove non-16S rRNA sequences, and plant DNA contamination was removed by suppressing reads identified as k__Bacteria (100), p__Cyanobacteria (100), c__Chloroplast (100),

o__Streptophyta (100), and unclassified (100). Taxonomic assignments were made using a Bayesian approach (Wang et al., 2007) with the Greengenes database (DeSantis et al., 2006). Finally, OTUs were derived using the Needleman distance and average neighbor clustering at a distance of 0.03.

Diversity parameters were calculated based on a dataset in which numbers of reads per sample were rarefied to 3,000 reads per sample. We calculated rarefaction curves at a 97 % similarity level to verify whether the amount of sequencing reflected the diversity of the original microbial communities. Rarefaction curves were created using the "rarefaction" function and the Vegan package in R (Work et al., 2010). A 2-dimensional non-metric multi-dimensional scaling (NMDS) plot was calculated using the Bray Curtis method (k = 3) based on the standardized (Wisconsin double) and square root transformation of OTU abundance using the "metaMDS" function in the Vegan package. We used ANalysis Of SIMilarities (ANOSIM) to obtain P (i.e., significance levels) and R (i.e., the strength of the factors on the samples) values. These results were paired with a heatmap of Spearman's correlations between the 20 most abundant OTUs in each habitat (a total 59 OTUs) created with "heatmap.2" from the "gplots" package. In addition, in the same pool of OTUs, we use the "multipatt" function of the "indicspecies" package to evaluate the best bioindicator OTUs (De Cáceres et al., 2012). OTU-based analysis of alpha diversity was performed with the following functions using Mothur calculators: "sobs", "chao", "shannoneven", "shannon", "invsimpson", "coverage" and "nseqs" (Schloss et al. 2009). These estimates included observed OTU richness, Chao estimation (Chao, 1949), Shannon diversity index, inverse of Simpson diversity index, and measure of evenness based on the Shannon index and coverage. The coverage calculator returns Good's coverage for an OTU definition (Good, 1953). Coverage was calculated using the following equation: $C=[1-(n/N)]*100$ (%), where n is the number of OTUs and N is the number of sequences. The numbers of OTUs that were shared

between habitats were visualized using Venn diagrams that were generated in Mothur with the function "venn". We considered an OTU to be present in a compartment if it was present in at least 25 % of the habitat samples.

2.4. Isolation of Hg-resistant bacterial strains

Bacterial strains were isolated from bulk and rhizospheric soils and from stem and leaf phyllospheric habitats of poplar trees. Soil properties and Hg concentrations were detailed in our previous study (Durand et al., 2017). Bulk soil samples (3.5 g) were taken far from the root system under the canopy and placed in a flask containing 35 ml of phosphate buffer (PBS: 8 g of NaCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 per l, pH 7.2). Roots with adhering soil (defined as the rhizospheric soil) were washed in a PBS bath in a rotatory shaker (30 min, 150 rpm). Roots were separated from the soil suspension and rhizospheric soil was recovered after centrifugation (14,000 g for 10 min), and 1 g of the rhizospheric precipitated soil was then resuspended by shaking in 35 ml of PBS. Phyllospheric epiphytes, defined as the microbes living on the surfaces of aboveground habitats, were isolated from leaves and stems. Three g of leaves or 20 g of stems were thoroughly washed with distilled water and suspended in 150 ml of PBS extraction solution, then placed on a rotatory shaker (150 rpm) for 1 h. Isolation of endophytes from root, stem and leaf habitats was carried out after surface sterilization with 2 % sodium hypochlorite for 2 min. Samples were thoroughly rinsed with sterile distilled water and then crushed in 35 ml of PBS and macerated for 1 h on a rotatory shaker (150 rpm). Soil and plant suspensions were diluted in 10-fold series and plated onto 1/10 rich medium (869) as well as onto environmental medium (284) (Eevers et al. 2015). Densities of Hg- resistant bacteria were determined using the same media supplemented with 0, 2 or 10 µM $HgCl_2$ (Francois et al., 2012). The agar plates were then incubated in the dark at 27°C for 6 days. The number of bacteria was expressed as log (CFU) g^{-1} dry weight (DW) of sample. Hg-resistant colonies were isolated and purified by subculture on Hg-enriched 1/10

869 or 284 medium. A bacterial collection was established by growing each previous isolated strains in 6 ml of Luria Bertani (LB) broth (Sigma-Aldrich, Steinheim, Germany) for 15 h on a rotatory shaker (150 rpm) at 30°C and stored in 25 % sterile glycerol containing 75 % brain heart infusion broth (Roth, Karlsruhe, Germany) at -80°C for further analyses.

2.5. Taxonomic identification

For taxonomic identification, each Hg-resistant isolate was cultured in 15 ml of LB medium for 24 h on a rotatory shaker (120 rpm) at 27°C. After centrifugation, DNA was extracted from the pellets using an EZNA bacterial DNA isolation kit (OMEGA Bio-tek, Inc, Norcross, Georgia, USA) according to the manufacturer's instructions. The BOX-PCR fingerprinting method was used to group genotypic profiles at a similarity level of 97 % as previously described (Becerra-Castro et al., 2011). One strain was selected for each BOX-group and R rDNA genes were amplified from 5 µl of bacterial DNA in a final reaction volume of 50 µl containing 25 µl of 2x PCR master mix (Thermo Fisher Scientific, Carlsbad, California, USA) and each universal primer at 0.5 µM (Eurofins Genomics, Paris, France), specifically 27f (5′- AGA GTT TGA TCM TGG CTC AG -3') and 1492r (5′- GGT TAC CTT GTT ACG ACT T -3′) (Mark Ibekwe et al., 2007). DNA amplification was carried out in a thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) under the following conditions: 4 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, with an additional 15-min cycle at 72°C (Durand et al., 2015). All 16S rRNA PCR products were sequenced by Sanger sequencing (Genewiz Beckman Coulter Genomics, United Kingdom). DNA sequences were edited with BioEdit software v7.2.6. (Hall, 1999) screened against the GenBank database using the BLASTn tool at the NCBI website (http://www.ncbi.nlm.nih.gov/) and then aligned with reference sequences of bacteria (LN551925, KR922297, HQ256858, KU587965, KU523561, JX827229, HQ256858, HQ224627, KT825724, NR_028626, LN774179, JF496259, KR476438, JQ956529,

FJ380956, and KC790245) using Clustal W (Thompson et al., 1994) as implemented in Bioedit. Neighbourg Joining (NJ) tree reconstructions were produced using MEGA 5 (Tamura et al., 2011). The most appropriate model of 16S rRNA sequence evolution was K2P model (Kimura, 1980).

2.6. Functional characterization

Each isolate of the bacterial collection was screened for the following plant growth-promoting traits: siderophore production, IAA synthesis and phosphate solubilization. The siderophore production of each bacterial strain was determined using chrome azurol sulfonate (CAS) agar medium (Alexander and Zuberer, 1991; Schwyn and Neilands, 1987). After 5 days of incubation on CAS medium at 30°C, a red-orange halo around colonies indicated the production of siderophores. For each strain, the ratio between the diameter of the halo and the diameter of colony was recorded. The IAA synthesis of each bacterial strain was determined using Salkowski's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35 % $HClO_4$) as described by (Barillot, 2012). The phosphate-solubilizing capacity of bacterial strains was determined on Pikovskaya medium (Mehta and Nautiyal, 2001). After 5 days of incubation at 30°C, a discolored halo around colonies demonstrated phosphate solubilization. For each strain, the ratio between the halo diameter and the diameter of colony was recorded. Additionally, MICs (minimal inhibitory concentrations) of Hg were determined for each isolated strain. Briefly, microtitration plates were prepared using 2-fold dilutions of Hg in LB liquid broth, from 0 to 256 µM Hg. The strain *Pseudomonas aeruginosa* PA14, which is resistant to Hg above 256 µM, was used as a positive control. Each well was inoculated with 2 µl of a bacterial suspension, and plates were incubated for 48 h at 27 °C on a rotatory shaker table (180 rpm). After incubation, the bacterial growth was evaluated by measuring the optical density at 600 nm (OD_{600}). Nitrogen fixation ability was evaluated using N-free semisolid malate medium (NFb) (Baldani and Döbereiner, 1980) inoculated with the assayed strains. After 10 days,

strains that successfully grew were transferred to fresh NFb medium, and after 7 days of incubation, the characteristic pellicles of diazotrophic bacteria were observed.

