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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 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 contaminated 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 inoculation 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 (OD₆₀₀) 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 leaf) 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, leaf, 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 (Zappellini 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. Desgarnes 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.


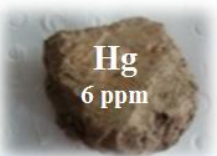



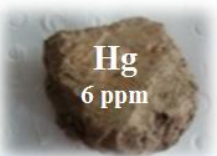







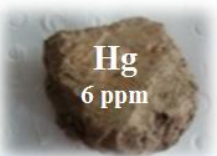





L189: Still the 16S rRNA gene even if you targeted DNA
Corrected throughout the manuscript

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.

Tavaux poplar plantation	Habitats studied	Illumina MiSeq sequencing						
	<table border="1"><tbody><tr><td data-bbox="703 187 938 361"> Soil</td><td data-bbox="938 187 1186 361"> Root</td></tr><tr><td data-bbox="703 444 938 618"> Stem</td><td data-bbox="938 444 1186 618"> Leaf</td></tr></tbody></table>	 Soil	 Root	 Stem	 Leaf	<table border="1"><tbody><tr><td data-bbox="1219 187 1719 425"></td></tr><tr><td data-bbox="1219 429 1719 728">Bacterial isolation </td></tr></tbody></table>		Bacterial isolation 
 Soil	 Root							
 Stem	 Leaf							
								
Bacterial isolation 								

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

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

4

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21

22

23 Abstract

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

48 | ~~to increased its root dry biomass but did not increase Hg concentration in leaves.~~ were
49 | ~~incubated and increased root dry biomass but didn't increased Hg concentration in leaves.~~ The
50 | N₂-fixing and Hg-resistant species *Pseudomonas graminis*, observed in the poplar
51 | phyllosphere, may be a key microorganism for the restoration of industrial tailings dumps.

52 | **Keywords:** Phytomanagement, Poplar habitats, Trace elements, Plant growth-promoting
53 | bacteria, Mercury-resistant bacteria

54 | 1. Introduction

55 | Soil contamination by metal(loids) and trace elements (TEs) such as Ag, As, Cd, Cr,
56 | Cu, Hg, Ni, Pb, Se, or Zn is a threat to ecosystem stability and a risk to human health. In
57 | 2011, 127,000 sites in 33 European Union countries were confirmed to have contaminated
58 | soils, and approximately 45 % of these sites (58,000) were under remediation project
59 | management to reduce associated risk (~~remediation~~) (Panagos et al., 2013). Data published in
60 | 2016 showed that 6.24 % (137,000 km²) of European agricultural lands were unsafe for food
61 | production, requiring local assessment and eventual remediation (Toth et al., 2016). Due to
62 | the frequent lack of a plant cover at industrial dumps, processes such as wind erosion or
63 | leaching can led to the contamination of adjacent agriculture lands leading to the decrease in
64 | soil productivity.

65 | Phytomanagement-based restoration programs for contaminated soils may use woody
66 | plant species that are ~~readily~~ colonized by essential microorganisms (Bell et al., 2014; Partida-
67 | Martínez and Heil, 2011; Yergeau et al., 2012), especially in highly stressed and nutrient-poor
68 | environments, such as heavy metals TE- contaminated sites (Durand et al., 2017; Foulon et al.,
69 | 2016a, 2016b) (~~Partida-Martínez and Heil, 2011~~). However, knowledge of these associated
70 | microbial communities remains scarce, particularly with respect to aboveground tissues.
71 | Among the microorganisms that colonize the plant rhizosphere and phyllosphere, some are

72 known to play important roles in determining plant fitness and are collectively described as
73 plant growth-promoting bacteria (PGPB) ~~Among the microorganisms that colonize the~~
74 ~~rhizosphere and the phyllosphere of a plant, some play important roles in plant fitness and are~~
75 ~~collectively described as plant growth-promoting bacteria (PGPB)~~ (Glick, 2014; Lindow and
76 Brandl, 2003; Pieterse et al., 2016). PGPB directly benefit host plants via hormone production
77 (e.g., ~~Correction has been done~~indole acetic acidindoleacetic acid, cytokinin, and zeatin) and
78 improved nutrition and indirectly by acting as biocontrol agents, inducing systemic resistance
79 and ethylene stress resistance (Bringel and Couée, 2015; Glick, 2005; Lindow and Brandl,
80 2003; Maignien et al., 2014; Trouvelot et al., 2014; Vorholt, 2012). Belowground and
81 aboveground plant , plant tissues provide various habitats, which can be easily colonized by
82 specific PGPB for bacteria growth/colonization and may host specific PGPB in or on those
83 tissues ~~Belowground, various bacteria live in, on, or around plant tissues and may stimulate~~
84 ~~plant growth, nutrition and health~~ (Vessey, 2003), ~~while aboveground, leaves and stems~~
85 ~~provide a broad habitat for bacteria and may host specific PGPB in or on tissues~~ (Vorholt,
86 2012). In the soil, free-living bacteria of various genera exert beneficial effects on plants such
87 as *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*,
88 *Curtobacterium*, *Klebsiella*, *Enterobacter*, *Pseudomonas*, and *Serratia* exert beneficial effects
89 on plants (Kloepper et al., 1989 ; Glick et al., 1999 ; Benizri et al., 2001) and are classified as
90 rhizospheric PGPB. ~~Widely, p~~Plant-associated bacteria also include endophytic bacteria,
91 which colonize internal plant tissues ~~of plants~~. They are originating from the epiphytic
92 bacterial communities of the rhizosphere and phyllosphere ~~as well as from~~ but may also arise
93 from transmission through seeds or plant vegetative parts ~~endophyte infested seeds or~~
94 ~~planting materials~~ (Hallmann et al., 1997). ~~These bacterial communities may host endophytic~~
95 ~~PGPB which are hosted by numerous genera such as~~ Members of the genera such as
96 *Acinetobacter*, *Aminobacter*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Curtobacterium*,