Using the previous extracted bacterial DNA, the presence of the genes *merA* and *nifH* in the genome of each strain was assayed. PCR amplification of the *merA* gene was performed using 3 µl of bacterial DNA in a final volume of 20 µl that also contained 10 µl of 2x PCR master mix (Thermo Fisher Scientific, Carlsbad, California, USA) and each primer at 0.5 µM, specifically merA2F (5'- CCT GCG TCA ACG TCG GCT G -3') and merA2R (5'- GCG ATC AGG CAG CGG TCG AA -3') (Poulain et al., 2015). DNA amplification was carried out in a thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) using the following conditions: 10 min at 95°C, followed by 35 cycles of 30 sec at 95 °C, 15 sec at 63 °C, and 30 sec at 72°C, with an additional 5-min incubation at 72 °C. PCR amplification of the *nifH* gene was performed in a final volume of 25 µl containing 5 µl of bacterial DNA, 12.5 µl of 2x PCR master mix, and each primer at 0.5 µM including nifHF (5'- TAC GGN AAR GGS GGN ATC GGC AA -3') and nifHI (5'- AGC ATG TCY TCS AGY TCN TCC A -3') (Laguerre et al., 2001). DNA amplification was carried out in a thermocycler under the following conditions: 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 53.5 °C, and 2 min at 72 °C, with an additional 15-min incubation at 72 °C.

2.7. Pot experiment with Hg-resistant bacteria and pepper

Capsicum annuum seeds (Yolo Wonder Pepper) were planted in peat (Brill, Germany) and grown for 68 days. As part of a bioindicator programme implemented at the chlor-alkali site, we found out that *Capsicum annuum* was the highest Hg-accumulator among other edible (*Solanaceae* or *Brassicaceae*) or woody (poplar, willow) species, well reflecting the actual Hg atmospheric content (Assad, unpublished data). These edible species were chosen, as they are currently cultivated in the vicinity of the chlor-alkali site in private gardens. The 68-day-old plants were used to start a 46-day pot experiment. Each mesocosm was defined as a pepper

plant growing in a 0.5 liter pot in a growth chamber (photoperiod, 16 h; temperature, 18 °C (night) and 22 °C (day); relative humidity, 60-80 %; photosynthetic photon flux density, 250 mmol m^{-2} s^{-1}). In total, 16 *Capsicum annuum* plants were used for each of the following treatments: non-inoculated and Hg-unexposed (ni-); inoculated and Hg-unexposed Hg (i-); uninoculated and Hg-exposed (ni+); and inoculated and Hg-exposed (i+). Hg-unexposed plants (ni- and i-) were grown in the same growth chamber, while Hg-exposed plants (ni+ and i+) were grown in a separate growth chamber. Hg-enriched soil (6.38 mg kg^{-1} Hg) from the industrial tailings described above was added to the Hg-contaminated growth chamber to create an Hg-enriched atmosphere as previously described (Assad et al., 2016). The soil was not in direct contact with the plants in any way but provided an atmospheric enrichment with Hg, similar to that observed in the natural environment. Atmospheric Hg in the contaminated and uncontaminated growth chambers was measured at 21.0 and 3.48 ng m^{-3}, respectively. Using the previously isolated bacteria stored at -80 °C, two bacterial consortia were produced to inoculate both the belowground and aboveground compartments of *Capsicum annuum*.

The bacteria were grown at 30 °C with shaking at 200 rpm until maximum optical density (OD_{600}) in the liquid 869 media was reached. To generate the final phyllospheric consortium (PC), four *Pseudomonas graminis* strains (AT3, AT7, AT8 and AT9), isolated from the phyllosphere were added in equal proportions and adjusted to a final OD_{600} of 1 in PBS. To generate the final belowground consortium (BC), two *Pseudomonas* sp. strains (FT79, FT82) isolated from the rhizospheric soil and two *Bacillus subtilis* strains isolated from the bulk soil (FT97 and FT50) were used in equal proportions and adjusted to a final OD_{600} of 1 in PBS. For each plant, 4.8 ml of PC was sprayed onto the leaves, and 10 ml of BC was added near the base of the stems. The same amounts of PBS were added using the same methods with uninoculated plants. In addition, unplanted control pots were prepared.

Stem lengths and Hg concentrations in older leaves were measured at 0, 15, 32 and 46 days. In addition, at harvest (46 days), dry biomass of belowground and aboveground tissues was measured as well as the total Hg content in younger leaves, fruits, stem and roots. All samples were dried at room temperature and ground into a homogenous powder in a Mixer Mill for 3 min at 30 Hz (model MM400; Retsch Inc., Newtown, Pennsylvania, USA). We used Biolog Ecoplate™ microplates (Biolog Inc., Hayward, CA.) following the manufacturer's recommendations to determine the metabolic capacity of soil microbial communities under the different treatments.

2.8. Hg analysis in the substrate and biomass

Hg was measured in the soil and plant samples using an AMA-254 (Advanced Mercury Analyzer) cold vapor atomic absorption (CV-AAS) Hg analyzer (Altec Co., Czech Republic) under the standard conditions recommended by the manufacturer (120 sec drying, 150 sec heating, and 45 sec cooling). The validity of the analytical method was assessed using certified reference material (CRM), specifically Oriental Basma Tobacco Leaves (INCT-OBTL-5) with a certified Hg content of 20.9 ± 1.3 ng g^{-1} DM (Samczyński et al., 2012). Quality controls were regularly performed as described elsewhere (Maillard et al., 2016).

2.9. General statistical analysis

The Shapiro test and the Bartlett test were used to check the normality and homoscedasticity of the data, respectively. Our data verifying normality and homoscedasticity were assessed using analysis of variance (ANOVA). In other cases the data were examined using the Kruskal-Wallis test. Statistical analyses were performed with R software, version 3.3.2 (Team R, 2014).

3. Results

342 *3.1. Contrasting microbial communities in poplar tree habitats revealed by metabarcoding*

343 Illumina MiSeq sequencing of bacterial 16S rRNA genes from environmental DNA
344 isolated from four habitats (soil, root, stem and leaf of poplar trees) yielded 4,437,316
345 sequences. After filtering artifactual, chimeric and unaligned sequences, 76 % (3,222,285) of
346 the initial sequences were retained, distributed into 2,699 OTUs. After the removal of non-
347 bacterial OTUs, 2,661,227 bacterial sequences remained and composed the operational
348 dataset. Subsampling of the dataset was adjusted to 3,000 reads clustered in 2,414 OTUs
349 (Table S1). Our results showed large variations in total OTU numbers in aboveground *versus*
350 belowground samples.

351 Rarefaction curve analysis, which assesses species richness, showed that all curves from
352 each habitat were almost parallel with the x-axis, indicating that overall bacterial diversity
353 was well represented (Fig. S1). Visualization of a global analysis of the four habitats using
354 NMDS plots was difficult due to the gap between the aboveground and belowground habitats;
355 therefore, they were represented separately. The NMDS plot of the belowground habitats
356 (root and soil habitats) showed clear clustering of the two microbial communities (Fig. 1a),
357 while the bacterial communities of aboveground tissues (stem and leaf habitat) clustered more
358 closely (Fig. 1b). ANOSIM analyses confirmed a clear separation between the root and soil
359 bacterial communities ($R = 1$; P value = 0.001) while a less marked dissimilarity between the
360 stem and leaf communities was observed ($R = 0.61$; P value = 0.001). Roots (Fig. 1a) and
361 stems (Fig. 1b) data exhibited the greatest between-sample variations, evidenced by their
362 higher scattering.

363 Diversity and richness indices were calculated for each habitat (Table 1). Most indices
364 occurred in the order soil > root > leaf ≥ stem, revealing that aboveground habitats were
365 significantly less rich than belowground habitats. Comparing within belowground habitats,

soil appeared to be significantly more diverse and rich than the root habitat, while that when comparing the aboveground compartments stem habitat was as rich or richer than the leaf habitat but less diverse. Indeed, the Chao1 and Shannon indices indicated that the highest richness and diversity of the microbial community were present in soil samples, followed by the root. Soil reads were evenly spread among diverse OTUs, as revealed by high Simpson indices and high evenness.

The Venn diagram highlights shared and unique OTUs for each habitat (Fig. S2). The sums of the total observed bacterial OTUs in the four sample types in the poplar tree plots were 475, 337, 94 and 77 for soil, root, stem and leaf samples, respectively (Fig. S2). Overall, 9 OTUs (1.4 %) were shared by all compartments, and only 4.2 % of OTUs were found in both belowground and aboveground habitats. Moreover, aboveground habitats shared 50.8 % of their OTUs, while belowground habitats shared 43.9 % of their OTUs. Soil samples contained the highest proportions of unique OTUs (47.3 %), followed by leaf (31.2 %), root (25.2 %) and stem (18.1 %) samples.

Through environmental 16S rRNA sequencing, bacterial communities from the four habitats (soil, root, stem and leaf) were categorized into 30 phyla and 80 classes, the most abundant of which are shown in Fig. 2. *Actinobacteria* (28 %) and *Alphaproteobacteria* (26 %) dominated the entire dataset. The largest proportion of *Actinobacteria* was found in the root habitat (41.7 %) compared to the other compartments (Kruskal-Wallis $X^2 = 45.8$; $P < 1.3 \times 10^{-11}$), and the aboveground samples contained more *Alphaproteobacteria* (35.7 %) compared to belowground samples (Kruskal-Wallis $X^2 = 59.7$; $P < 1.1 \times 10^{-14}$). Altogether, belowground habitats were enriched for *Acidobacteria* (Kruskal-Wallis $X^2 = 68.9$; $P < 2.2 \times 10^{-16}$), *Chloroflexi* (Kruskal-Wallis $X^2 = 66.9$; $P < 2.9 \times 10^{-16}$), *Betaproteobacteria* (Kruskal-Wallis $X^2 = 42.7$; $P < 6.3 \times 10^{-11}$), *Deltaproteobacteria* (Kruskal-Wallis $X^2 = 66.7$; $P < 3.2 \times 10^{-16}$) and *Gammaproteobacteria* (Kruskal-Wallis $X^2 = 66.6$; $P < 3.2 \times 10^{-16}$), whereas

aboveground habitats were enriched for *Deinococcus-Thermus* (Kruskal-Wallis $X^2 = 68.0$; $P < 2.2 \times 10^{-16}$) and *Bacteroidetes* (Kruskal-Wallis $X^2 = 59.1$; $P < 1.5 \times 10^{-14}$). Although soil and root habitats were closely related to each other compared to aboveground habitats, both root and soil samples demonstrated also specificity in terms of the composition of their bacterial communities. The soil habitat was distinguished from the root habitat by a higher proportion of *Chloroflexi* (Kruskal-Wallis $X^2 = 66.6$; $P < 3.2 \times 10^{-16}$) and *Gemmatimonadetes* (Kruskal-Wallis $X^2 = 34.6$; $P < 4.1 \times 10^{-9}$) and lower *Actinobacteria* (Kruskal-Wallis $X^2 = 33.1$; $P < 8.6 \times 10^{-9}$). Additionally, soil was the only habitat containing bacteria from the phylum *Nitrospirae*. Bacterial communities inhabiting aboveground habitats were similar in both stem and leaf samples, although *Firmicutes* was only found in stems (8.5 %). A heatmap constructed with the most abundant OTUs in each of the 4 habitats (Fig. 3, 59 OTUs in total) indicated a net clustering between bacterial communities in belowground and aboveground habitats (Fig. 3). Moreover, the "multipatt" function of the "indicspecies" package identified the most specific OTUs for soil, root and phyllosphere habitats (Table S3). Among these 59 OTUs, only 4 were shared by all four habitats, exemplified by OTU00025 assigned to *Pseudomonas* and OTU00023 assigned to *Comamonadaceae*, with both OTUs comprising between 0.5 and 1.5 % of the relative abundance of each habitat. A total of 34 OTUs were specific to belowground samples, and 16 were specific to aboveground samples. Five OTUs, all from the phylum *Firmicutes*, were essentially found in the stem habitat, exemplified by OTU00083 assigned to *Paenibacillus amyloticus*. Among the 16 OTUs shared by stem and leaf communities, the most abundant were species from the genera *Hymenobacter*, *Deinococcus*, *Sphingomonas*, *Kineococcus* and *Methylobacterium*. Among the 34 OTUs shared in belowground habitats, several were more specific to the root habitat, such as *Streptomyces* (OTU00003) and *Micromonosporaceae* (OTU00005 and OTU00014). Indeed, *Streptomyces* was most specific to the root community, accounting for 24.4 % of the relative

abundance, but represented only 0.8 % of the soil community. In contrast, OTUs affiliated to the *Chloroflexi* phylum were the most abundant in the soil community.

3.2. Mercury resistance and functional traits of bacteria isolated from belowground and aboveground habitats

Table 2 shows densities of culturable bacteria from the four habitats and the densities of Hg-resistant bacteria. In the media without Hg, a higher number of CFUs were isolated in all habitats when using 1/10 869 medium compared to 284 medium, and followed this gradient: rhizospheric soil > bulk soil > root endosphere > stem endosphere > leaf episphere > stem episphere > leaf endosphere. The sizes of the cultivable microbial communities were compared to the sizes of communities exposed to 10 µM $HgCl_2$ for all habitats. A decrease in all cultivable bacterial communities was observed after exposure to 10 µM $HgCl_2$. The bacteria living in the phyllosphere demonstrated the lower density when exposed to 10 µM $HgCl_2$ compared to bacteria from the belowground habitats. Hg-resistant bacteria were isolated from bulk soil, rhizospheric soil, epiphytic stem and epiphytic leaf samples, whereas no Hg-resistant endophytic bacteria were found between the isolated strains. A few root endophytic bacteria strains were isolated initially but were not confirmed in subsequent steps. Among all habitats, the bulk soil hosted the highest densities of Hg-resistant bacteria (at 10 µM $HgCl_2$).

The list of isolated Hg-resistant species in belowground and aboveground habitats is shown in Fig. 2. Phenotypic and genotypic characterizations were performed for the 56 identified Hg-resistant strains (34 isolated from aboveground habitats and 22 from belowground habitats) (Fig. 4 and Table S4). All strains were identified after BLAST analysis of 16S rRNA sequences, and NJ-phylogenetic trees were subsequently established. For example, in the phyllosphere we identified strains from the genera *Pseudomonas*,

Sphingomonas, *Frigoribacterium*, *Curobacterium*, *Micrococcus*, *Paenibacillus* and *Staphylococcus* (Fig. 4). For those 56 identified strains, the Hg resistance of bacteria from belowground habitats was higher than that of bacteria from aboveground habitats (Table S4). Indeed, the strains found in aboveground habitats presented MIC values between 4 and 64 µM $HgCl_2$ (Fig. 4), whereas the strains from belowground habitats exhibited MIC values between 16 and 254 µM $HgCl_2$. In bacteria from various habitats, the presence of the *merA* gene did not consistently correlate with their Hg MICs (Table S4).

Regarding the study of PGP traits, surprisingly, the *nifH* gene was observed in all 13 aboveground *Pseudomonas graminis* strains (Fig. 4 and Table S4), and an experiment in semi-solid nitrogen-free medium confirmed the ability of these strains to fix N_2. In both aboveground and belowground habitats, all strains demonstrated IAA production, with variations in the amount of IAA produced (mg l^{-1}) between strains. The lowest IAA producers were AT38 (*Paenibacillus amylolyticus*) in the phyllosphere (with 0.9 mg l^{-1} IAA) and FT43 (*Pseudomonas fluorescens*) in the belowground samples (with 2.1 mg l^{-1} IAA), while the best IAA producers in the phyllosphere were AT8 (*Pseudomonas graminis*), with 12 mg l^{-1} IAA, and FT97 (*Bacillus subtilis*), with 12.5 mg l^{-1} IAA (Table S4). In belowground habitats, all strains with the exception of ST81 (*Bacillus licheniformis*) showed siderophore activity, reported as the ratio of the diameter of the orange-colored zone compared to the diameter of the colony; ratios ranged from 1.8 to 4.8. For the belowground bacterial consortium, we selected strains with ratios higher than 3.0. In aboveground samples, 28 of the 34 colonies demonstrated siderophore production, with ratios ranged from 1.1 to 3.0. Phosphate solubilization ability (ratio > 1) was more frequently detected in aboveground bacteria (31 of 34 colonies) than in belowground bacteria (6 of 22 colonies) (Table S4). Finally, the strains with the best profiles in terms of Hg resistance and PGP potential were selected for inoculation (IAA > 4 mg l^{-1} and siderophore ratio > 2.4, plus *nifH* detection, high Hg

resistance, phosphate solubilization potential). The strains AT3, AT7, AT8 and AT9 were part of the consortium to inoculate the phyllosphere, and the strains FT79, FT82, FT97 and FT50 were part of the consortium to inoculate belowground.

3.3. Influence of the Hg-resistant bacteria PGPB consortia on plant growth and Hg accumulation in pepper

The Hg-contaminated atmosphere significantly lowered the stem length in both inoculated and uninoculated pepper plants after 32 days compared to the non-contaminated atmosphere, but no differences were observed after 46 days of growth (data not shown) and at harvest, Hg had no impact on plant dry biomass. However, inoculation significantly increased the dry root biomass of Hg-unexposed plants (increase of 24%), and the same trend appeared for Hg-exposed plants (increase of 8%) (Fig. 5a). We did not observe significant differences between various treatments in terms of shoot and total dry biomasses (Fig. 5b and c). Plants from Hg-enriched atmosphere chamber had significantly higher Hg concentrations in leaves at harvest (4 fold higher) than Hg-unexposed plants (Fig. 6). However, inoculation with the selected bacterial consortia had no impact on Hg accumulation. Hg amounts in root samples (expressed in ng mg^{-1} DW) were below quantification limit (0.01 ng Hg). The metabolic activities estimated using Biolog Ecoplate™ microplates revealed that planted soils had higher metabolic activities than unplanted soils and no differences between Hg-exposed or Hg-unexposed pots were observed (Fig. S3). Among the 31 substrates tested, we observed significant differences for L-phenylalanine, which was metabolized to a greater degree by soil microorganisms of the inoculated plants compared to uninoculated plants.