97 Devosia, Enterobacter, Gluconacetobacter, Herbaspirillum, Methylobacterium,
98 Microbacterium, Ochroactrum, Paenibacillus, Pseudomonas, Phyllobacterium, Rahnella,
99 Shinella, Sphingomonas and Staphylococcus have been described as plant endophytes ~~which~~
100 ~~are represented by numerous genera~~ (Compant et al., 2009; De Meyer et al., 2015; Doty et al.,
101 2009). Other bacterial endophytes that are only localized in specific structures ~~_, termed~~
102 ~~nodules,~~ are referred to as symbiotic PGPB ~~belonging such as members of totof the- genera~~
103 ~~such as~~ Azorhizobium, Bradyrhizobium, Devosia, Ensifer, Frankia, Mesorhizobium,
104 Microvirga, Ochroactrum, Phyllobacterium and Rhizobium (Glick 2014, Kloepper et al.
105 1989; Wang et al. 2012; De Meyer et al. 2015). Aboveground, phyllospheric PGPB are
106 subjected to fitness stresses (~~such as e.g. direct solar radiation?, drought~~), that are very
107 different than those found belowground, suggesting a specific selection pressure (Lindow and
108 Brandl, 2003). ~~and may result in~~ It has been demonstrated for Pseudomonas fluorescens
109 SBW25 that the ecological success/successful colonizing/survival capacity ?-of- Pseudomonas
110 fluorescens SBW25 in phyllospheric environment the phyllosphere is dependent ~~ofon the~~
111 expression of habitat specific genes. Indeed, those that genes are significantly more correlated
112 to the fitness of the bacteria in the phyllosphere significantly more- that in the soil or the
113 rhizosphere -genetic adaptations- (Gal et al., 2003). ~~Whereas our u~~ Albeit, a full understanding
114 of these bacterial communities ~~is- has not been reached- limited-~~ (Vacher et al., 2016), several
115 phyllospheric PGPB have been already previously described such as members of
116 Arthrobacter, Azotobacter, Beijerinckia, Klebsiella,- Methylobacterium, Nostoc,- Pantoea,
117 Pseudomonas,- Scytonema, Sphingomonas and Stigonema (Lindow and Brandl, 2003; Vacher
118 et al., 2016). However, gaining an understanding of the structure and composition of
119 microbial communities *in situ* is difficult due to the diversity and complexity of these
120 communities. Metabarcoding using high-throughput sequencing technologies offers a solution
121 to describe the bacterial communities associated with environmental habitats with high

122 resolution, as exemplified recently in our previous work (Durand et al., 2017; Foulon et al.,
123 2016a, 2016b). We demonstrated for instance that *Alphaproteobacteria* and *Actinobacteria*
124 dominated root bacterial communities, whereas soil samples were dominated by
125 *Alphaproteobacteria* and *Acidobacteria* (Foulon et al., 2016b). -These innovative approaches
126 still need complementary culture-dependent approaches to allow for the functional
127 characterization and selection of the best-performing PGPB.

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

133 In this study, we combined a metabarcoding approach that employs a high-throughput
134 Illumina MiSeq platform (Durand et al., 2017; Foulon et al., 2016a, 2016b; Schmidt et al.,
135 2013; Wu et al., 2015) and a culture-dependent approaches to investigate the composition and
136 assembly of ~~characterize bacterial communities investigate bacterial diversity~~ and isolate Hg-
137 resistant PGPB ~~in the rhizosphere, endosphere and phyllosphere of a poplar plantation~~
138 at a Hg-contaminated site. ~~We hypothesized that the presence of a poplar tree plantation on an~~
139 ~~industrial dump would significantly and differentially shape the bacterial communities from~~
140 ~~the different soils and poplar habitats. We expected that we would observe clear differences in~~
141 ~~the relative abundance and composition of bacterial communities across poplar habitats that~~
142 ~~may improve our understanding of the microbial ecology of these environments. We also~~
143 ~~aimed at better understanding the role of isolated bacterial strains in Hg transfer from the~~
144 ~~atmosphere to plant tissues using PGPB inoculation of the model plant *Capsicum annum*.~~