4. Discussion

The bacterial communities of the 4 habitats (soil, root, stem and leaf) associated with poplars growing at an Hg-contaminated site were characterized using metabarcoding tools and traditional isolation-based techniques. In the present study, analyses of metabarcoding data made no distinction between epiphytic and endophytic microbial communities of the plant habitats. Previous studies showed that only a limited proportion of the microbiome was specific of the epiphytic or endophytic habitats (Coleman-Derr et al., 2015). We sampled communities under trees during the growing season (from leaf emergence to leaf fall). Notably, sampling was carried out in mid-season (July-August), and we did not attempt to study the seasonal dynamics of our habitats. According to previous studies, the mid-season community in the phyllospheric habitat is more diverse and contains more unique species than the early (May-Jun) or late-season (September-October) communities (Redford and Fierer, 2009).

Illumina-based sequencing of bacterial communities from plant habitats, such as roots, stems and leaves can be problematic due to unwanted chloroplast DNA amplification. A similar study investigating oak phyllospheric bacterial communities in oak trees using 16S rRNA amplification of the V6 region with Illumina technologies, revealed higher proportions of oak chloroplast sequences (up to 92.1 % from the total sequences) than bacterial sequences (Jakuschkin et al., 2016) when using a previously described amplification protocol (Gloor et al., 2010). Using a different amplification protocol described by Kembel et al. (2014) with specific primers (799F-1115R, V5-V6 region) and optimized hybridization conditions (64°C), we still observed chloroplast DNA amplification for the stem and leaf habitats. However, the majority of amplified sequences were of bacterial origin, and the total pool of sequences (2,661,227 effective sequences) was consistent with high Good's coverage and satisfactory taxonomical assignment. In our study, Illumina MiSeq sequencing allowed us to obtain a

greater number of reads per sample, and the alpha diversity indices were comparable to those found in a previous study (Beckers et al., 2017).

Analysis of our datasets revealed that bacterial communities were habitat-specific. Moreover, aboveground and belowground habitats were clearly different between them, as shown in Fig. 3. Similar results were found by Knief et al. (2012), who demonstrated that the rhizosphere of rice hosts a bacterial community that is distinct to that in the phyllosphere regarding composition and complexity. The aboveground and belowground communities shared few OTUs, and the aboveground communities were less enriched and diverse than the belowground communities. Similarly, a recent study carried out on 7 tree species revealed lower alpha diversity in aboveground habitats compared to belowground habitats, which is consistent with our results (Lambais et al., 2014). On the other side, this results seems to be plant species-specific since Coleman-Derr et al., (2015) found that the phyllosphere of the agave plant hosts a diversity of prokaryotes, comparable to the rhizosphere.

The soil and root habitats shared approximately half of their OTUs, which was lower than the proportions previously observed for *Arabis alpina* (> 64 % for the soil, rhizosphere and root communities) (Dombrowski et al., 2016). However, evidence of a lower alpha diversity in the roots compared to the soil was highlighted in our results. The dataset obtained for root and soil samples in the present work corroborates our previous studies, which was carried out at the same site on native tree species (poplar and willow), revealing the dominance of *Proteobacteria* and *Actinobacteria* (Zappelini et al., 2015). *Proteobacteria* and *Actinobacteria* also dominate in root bacterial communities from other polluted (Foulon et al., 2016b) or non polluted (Beckers et al., 2016) soils, although these authors used 454 pyrosequencing in their study. The dominance of the phylum *Chloroflexi* in soil samples from the present study may be explained by the adaptability of members of this phylum to oligotrophic environments (Barton et al., 2014). Moreover, this phylum has been found to be

dominant within the *Alyssum murale* rhizosphere growing on ultramafic soils (Lopez et al., 2017) and also geothermal soils, low-temperature meadow soils, sea and lake sediments, and hydrothermally active sediments (Yamada and Sekiguchi, 2009). These findings suggest that this phylum may be particularly abundant in extreme environments.

The root habitat was significantly enriched for spore-forming bacteria, such as those from the genus *Streptomyces*, compared with the other three habitats, this feature has been observed in studies focusing on the cultivable bacterial communities in contaminated soils (Kuffner et al., 2008; Sas-Nowosielska et al., 2008; Schmidt et al., 2009). The predominance of these spore-forming bacteria on or inside the roots could be explained by the fact that the abundance of r-strategists (bacteria capable of rapid growth and utilization of resources) decreases in the root area in disturbed soils (such as metal-contaminated soils), while k-strategists (bacteria with reduced growth and metabolic activity) such as *Streptomyces* may be privileged (Kunito et al., 2001). In addition, the *Streptomyces* genus contains many strains that are highly resistant to several metals (Álvarez et al., 2013) and thus may be more competitive than other bacteria in TE-enriched environments such as the tailings dump investigated in the present study.

In the leaf and stem habitats, OTUs from the phylum *Deinococcus-Thermus*, such as those from the genus *Deinococcus* (Figs. 2 & 3), were among the most abundant OTUs and were not detected in either the soil or root habitats. This group of bacteria consists of poly-extremophile bacteria that are resistant to very high doses of radiation and long periods of desiccation (Mattimore and Battista, 1996). The genus *Methylobacterium* was also identified in the phyllosphere of the plantation; this genus constitutes a considerable and generally stable fraction of the phyllospheric bacterial communities of terrestrial plants under varying environmental conditions (Knief et al., 2010). The other taxa that were more characteristic of the phyllosphere habitats belonged to the genera *Hymenobacter* and *Sphingomonas*. Members

of these bacterial genera were previously identified as more characteristic of the urban ivy (*Hedera helix*) phyllosphere than that of non-urban ivy (Smets et al., 2016). Atmospheric contamination may promote the growth of these specific taxa to a greater degree than other taxa.

Our study is one of the first to compare bacterial communities of leaf and stem habitats in trees using Illumina-based MiSeq sequencing; thus, little data is available in the literature for comparison. Both habitats clustered closely together (Table S2), but the stem appeared to be significantly less diverse than the leaf. The stem appeared to host a number of specific bacteria that were not detected in the leaf habitat. Indeed, *Firmicutes* were essentially only detected in the stem samples, and the genus *Paenibacillus* was the dominant member of this phylum. An abundance of this genus in the stem was previously observed for endophytes isolated from *Populus* cv. Hazendans but not for endophytes isolated from *Populus* cv. Hoogvorst (Moore et al., 2006). Several dominant bacterial OTUs in the stem group were assigned to *Paenibacillus amylolyticus* (Table S3). Some environmental or physiological specific factors associated with the stem are likely responsible for the presence of this bacterium. This species was previously identified in the bract phyllosphere of *Phoenix dactylifera,* which dries out and remains on the plant (Rivas et al., 2005). Other studies investigating the rhizosphere (Hosoda et al., 2003) or the phyllosphere (Rivas et al., 2005) revealed that most of the plant polysaccharide-degrading bacteria isolated belonged to the *Paenibacillus* genus. These bacteria readily digest polysaccharides, such as cellulose, pectin, starch, esculin and xylan. Moreover, we tested our phyllospheric-isolated strains for pectinase and cellulase activities (according previously described protocols (Verma et al., 2001) and we observed that the isolated *Paenibacillus amylolyticus* strain was positive for pectinase activity and negative for cellulase activity (data not shown). Based on these results, it seems that tissues of terrestrial plants represent different habitats that host specific microbial

communities. It may be interesting to decipher the structure and composition of these communities using both DNA sequencing and culture-dependent methods, each of which has constraints and advantages.

We further assessed densities of bacteria isolated from various habitats of the poplar tree plantation when Hg was added as a stressor. The bacterial communities from the phyllospheric habitats were altogether less resistant than the communities hosted in the belowground habitats, potentially due to the direct contact between soil bacterial communities and higher amounts of Hg in the soil. The rhizospheric soil hosted a bacterial community that was richer than that of the bulk soil, which was expected. Indeed, previous works have shown that planted soils host significantly richer bacterial communities compared to unplanted soils (Durand et al., 2015). This is explained by the presence of plant exudates in the soil surrounding the roots, which play a role in bacterial growth (Baudoin et al., 2003). However, bacteria isolated from the bulk soil were more resistant to Hg than bacteria isolated from the rhizospheric soil. We isolated Hg-resistant strains harboring PGP traits, such as isolates of *Bacillus subtilis* and *Pseudomonas* sp. from each habitat. The species *Bacillus subtilis* is known for its antifungal properties, siderophores, IAA production and biocontrol effects on *Fusarium wilt* (Zaidi et al. 2006; Chebotar' et al. 2009). This species was previously tested as a bioinoculant to improve Ni bioaccumulation by *Brassica juncea* and promoted the growth of pepper plants (Yu et al., 2011). Many plant-associated *Pseudomonas* species directly and indirectly promote plant growth, a phenomenon that has previously been reviewed (Preston, 2004). These species compete with and suppress pathogenic microorganisms to promote plant growth.