145

146

147 2. Materials and Methods

148 2.1. Site description

149 The ~~chlor-alkali study~~ site is located in Tavaux, France, in Western Europe along a
150 ~~major~~ the Saône river (Saône) and is surrounded by several urban aggregates. The study site is
151 a dump/This ~~The study was carried out at an industrial dump which was exploited as a~~
152 sediment storage area from the 1950s to 2003. These sediments were originated from the
153 effluents produced during the chlor-alkali Hg-cell process used during for the electrolysis of
154 NaCl ~~tie processes associated with a Hg cell chlor-alkali process.~~ –A detailed map of the
155 industrial site is was provided by Maillard et al. (2016). ICP-AES analyses revealed that total
156 Hg and total As were significantly increased in the dump compared to the levels in ~~control~~
157 soil from an adjacent undisturbed forest nearby. Further details ~~of soil analyses on soil~~
158 characteristics of the ~~lagoon dump~~ are provided in Zappellini et al. (2015). The poplar cultivar
159 Skado (*P. trichocarpa* x *P. maximowiczii*) was planted in 2011 as a short rotation coppice
160 (2200 stems ha⁻¹) as part of a phytomanagement strategy, fully described in Durand et al.
161 (2017).

162

163 2.2. Sampling design ~~Metabarcoding analysis~~

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

168 from under the canopy of the poplars were sieved to <4 mm. In more details, the roots were
169 collected after litter removal from the upper 20 cm layer of soil from under the canopy of the
170 trees. They were separated from the soil via 2 distilled water baths, and the smallest roots
171 were selected and separated from larger roots by cutting them with a scalpel. Woody samples
172 were collected from poplar branches of the axe 2 (0.8-1.2 cm in diameter) at a ca. 5 m height,
173 corresponding to the half-crown of the poplar. Leaf samples were composed of 3 leaves
174 collected from the branches collected as described above. All samples were obtained over a
175 one-day period to reduce any heterogeneity imparted by climatic conditions. The samples
176 were either freeze-dried and stored at -20°C for molecular analysis or dried at ambient
177 temperature (24°C ± 1) for physico-chemical analyses. Thus, we considered the belowground
178 and aboveground habitats to include both endophytic and epiphytic fungi. Detailed
179 information about sampling procedure is described in Durand et al 2017

180 2.3. Metabarcoding analysis

181 ~~Sampling for metabarcoding analysis was carried out in August 2014 on the 4~~
182 ~~different habitats: bulk soil, root, stem and leaf. For each habitat, 4 samples~~ Samples were
183 ~~collected from each of the six random trees that were selected on three replicated plots (2~~
184 ~~trees per plot, 24 samples per habitat), with DNA extracted as previously described in Durand~~
185 ~~et al. (2017). Briefly, environmental DNA was extracted from biological samples within 2~~
186 ~~months after sampling. Plant material (root, stem and leaf samples), was thoroughly washed~~
187 ~~with distilled water and DNA was extracted from XX20 mg of fresh material~~ using an
188 adapted cetyl trimethylammonium bromide/chloroform/isoamyl alcohol protocol (Lefort and
189 Douglas 1999; Healey et al. 2014) for root, stem and leaf samples, whereas environmental
190 Soil DNA from soil samples was extracted from 100 mg of sieved fresh material soil, using a
191 PowerSoil DNA Isolation Kit following the manufacturer's instructions (MoBio Laboratories,
192 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 $10 \text{ ng } \mu\text{l}^{-1}$, 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 sumacust (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 %

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

240 2.43. *Isolation ~~and characterization~~ of Hg-resistant bacterial strains*

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

243 our previous study (Durand et al., 2017). Bulk soil samples (3.5 g) were taken far from the
244 root system under the canopy and placed in a flask containing 35 ml of phosphate buffer
245 (PBS: 8 g of NaCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄ per l, pH 7.2). Roots with
246 adhering soil (defined as the rRhizospheric soil) ~~Rhizospheric soil, defined as root-adhering~~
247 ~~soil, was separated from roots after were washed in a 30-min~~ PBS bath in a rotatory shaker
248 (30 min, 150 rpm). Roots were separated from the soil suspension and then rRhizospheric soil
249 ~~solutions werewas recovered after~~ centrifugation ~~at~~ (14,000 g for 10 min), and 1 g of the
250 rhizospheric precipitated soil was then rediluted-suspended by shaking in 35 ml of PBS
251 ~~(shaking?)~~. Phyllospheric epiphytes, defined as the microbes living on the surfaces of
252 aboveground habitats, were isolated from leaves and stems. ~~Three g of leaves or 2020 g of~~
253 ~~stems or 3g leaves and added~~ were thoroughly washed with distilled water and suspended in
254 to 150 ml of PBS extraction solution, then placed on a rotatory shaker (150 rpm) for 1 h.
255 Isolation of endophytes from root, stem and leaf habitats was carried out using the same
256 ~~tissues sampled for epiphytic isolation~~ after surface sterilization with 2 % sodium
257 hypochlorite for 2 min. Samples were thoroughly rinsed with sterile distilled water and then
258 ~~were crushed~~ in 35 ml of PBS and macerated for 1 h on a rotatory shaker (150 rpm). Soil and
259 plant suspensions were diluted in 10-fold series and plated onto 1/10 rich medium (869) as
260 well as onto environmental medium (284) (Eevers et al. 2015). ~~Cultivabilities-Densities~~ of
261 Hg- resistant bacteria were determined using the same media supplemented with 0, 2 or 10
262 µM HgCl₂ (Francois et al., 2012). The agar plates were then incubated in the dark at 27°C for
263 6 days. The number of bacteria was expressed as log(log (CFU) g⁻¹ dry weight (DW) of
264 sample. Hg-resistant colonies were isolated and purified by subculture on Hg-enriched 1/10
265 869 or 284 medium. A bacterial collection was ~~further~~ established by growing each previous
266 isolated strains in 6 ml of Luria Bertani (LB) broth (Sigma-Aldrich, Steinheim, Germany) for

267 15 h on a rotatory shaker (150 rpm) at 30°C and stored in 25 % sterile glycerol containing 75
268 % brain heart infusion broth (Roth, Karlsruhe, Germany) at -80°C for further analyses.