The stem episphere exhibited a lower cultivable bacterial community than the stem endosphere, and in contrast, the leaf episphere hosted more cultivable bacteria than its endosphere. The structures of these plant organs might play a role in the development of these

communities/be correlated with these results. In our study, several *Pseudomonas sp.* (including *P. graminis*) were isolated from the phyllosphere of poplar in the Hg-enriched growth medium. The presence of this genus was also observed using the metabarcoding technique essentially with the OTU00025 (Fig. 3 and Table S3), which were mostly detected in the leaf (0.96 %), the root (0.66 %) and soil (0.73 %) habitats. *Pseudomonas* are ubiquitous bacteria that survive on plant surfaces and inside plant tissues in both aboveground and belowground habitats (Schreiber et al., 2004). Specifically, *Pseudomonas graminis* is found in phyllospheric habitats (Behrendt et al., 1999) and was previously isolated from poplar (Doty et al., 2009). Moreover, this strain was also defined as an endophyte but unable to grow in N-free media (Hutner, 1972). However, we observed the presence of the *nifH* gene in all *Pseudomonas graminis* strains, and we confirmed that these strains were able to reduce atmospheric N_2 in nitrogen-free medium. A previous study showed that a *Pseudomonas graminis* strain isolated from poplar and re-inoculated on sterile *Populus trichocarpa* as part of a microbial consortium promoted root production, improved total biomass production and increased leaf area (Knoth et al., 2014). In addition, inoculation resulted in a 25 % increase in the total nitrogen content. The presence of high Hg resistance and the potential to fix atmospheric N_2 such as the *Pseudomonas graminis* strains identified in this study might be valuable traits to further investigate in a future study and may therefore be key microorganisms for the restoration of disturbed environments. We were unable to isolate endophytic Hg resistant bacteria from any of the three habitats studied; suggesting the internal plant tissues may not host resistant bacteria.

As previously reviewed, the microbial communities of the phyllosphere have a central role in plant function and atmospheric trace gas dynamics (Bringel and Couée, 2015). Thus, using PGPB inoculation in both the rhizosphere and phyllosphere, we sought to improve the uptake of Hg by an Hg-accumulating plant, specifically, pepper. However, our results

revealed that inoculation did not affect the rate of Hg accumulation in pepper leaves, although it increased root growth. This growth may be related to a significant increase in the metabolism of L-phenylalanine by microbial communities of the inoculated plants. Indeed, the increased activity of phenylalanine ammonia-lyase in *Bacillus subtilis* leads to the release of ammonia into the soil, which then acts as a fertilizer (Podile and Laxmi, 1998).

In conclusion, the application of a metabarcoding approach to the study of bacterial communities associated to the poplar cultivar Skado (*P. trichocarpa* x *P. maximowiczii*) planted at an industrial phytomanaged site revealed contrasting microbial communities in different poplar habitats. In parallel, a more conventional approach allowed us to isolate strains with functional traits of interest. We believe that the present findings will be instructive for the design of future restoration practices for industrial dumps.

Acknowledgments

This work was supported by the French Environment and Energy Management Agency [PROLIPHYT 1172C0053], the Région Franche-Comté [Environnement-Homme-Territoire 2014-069], the Pays de Montbéliard Agglomération [13/070-203-2015], and the French national programme EC2CO/MicrobiEen FREIDI-Hg. A.D. received a PhD grant from the Région Franche-Comté. V.A.L. received a post-doc grant from the Région Franche-Comté.

Figure legends

Figure 1. NMDS plot of bacterial communities associated with the leaf, stem, root and soil habitats using the Bray-Curtis dissimilarity measure and 1,000 iterations. Each point represents the bacterial community of a given sample, a: belowground habitats; b: aboveground habitats.

Figure 2. A barplot showing the bacterial community composition at the phylum or class level for each habitat (leaf, stem, root and soil) based on 16S rRNA sequencing of samples using the metabarcoding approach. The species of Hg-resistant strains isolated from belowground and aboveground habitats are indicated.

Figure 3. A heatmap comparing the abundance of bacterial OTUs among the 20 most abundant OTUs in at least one of the four poplar habitats. The dendrogram represents linkage clustering using Euclidean distance measures. OTU delineation was based on a cutoff of < 97 % sequence similarity. Assignments between brackets indicate the lower taxonomic level associated with the OTU using the Greengenes database, k: kingdom, p: phylum, o: order, c: class, f: family, s: genus and species.

Figure 4: Phylogeny of the Hg-resistant bacteria isolated from the phyllosphere in 1/10 869 medium containing 2 µM $HgCl_2$. The phylogenic tree was constructed using a K2P Neighbor-joining model based on aligned DNA sequences (697 bp) with 1,000 iterations. The parentage of branches is given in the tree. Phenotypic and genotypic characterizations for each strain are also shown.

Figure 5: Violin plot of the root, shoot and total dry biomasses at harvest (46-day mesocosms) for uninoculated and Hg-unexposed (ni-), uninoculated and Hg-exposed (ni+), inoculated and Hg-unexposed (i-) and inoculated and Hg-exposed (i+) plants. Different letters indicate significant differences, P value < 0.01.

Figure 6: Hg concentration (ng mg DM^{-1}) in the dry biomass of older leaves of peppers grown for 46 days with the following treatments: uninoculated and Hg-unexposed (ni-), uninoculated and Hg-exposed (ni+), inoculated and Hg-unexposed (i-) and inoculated and Hg-exposed (i+). Different letters indicate significant differences, P value < 0.01.

Tables

Table 1. Alpha diversity estimations of bacterial communities from the four poplar habitats. All diversity statistics were calculated using an OTU threshold of ≥ 97 % sequence similarity on randomly subsampled data at the lower sample size (3,000 reads). Mean values followed by different letters are significantly different at P < 0.05 (Kruskal-Wallis comparison test). Numbers between brackets indicate the standard error (SE).

Table 2: Cultivable colony forming units per gram dry weight soil (CFUs g^{-1}) on solid media and percentage (%) of Hg-resistant cultivable bacteria to 2 and 10 µM $HgCl_2$ in the different habitats.

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951

Table and Figure

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Figure 1

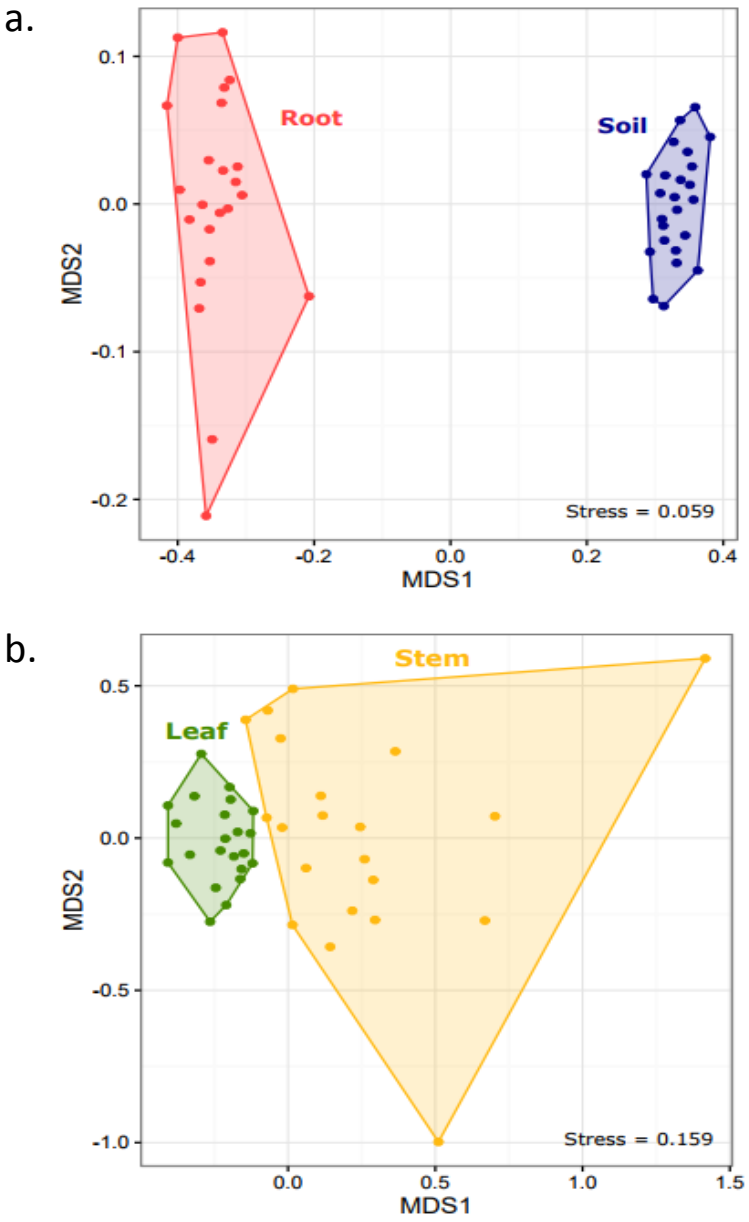


Table 1

Habitats	Soil	Root	Stem	Leaf
Mean reads per sample	41,700	34,090	17,741	13,793
Subsample size (number of samples)	3,000 (24)	3,000 (23)	3,000 (21)	3,000 (22)
Observed OTUs	671[a] (± 4)	455[b] (± 7)	90[c] (± 1)	93[c] (± 3)
Chao1 OTUs definition	1,112[a] (±15)	750[b] (± 15)	138[c] (± 2)	128[c] (± 7)
Shannon Index (H)	5.50[a] (± 0.02)	4.79[b] (± 0.04)	2.94[d] (± 0.01)	3.13[c] (± 0.04)
Inverse Simpson index (1/D)	75.70[a] (± 2.60)	40.56[b] (± 2.75)	11.83[d] (± 0.13)	14.18[c] (± 0.60)
Shannon Index Evenness (E)	0.846[a] (± 0.001)	0.782[b] (± 0.005)	0.655[d] (± 0.001)	0.694[c] (± 0.006)