269 2.5. Taxonomic identification

270 For taxonomic identification, Each Hg-resistant isolate was cultured in 15 ml of LB
271 medium for 24 h on a rotatory shaker (120 rpm) at 27°C. After centrifugation, DNA was
272 extracted from the pellets using an EZNA bacterial DNA isolation kit (OMEGA Bio-tek, Inc,
273 Norcross, Georgia, USA) according to the manufacturer's instructions. The BOX-PCR
274 fingerprinting method was used to group genotypic profiles at a similarity level of 97 % as
275 previously described (Becerra-Castro et al., 2011). One strain was selected for each BOX-
276 group and R16S rDNA genes were amplified from 5 µl of bacterial DNA in a final reaction
277 volume of 50 µl containing 25 µl of 2x PCR master mix (Thermo Fisher Scientific, Carlsbad,
278 California, USA) and each universal primer at 0.5 µM (Eurofins Genomics, Paris, France),
279 specifically 27f (5'- AGA GTT TGA TCM TGG CTC AG -3') and 1492r (5'- GGT TAC CTT
280 GTT ACG ACT T -3') (Mark Ibekwe et al., 2007). DNA amplification was carried out in a
281 thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) under the following
282 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
283 an additional 15-min cycle at 72°C (Durand et al., 2015). All 16S rRDNA PCR products were
284 sequenced by Sanger sequencing (Genewiz Beckman Coulter Genomics, United Kingdom).
285 DNA sequences were edited with BioEdit software v7.2.6. (Hall, 1999) screened against the
286 GenBank database using the BLASTn tool at the NCBI website
287 (<http://www.ncbi.nlm.nih.gov/>) and then aligned with reference sequences of bacteria
288 (LN551925, KR922297, HQ256858, KU587965, KU523561, JX827229, HQ256858,
289 HQ224627, KT825724, NR_028626, LN774179, JF496259, KR476438, JQ956529,
290 FJ380956, and KC790245) using Clustal W (Thompson et al., 1994) as implemented in
291 Bioedit. Neighbour Joining (NJ) tree reconstructions were produced using MEGA 5 (Tamura

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

294 | 2.6. Functional characterization

295 | Each isolate of the bacterial collection was screened for the following plant growth-
296 | promoting traits: siderophore production, IAA synthesis and phosphate solubilization. The
297 | siderophore production of each bacterial strain was determined using chrome azurol sulfonate
298 | (CAS) agar medium (Alexander and Zuberer, 1991; Schwyn and Neilands, 1987). After 5
299 | days of incubation on CAS medium at 30°C, a red-orange halo around colonies indicated the
300 | production of siderophores. For each strain, the ratio between the diameter of the halo and the
301 | diameter of colony was recorded. The IAA synthesis of each bacterial strain was determined
302 | using Salkowski's reagent (1 ml of 0.5 M FeCl₃ in 50 ml of 35 % HClO₄) as described by
303 | (Barillot, 2012). The phosphate-solubilizing capacity of bacterial strains was determined on
304 | Pikovskaya medium (Mehta and Nautiyal, 2001). After 5 days of incubation at 30°C, a
305 | discolored halo around colonies demonstrated phosphate solubilization. For each strain, the
306 | ratio between the halo diameter and the diameter of colony was recorded. Additionally, MICs
307 | (minimal inhibitory concentrations) of Hg were determined for each isolated strain. Briefly,
308 | microtitration plates were prepared using 2-fold dilutions of Hg in LB liquid broth, from 0 to
309 | 256 µM Hg. The strain *Pseudomonas aeruginosa* PA14, which is resistant to Hg above
310 | 256 µM, was used as a positive control. Each well was inoculated with 2 µl of a bacterial
311 | suspension, and plates were incubated for 48 h at 27 °C on a rotatory shaker table (180 rpm).
312 | After incubation, the bacterial growth was evaluated by measuring the optical density at 600
313 | nm (OD₆₀₀). Nitrogen fixation ability was evaluated using N-free semisolid malate medium
314 | (NFb) (Baldani and Döbereiner, 1980) inoculated with the assayed strains. After 10 days,
315 | strains that successfully grew were transferred to fresh NFb medium, and after 7 days of
316 | incubation, the characteristic pellicles of diazotrophic bacteria were observed.