Figure 2

Mercury resistant bacteria isolated from belowground habitats

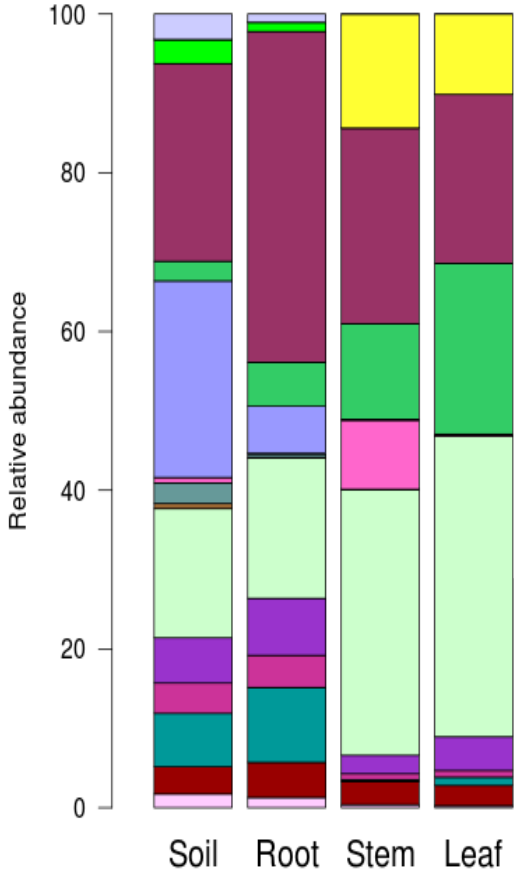
Firmicutes
Bacillus licheniformis
Staphylococcus epidermidis
Bacillus subtilis
Bacillus pimilus

Alphaproteobacteria
Sphingomonas sp.

Betaproteobacteria
Variovorax sp.

Gammaproteobacteria
Pseudomonas sp.
Pseudomonas migulae
Pseudomonas putida
Pseudomonas vancouverensis
Pseudomonas fluorescens
Erwinia sp.
Pseudomonas moraviensis

Relative abundance of bacterial OTUs



Mercury resistant bacteria isolated from aboveground habitats

Actinobacteria
Frigoribacterium faeni
Curtobacterium flaccumfaciens
Micrococcus yunnanensis
Arthrobacter scleromaes

Firmicutes
Staphylococcus capitis
Staphylococcus xylosus
Paenibacillus amylolyticus
Pseudomonas luria
Eubacterium sp.

Alphaproteobacteria
Sphingomonas melonis

Gammaproteobacteria
Pseudomonas graminis
Pseudomonas alkaligenes
Stenotrophomonas rhizophila
Erwinia persicina
Erwinia rhapontici
Erwinia aphidicola
Pseudomonas moraviensis