317 Using the previous extracted bacterial DNA, the presence of the genes *merA* and *nifH*
318 in the genome of each strain was assayed. PCR amplification of the *merA* gene was performed
319 using 3 µl of bacterial DNA in a final volume of 20 µl that also contained 10 µl of 2x PCR
320 master mix (Thermo Fisher Scientific, Carlsbad, California, USA) and each primer at 0.5 µM,
321 specifically *merA2F* (5'- CCT GCG TCA ACG TCG GCT G -3') and *merA2R* (5'- GCG
322 ATC AGG CAG CGG TCG AA -3') (Poulain et al., 2015). DNA amplification was carried
323 out in a thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) using the
324 following conditions: 10 min at 95°C, followed by 35 cycles of 30 sec at 95 °C, 15 sec at 63
325 °C, and 30 sec at 72°C, with an additional 5-min incubation at 72 °C. PCR amplification of
326 the *nifH* gene was performed in a final volume of 25 µl containing 5 µl of bacterial DNA,
327 12.5 µl of 2x PCR master mix, and each primer at 0.5 µM including *nifHF* (5'- TAC GGN
328 AAR GGS GGN ATC GGC AA -3') and *nifHI* (5'- AGC ATG TCY TCS AGY TCN TCC A
329 -3') (Laguerre et al., 2001). DNA amplification was carried out in a thermocycler under the
330 following conditions: 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 53.5
331 °C, and 2 min at 72 °C, with an additional 15-min incubation at 72 °C.

332

333 2.74. Pot experiment with Hg-resistant bacteria and pepper

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

341 ~~the other edible or woody crops tested and thus may be the most sensitive to any changes of~~
342 ~~Hg accumulation. This plant was considered as an adequate candidate for their use (in the~~
343 ~~adjacent areas of industrial sites) as bioindicator of atmospheric Hg pollution. Next~~The 68-
344 day-old plants; were used to start a 46-day pot experiment ~~was performed~~. Each mesocosm
345 was defined as a pepper plant growing in a 0.5 liter pot in a growth chamber (photoperiod, 16
346 h; temperature, 18 °C (night) and 22 °C (day); relative humidity, 60-80 %; photosynthetic
347 photon flux density, 250 mmol m⁻² s⁻¹). In total, 16 *Capsicum annuum* plants were used for
348 each of the following treatments: ~~non-~~uninoculated and Hg-unexposed (ni-); inoculated and
349 Hg-unexposed Hg (i-); uninoculated and Hg-exposed (ni+); and inoculated and Hg-exposed
350 (i+). Hg-unexposed plants (ni- and i-) were grown in the same growth chamber, while Hg-
351 exposed plants (ni+ and i+) were grown in a separate growth chamber. Hg-enriched soil (6.38
352 mg kg⁻¹ Hg) from the industrial tailings described above was added to the Hg-contaminated
353 growth chamber to create an Hg-enriched atmosphere as previously described (Assad et al.,
354 2016). The soil was not in direct contact with the plants in any way but provided an
355 atmospheric enrichment with Hg, similar to that observed in the natural environment.
356 Atmospheric Hg in the contaminated and uncontaminated growth chambers was measured at
357 21.0 and 3.48 ng m⁻³, respectively. Using the previously isolated bacteria stored at -80 °C, two
358 bacterial consortia were produced to inoculate both the belowground and aboveground
359 compartments of *Capsicum annuum*.

360 -The bacteria were grown at 30 °C with shaking at 200 rpm to until maximum optical
361 density (OD₆₀₀) in saturation in the liquid 869 media ~~for was reached~~24 h at 30 °C with
362 shaking at 200 rpm. To generate the final phyllospheric consortium (PC), four *Pseudomonas*
363 *graminis* strains (AT3, AT7, AT8 and AT9), isolated from the phyllosphere were added in
364 equal proportions and adjusted to a final OD₆₀₀ of 1 in ~~0.5 l of~~ PBS. To generate the final
365 belowground consortium (BC), two *Pseudomonas* sp. strains (FT79, FT82) isolated from the

366 | rhizospheric soil and two *Bacillus subtilis* strains isolated from the bulk soil (FT97 and FT50)
367 | were used in equal proportions and adjusted to a final OD₆₀₀ of 1 in ~~0.5 l of~~ PBS. For each
368 | plant, 4.8 ml of PC was sprayed onto the leaves, and 10 ml of BC was added near the base of
369 | the stems. The same amounts of PBS were added using the same methods with uninoculated
370 | plants. In addition, unplanted control pots were prepared.

371 | Stem lengths and ~~the amounts of~~ Hg concentrations in older leaves were measured at
372 | 0, 15, 32 and 46 days. In addition, at harvest (46 days), dry biomass of belowground and
373 | aboveground tissues ~~plants werewas~~ measured as well as the total Hg content in younger
374 | leaves, fruits, stem and roots. All samples were dried at room temperature and ground into a
375 | homogenous powder in a Mixer Mill for 3 min at 30 Hz (model MM400; Retsch Inc.,
376 | Newtown, Pennsylvania, USA). We used Biolog Ecoplate™ microplates (Biolog Inc.,
377 | Hayward, CA.) following the manufacturer's recommendations to determine the metabolic
378 | capacity of soil microbial communities under the different treatments.

379 | 2.85. Hg analysis in the substrate and biomass

380 | Hg was measured in the soil and ~~pepper~~ plant samples using an AMA-254 (Advanced
381 | Mercury Analyzer) cold vapor atomic absorption (CV-AAS) Hg analyzer (Altec Co., Czech
382 | Republic) under the standard conditions recommended by the manufacturer (120 sec drying,
383 | 150 sec heating, and 45 sec cooling). The validity of the analytical method was assessed using
384 | certified reference material (CRM), specifically Oriental Basma Tobacco Leaves (INCT-
385 | OBTL-5) with a certified Hg content of $20.9 \pm 1.3 \text{ ng g}^{-1} \text{ DM}$ (Samczyński et al., 2012).
386 | Quality controls were regularly performed as described elsewhere (Maillard et al., 2016).

387 | 2.96. General statistical analysis

388 The Shapiro test and the Bartlett test were used to check the normality and
389 homoscedasticity of the data, respectively. Our data verifying normality and homoscedasticity
390 were assessed using analysis of variance (ANOVA). In other cases the data were examined
391 using the Kruskal-Wallis test. Statistical analyses were performed with R software, version
392 3.3.2 (Team R, 2014).

393

394 **3. Results**

395 *3.1. Contrasting microbial communities in poplar tree habitats revealed by metabarcoding*

396 Illumina MiSeq sequencing of bacterial 16S rRNA genes from environmental DNA
397 isolated from four habitats (soil, root, stem and leaf of poplar trees) yielded 4,437,316
398 sequences. After filtering ~~artifactual~~artiefactual, chimeric and unaligned sequences, 76 %
399 (3,222,285) of the initial sequences were retained, distributed into 2,699 OTUs. After the
400 removal of non-bacterial OTUs, 2,661,227 bacterial sequences remained and composed the
401 operational dataset. Subsampling of the dataset was adjusted to 3,000 reads clustered in 2,414
402 OTUs (Table S1). Our results showed large variations in total OTU numbers in aboveground
403 *versus* belowground samples.

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

412 confirmed a clear separation between the root and soil bacterial communities ($R = 1$; P value
413 = 0.001) while a less marked dissimilarity between the stem and leaf communities was
414 observed ($R = 0.61$; P value = 0.001). Roots (Fig. 1a) and stems (Fig. 1b) data exhibited the
415 greatest between-sample variations, evidenced by their higher scattering.

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

425 The Venn diagram highlights shared and unique OTUs for each habitat (Fig. S2). The
426 sums of the total observed bacterial OTUs in the four sample types in the poplar tree plots
427 were 475, 337, 94 and 77 for soil, root, stem and leaf samples, respectively (Fig. S2). Overall,
428 9 OTUs (1.4 %) were shared by all compartments, and only 4.2 % of OTUs were found in
429 both belowground and aboveground habitats. Moreover, aboveground habitats shared 50.8 %
430 of their OTUs, while belowground habitats shared 43.9 % of their OTUs. Soil samples
431 contained the highest proportions of unique OTUs (47.3 %), followed by leaf (31.2 %), root
432 (25.2 %) and stem (18.1 %) samples.

433 Through environmental 16S [rDNA](#) sequencing, bacterial communities from the four
434 habitats (soil, root, stem and leaf) were categorized into 30 phyla and 80 classes, the most
435 abundant of which are shown in Fig. 2. [The entire dataset was dominated by Actinobacteria](#)

436 | (28 %) and *Alphaproteobacteria* (26 %) dominated the entire dataset. The largest proportion
437 | of *Actinobacteria* was found in the root habitat (41.7 %) compared to the other compartments
438 | (Kruskal-Wallis $X^2 = 45.8$; $P < 1.3 \times 10^{-11}$), and the aboveground samples contained more
439 | *Alphaproteobacteria* (35.7 %) compared to belowground samples (Kruskal-Wallis $X^2 = 59.7$;
440 | $P < 1.1 \times 10^{-14}$). Altogether, belowground habitats were enriched for *Acidobacteria* (Kruskal-
441 | Wallis $X^2 = 68.9$; $P < 2.2 \times 10^{-16}$), *Chloroflexi* (Kruskal-Wallis $X^2 = 66.9$; $P < 2.9 \times 10^{-16}$),
442 | *Betaproteobacteria* (Kruskal-Wallis $X^2 = 42.7$; $P < 6.3 \times 10^{-11}$), *Deltaproteobacteria*
443 | (Kruskal-Wallis $X^2 = 66.7$; $P < 3.2 \times 10^{-16}$) and *Gammaproteobacteria* (Kruskal-Wallis $X^2 =$
444 | 66.6 ; $P < 3.2 \times 10^{-16}$), whereas aboveground habitats were enriched for *Deinococcus-Thermus*
445 | (Kruskal-Wallis $X^2 = 68.0$; $P < 2.2 \times 10^{-16}$) and *Bacteroidetes* (Kruskal-Wallis $X^2 = 59.1$; $P <$
446 | 1.5×10^{-14}). Although soil and root habitats were closely related to each other compared to
447 | aboveground habitats, both root and soil samples demonstrated also specificity in terms of the
448 | composition of their bacterial communities. The soil habitat was distinguished from the root
449 | habitat by a higher proportion of *Chloroflexi* (Kruskal-Wallis $X^2 = 66.6$; $P < 3.2 \times 10^{-16}$) and
450 | *Gemmatimonadetes* (Kruskal-Wallis $X^2 = 34.6$; $P < 4.1 \times 10^{-9}$) and lower *Actinobacteria*
451 | (Kruskal-Wallis $X^2 = 33.1$; $P < 8.6 \times 10^{-9}$). Additionally, soil was the only habitat containing
452 | bacteria from the phylum *Nitrospirae*. Bacterial communities inhabiting aboveground habitats
453 | were similar in both stem and leaf samples, although *Firmicutes* was only found in stems (8.5
454 | %). A heatmap constructed with the most abundant OTUs in each of the 4 habitats (Fig. 3, 59
455 | OTUs in total) indicated a net clustering between bacterial communities in belowground and
456 | aboveground habitats (Fig. 3). Moreover, the “multipatt” function of the “indicspecies”
457 | package identified the most specific OTUs for soil, root and phyllosphere habitats (Table S3).
458 | Among these 59 OTUs, only 4 were shared by all four habitats, exemplified by OTU00025
459 | assigned to *Pseudomonas* and OTU00023 assigned to *Comamonadaceae*, with both OTUs
460 | comprising between 0.5 and 1.5 % of the relative abundance of each habitat. A total of 34