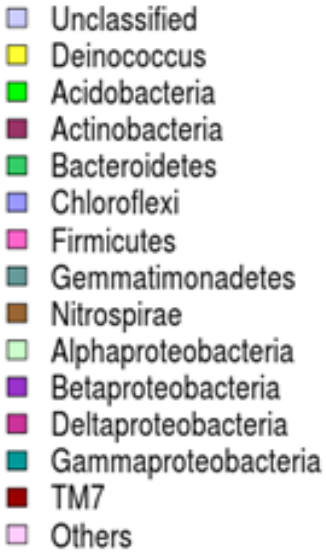


Figure 3

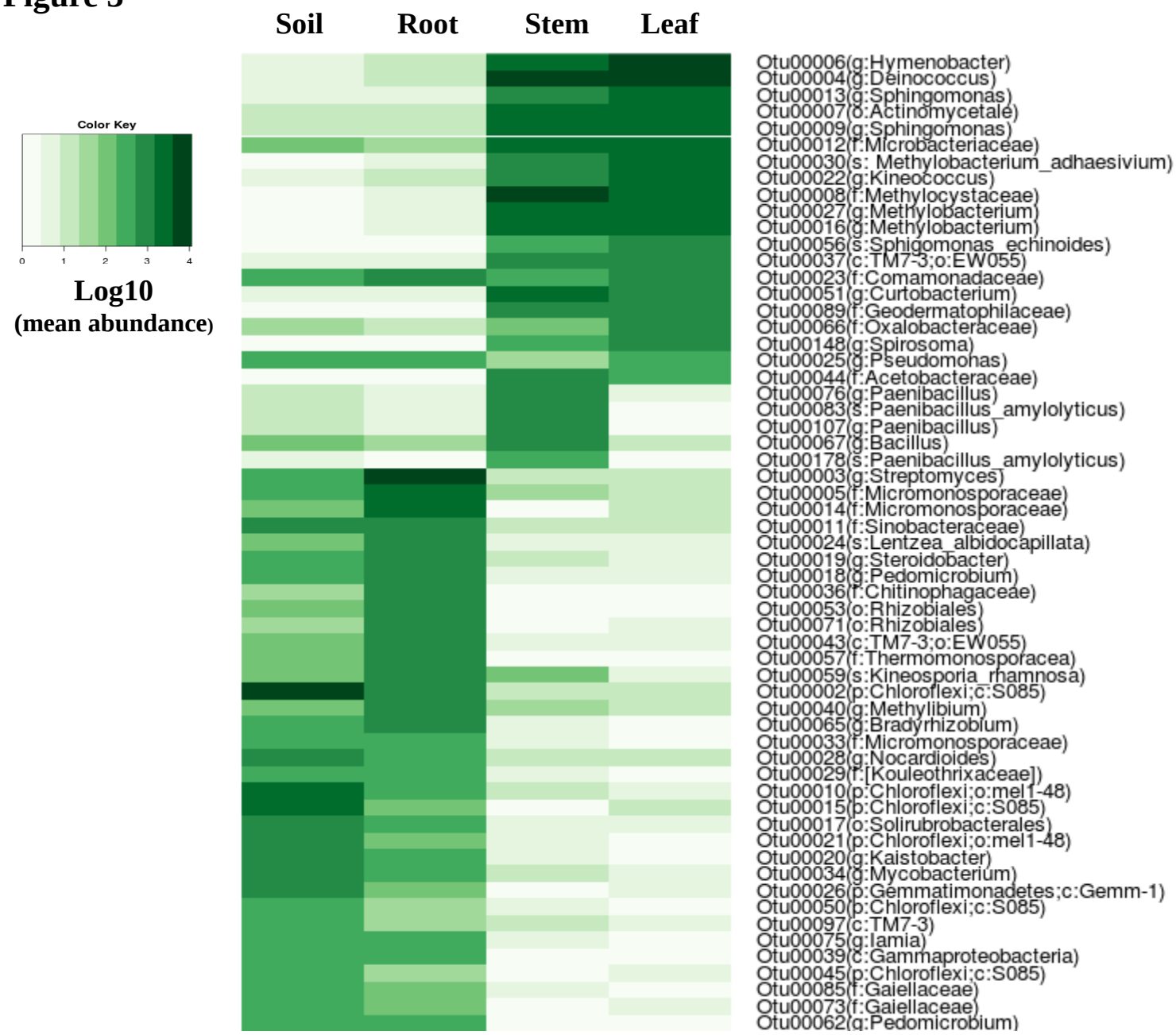

Soil
Root
Stem
Leaf
Color Key
0
1
2
3
4
Log10
(mean abundance)
Otu00006(g:Hymenobacter)
Otu00004(g:Deinococcus)
Otu00013(g:Sphingomonas)
Otu00007(o:Actinomycetale)
Otu00009(g:Sphingomonas)
Otu00012(f:Microbacteriaceae)
Otu00030(s:_Methylobacterium_adhaesivium)
Otu00022(g:Kineococcus)
Otu00008(f:Methylocystaceae)
Otu00027(g:Methylobacterium)
Otu00016(g:Methylobacterium)
Otu00056(s:Sphigomonas_echinoides)
Otu00037(c:TM7-3;o:EW055)
Otu00023(f:Comamonadaceae)
Otu00051(g:Curtobacterium)
Otu00089(f:Geodermatophilaceae)
Otu00066(f:Oxalobacteraceae)
Otu00148(g:Spirosoma)
Otu00025(g:Pseudomonas)
Otu00044(f:Acetobacteraceae)
Otu00076(g:Paenibacillus)
Otu00083(s:Paenibacillus_amylolyticus)
Otu00107(g:Paenibacillus)
Otu00067(g:Bacillus)
Otu00178(s:Paenibacillus_amylolyticus)
Otu00003(g:Streptomyces)
Otu00005(f:Micromonosporaceae)
Otu00014(f:Micromonosporaceae)
Otu00011(f:Sinobacteraceae)
Otu00024(s:Lentzea_albidocapillata)
Otu00019(g:Steroidobacter)
Otu00018(g:Pedomicrobium)
Otu00036(f:Chitinophagaceae)
Otu00053(o:Rhizobiales)
Otu00071(o:Rhizobiales)
Otu00043(c:TM7-3;o:EW055)
Otu00057(f:Thermomonosporacea)
Otu00059(s:Kineosporia_rhamnosa)
Otu00002(p:Chloroflexi;c:S085)
Otu00040(g:Methylibium)
Otu00065(g:Bradyrhizobium)
Otu00033(f:Micromonosporaceae)
Otu00028(g:Nocardioides)
Otu00029(f:[Kouleothrixaceae])
Otu00010(p:Chloroflexi;o:mel1-48)
Otu00015(p:Chloroflexi;c:S085)
Otu00017(o:Solirubrobacterales)
Otu00021(p:Chloroflexi;o:mel1-48)
Otu00020(g:Kaistobacter)
Otu00034(g:Mycobacterium)
Otu00026(p:Gemmatimonadetes;c:Gemm-1)
Otu00050(p:Chloroflexi;c:S085)
Otu00097(c:TM7-3)
Otu00075(g:Iamia)
Otu00039(c:Gammaproteobacteria)
Otu00045(p:Chloroflexi;c:S085)
Otu00085(f:Gaiellaceae)
Otu00073(f:Gaiellaceae)
Otu00062(g:Pedomicrobium)

Table 2

Habitats		Medium 1/10 869			Medium 284	
		0 μM $HgCl_2$	2 μM $HgCl_2$	10 μM $HgCl_2$	0 μM $HgCl_2$	10 μM $HgCl_2$
Aboveground	leaf episphere	3.59 105 (100%)	9.28 104 (25.86%)	2.43 104 (6.77%)	2.14 105 (100%)	2.85 104 (13.31%)
	stem episphere	5.38 104 (100%)	1.21 104 (22.45%)	3.56 103 (6.62%)	0	0
	leaf endosphere	2.24 104 (100%)	ND	0	0	0
	stem endosphere	1.71 106 (100%)	ND	0	0	0
Belowground	root endosphere	4.77 106 (100%)	ND	9.54 102 (0.02%)	1.45 106 (100%)	0
	rhizospheric soil	6.28 108 (100%)	ND	9.84 107 (10.89%)	2.01 108 (100%)	4.02 106 (2.00%)
	bulk soil	1.34 107 (100%)	ND	9.02 106 (67.33%)	8.20 106 (100%)	3.18 106 (38.78%)