461 OTUs were specific to belowground samples, and 16 were specific to aboveground samples.
462 Five OTUs, all from the phylum *Firmicutes*, were essentially found in the stem habitat,
463 exemplified by OTU00083 assigned to *Paenibacillus amylocticus*. Among the 16 OTUs shared
464 by stem and leaf communities, the most abundant were species from the genera
465 *Hymenobacter*, *Deinococcus*, *Sphingomonas*, *Kineococcus* and *Methylobacterium*. Among the
466 34 OTUs shared in belowground habitats, several were more specific to the root habitat, such
467 as *Streptomyces* (OTU00003) and *Micromonosporaceae* (OTU00005 and OTU00014).
468 Indeed, *Streptomyces* was most specific to the root community, accounting for 24.4 % of the
469 relative abundance, but represented only 0.8 % of the soil community. In contrast, OTUs
470 affiliated to the *Chloroflexi* phylum were the most abundant in the soil community.

471 3.2. Mercury resistance and functional traits of bacteria isolated from belowground and 472 aboveground habitats

473 Table 2 shows ~~the cultivability density densities~~ of culturable bacteria from the four
474 habitats and the ~~densities cultivability~~ of Hg-resistant bacteria. In the media without Hg, a
475 higher number of CFUs were isolated in all habitats when using 1/10 869 medium compared
476 to 284 medium, and followed this gradient: rhizospheric soil > bulk soil > root endosphere >
477 stem endosphere > leaf episphere > stem episphere > leaf endosphere. The sizes of the
478 cultivable microbial communities were compared to the sizes of communities exposed to 10
479 μM HgCl_2 for all habitats. A decrease in all cultivable bacterial communities was observed
480 after exposure to 10 μM HgCl_2 . The bacteria living in the phyllosphere demonstrated the
481 lower -density cultivability when exposed to 10 μM HgCl_2 compared to bacteria from the
482 belowground habitats. Hg-resistant bacteria were isolated from bulk soil, rhizospheric soil,
483 epiphytic stem and epiphytic leaf samples, whereas no Hg-resistant endophytic bacteria were
484 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,](#) 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,

510 reported as the ratio of the diameter of the orange-colored zone compared to the diameter of
511 the colony; ratios ranged from 1.8 to 4.8. For the belowground bacterial consortium, we
512 selected strains with ratios higher than 3.0. In aboveground samples, 28 of the 34 colonies
513 demonstrated siderophore production, with ratios ranged from 1.1 to 3.0. Phosphate
514 solubilization ability (ratio > 1) was more frequently detected in aboveground bacteria (31 of
515 34 colonies) than in belowground bacteria (6 of 22 colonies) (Table S4). Finally, the strains
516 with the best profiles in terms of Hg resistance and PGP potential were selected for
517 inoculation (IAA > 4 mg l⁻¹ and siderophore ratio > 2.4, plus *nifH* detection, high Hg
518 resistance, phosphate solubilization potential). The strains AT3, AT7, AT8 and AT9 were part
519 of the consortium to inoculate the phyllosphere, and the strains FT79, FT82, FT97 and FT50
520 were part of the consortium to inoculate belowground.

521 *3.3. Influence of the Hg-resistant bacteria PGPB consortia on plant growth and Hg* 522 *accumulation in pepper*