Figure 4

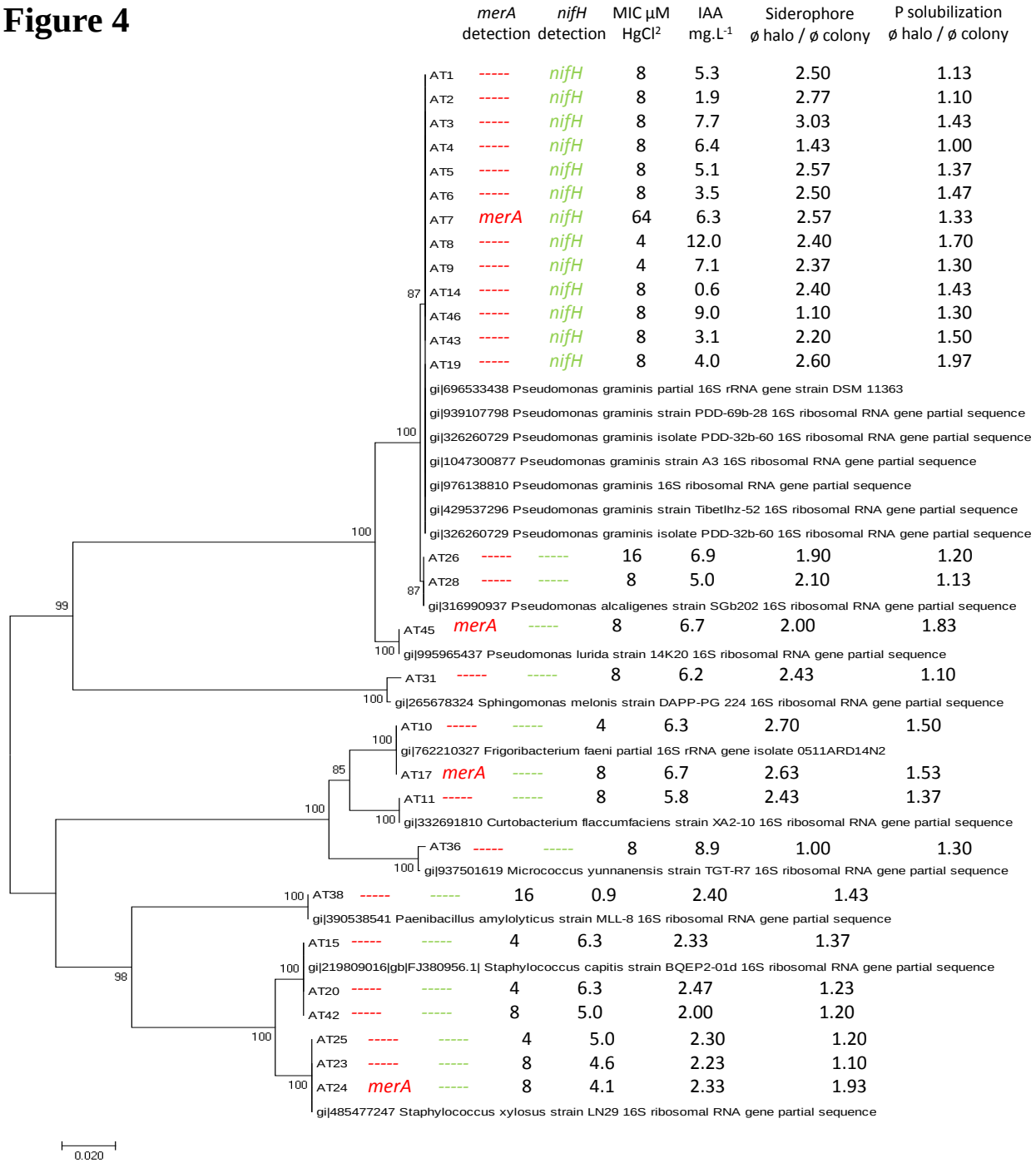

merA detection
nifH detection
MIC µM HgCl2
IAA mg.L-1
Siderophore ø halo / ø colony
P solubilization ø halo / ø colony
AT1 ----- nifH 8 5.3 2.50 1.13
AT2 ----- nifH 8 1.9 2.77 1.10
AT3 ----- nifH 8 7.7 3.03 1.43
AT4 ----- nifH 8 6.4 1.43 1.00
AT5 ----- nifH 8 5.1 2.57 1.37
AT6 ----- nifH 8 3.5 2.50 1.47
AT7 merA nifH 64 6.3 2.57 1.33
AT8 ----- nifH 4 12.0 2.40 1.70
AT9 ----- nifH 4 7.1 2.37 1.30
AT14 ----- nifH 8 0.6 2.40 1.43
AT46 ----- nifH 8 9.0 1.10 1.30
AT43 ----- nifH 8 3.1 2.20 1.50
AT19 ----- nifH 8 4.0 2.60 1.97
gi|696533438 Pseudomonas graminis partial 16S rRNA gene strain DSM 11363
gi|939107798 Pseudomonas graminis strain PDD-69b-28 16S ribosomal RNA gene partial sequence
gi|326260729 Pseudomonas graminis isolate PDD-32b-60 16S ribosomal RNA gene partial sequence
gi|1047300877 Pseudomonas graminis strain A3 16S ribosomal RNA gene partial sequence
gi|976138810 Pseudomonas graminis 16S ribosomal RNA gene partial sequence
gi|429537296 Pseudomonas graminis strain Tibetlhz-52 16S ribosomal RNA gene partial sequence
gi|326260729 Pseudomonas graminis isolate PDD-32b-60 16S ribosomal RNA gene partial sequence
AT26 ----- ----- 16 6.9 1.90 1.20
AT28 ----- ----- 8 5.0 2.10 1.13
gi|316990937 Pseudomonas alcaligenes strain SGb202 16S ribosomal RNA gene partial sequence
AT45 merA ----- 8 6.7 2.00 1.83
gi|995965437 Pseudomonas lurida strain 14K20 16S ribosomal RNA gene partial sequence
AT31 ----- ----- 8 6.2 2.43 1.10
gi|265678324 Sphingomonas melonis strain DAPP-PG 224 16S ribosomal RNA gene partial sequence
AT10 ----- ----- 4 6.3 2.70 1.50
gi|762210327 Frigoribacterium faeni partial 16S rRNA gene isolate 0511ARD14N2
AT17 merA ----- 8 6.7 2.63 1.53
AT11 ----- ----- 8 5.8 2.43 1.37
gi|332691810 Curtobacterium flaccumfaciens strain XA2-10 16S ribosomal RNA gene partial sequence
AT36 ----- ----- 8 8.9 1.00 1.30
gi|937501619 Micrococcus yunnanensis strain TGT-R7 16S ribosomal RNA gene partial sequence
AT38 ----- ----- 16 0.9 2.40 1.43
gi|390538541 Paenibacillus amylolyticus strain MLL-8 16S ribosomal RNA gene partial sequence
AT15 ----- ----- 4 6.3 2.33 1.37
gi|219809016|gb|FJ380956.1| Staphylococcus capitis strain BQEP2-01d 16S ribosomal RNA gene partial sequence
AT20 ----- ----- 4 6.3 2.47 1.23
AT42 ----- ----- 8 5.0 2.00 1.20
AT25 ----- ----- 4 5.0 2.30 1.20
AT23 ----- ----- 8 4.6 2.23 1.10
AT24 merA ----- 8 4.1 2.33 1.93
gi|485477247 Staphylococcus xylosus strain LN29 16S ribosomal RNA gene partial sequence
87
100
100
87
99
100
100
100
85
100
100
100
100
98
100
100
0.020

Figure 5

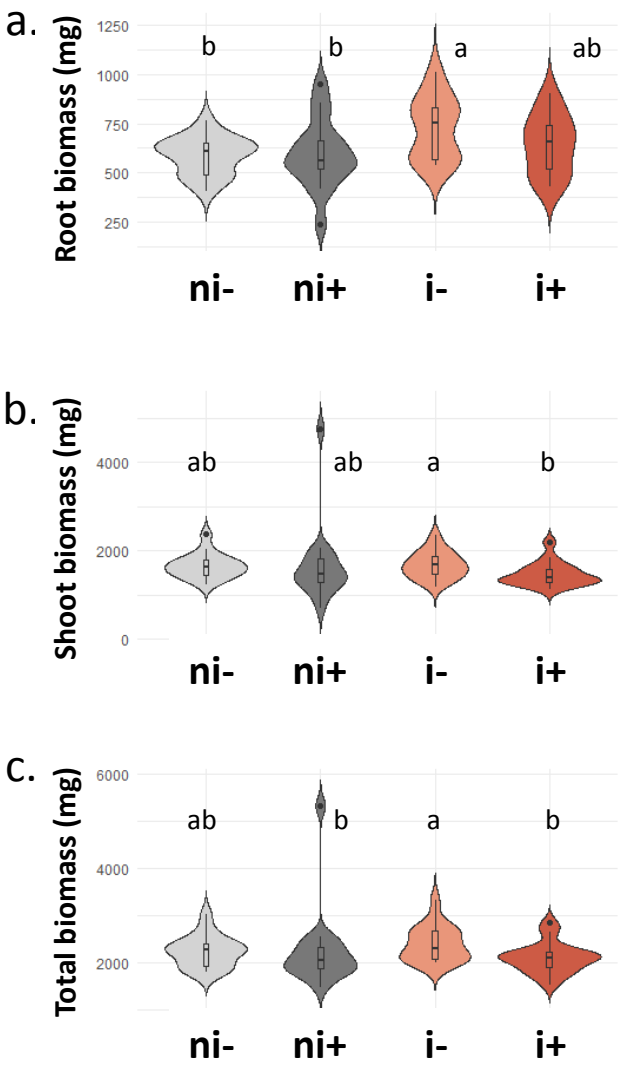
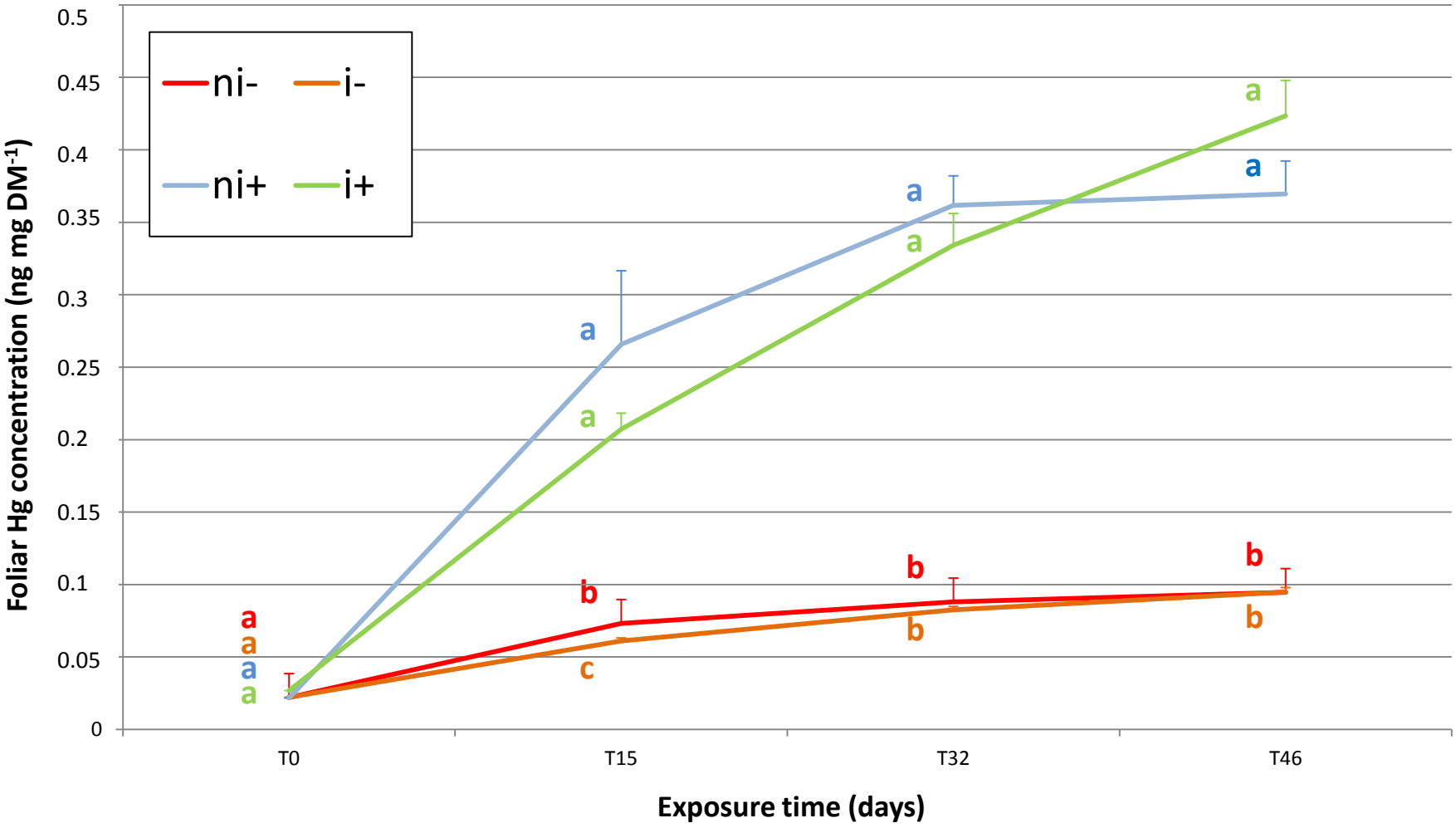

a.
Root biomass (mg)
1250
1000
750
500
250
b
b
a
ab
ni-
ni+
i-
i+
b.
Shoot biomass (mg)
4000
2000
0
ab
ab
a
b
ni-
ni+
i-
i+
c.
Total biomass (mg)
6000
4000
2000
ab
b
a
b
ni-
ni+
i-
i+

Figure 6


ni-
i-
ni+
i+
Foliar Hg concentration (ng mg DM-1)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
T0
T15
T32
T46
Exposure time (days)
a
a
a
a
b
a
a
b
c
a
a
b
b
b

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