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

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

539

540 4. Discussion

541 The bacterial communities in the four habitats (soil, root, stem and leaf)
542 associated with poplars growing at an Hg-contaminated site were characterized using ~~The~~
543 ~~experimental design provided us with the opportunity to characterize bacterial communities in~~
544 ~~4 habitats (soil, root, stem and leaf) at a poplar tree plantation on a Hg-contaminated site~~
545 ~~using~~ metabarcoding tools and traditional isolation-based techniques. In this the present study,
546 analyses of metabarcoding data made no distinction between epiphytic and endophytic
547 microbial communities of the plant habitats and considered the total community. ~~In~~
548 ~~other~~ There are other ~~Previous studies which showed that only a small limited part~~ proportion
549 of the microbiome were is was found to be specific of the epiphytic or endophytic habitats
550 suggesting a continuum of the microbiome between outside and inside the plant. ~~Regarding the~~
551 ~~metabarcoding approach, at each habitat, the community was considered in its entirety, and no~~
552 ~~distinction was made between endophytes and epiphytes for the three plant habitats.~~ ~~In other~~
553 ~~studies were both compartments were analyzed separately a major overlap between the two~~
554 ~~bacterial communities was observed~~ (Coleman-Derr et al., 2015). We sampled communities
555 under trees during the growing season (from leaf emergence to leaf fall). Notably, sampling
556 was carried out in mid-season (July-August), and we did not attempt to study the seasonal
557 dynamics of our habitats. According to previous studies, the mid-season community in the
558 phyllospheric habitat is more diverse and contains more unique species than the early (May-
559 Jun) or late-season (September-October) communities (Redford and Fierer, 2009).

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

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

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

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

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

610 | ~~feature that was observed in several studies performed on contaminated soils~~ (Kuffner et al.,
611 | 2008; Sas-Nowosielska et al., 2008; Schmidt et al., 2009). The predominance of these spore-
612 | forming bacteria on or inside the roots could be explained by the fact that the abundance of r-
613 | strategists (bacteria capable of rapid growth and utilization of resources) decreases in the root
614 | area in disturbed soils (such as metal-contaminated soils), while k-strategists (bacteria with
615 | reduced growth and metabolic activity) such as *Streptomyces* may be privileged (Kunito et al.,
616 | 2001). In addition, the *Streptomyces* genus contains many strains that are highly resistant to
617 | several metals (Álvarez et al., 2013) and thus may be more competitive than other bacteria in
618 | TE-enriched environments such as the tailings dump ~~of Tavaux~~[investigated in the present](#)
619 | [study](#).

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

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

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

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

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

677 ~~In endophytic habitats, our results of culturable bacterial densities regarding root and~~
678 ~~stems habitats were comparable with previous data from *Trifolium pretense* (Sturz et al.,~~
679 ~~1997); however, density of leaf endophytic cultivable bacteria was lower in our study. The~~
680 ~~epispheric habitat of the stem~~ ephisphere exhibited a lower cultivable bacterial community
681 than the ~~stem endospheric habitat~~endosphere, ~~whereas but contrary and in contrast,~~ the leaf
682 episphere hosted more cultivable bacteria than its endosphere. The structures of these plant
683 organs ~~might play a role in the development of these communities/~~ay be correlated with these
684 results. In ~~this our~~ study, several *Pseudomonas* sp. (including *P. graminis*) were isolated from

685 the phyllosphere of poplar in the Hg-enriched growth medium. The presence of this genus
686 was also observed using the metabarcoding technique essentially with the OTU00025 (Fig.
687 3 and Table S3), which were mostly detected in the leaf (0.96 %), the root (0.66 %) and soil
688 (0.73 %) habitats. *Pseudomonas* are ubiquitous bacteria that survive on plant surfaces and
689 inside plant tissues in both aboveground and belowground habitats (Schreiber et al., 2004).
690 Specifically, *Pseudomonas graminis* is found in phyllospheric habitats (Behrendt et al., 1999)
691 and was previously isolated from poplar (Doty et al., 2009). ~~However~~Moreover, this strain
692 was ~~also previously~~ defined as an endophyte ~~and was but~~ unable to grow in N-free media
693 (Hutner, 1972). ~~However, W~~we observed the presence of the *nifH* gene in all *Pseudomonas*
694 *graminis* strains, and we confirmed that these strains were able to reduce atmospheric N₂ in
695 nitrogen-free medium. A previous study showed that a *Pseudomonas graminis* strain isolated
696 from poplar and re-inoculated on sterile *Populus trichocarpa* as part of a microbial
697 consortium promoted root production, improved total biomass production and increased leaf
698 area (Knoth et al., 2014). In addition, inoculation resulted in a 25 % increase in the total
699 nitrogen content. The presence of high Hg resistance and the potential to fix atmospheric N₂
700 such as the *Pseudomonas graminis* strains identified in this study might be valuable traits to
701 further investigate in a future study and may therefore be key microorganisms for the
702 restoration of disturbed environments. We were unable to isolate endophytic Hg resistant
703 bacteria from any of the three habitats ~~tested, studied~~; suggesting the internal plant tissues may
704 not host resistant bacteria.

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

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

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

720

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727

728

729 **Figure legends**

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

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

738 Figure 3. A heatmap comparing the abundance of bacterial OTUs among the 20 most
739 abundant OTUs in at least one of the four poplar habitats. The dendrogram represents linkage
740 clustering using Euclidean distance measures. OTU delineation was based on a cutoff of < 97
741 % sequence similarity. Assignments between brackets indicate the lower taxonomic level
742 associated with the OTU using the Greengenes database, k: kingdom, p: phylum, o: order, c:
743 class, f: family, s: genus and species.

744 Figure 4: Phylogeny of the Hg-resistant bacteria isolated from the phyllosphere in 1/10 869
745 medium containing 2 μ M HgCl₂. The phylogenetic tree was constructed using a K2P Neighbor-
746 joining model based on aligned DNA sequences (697 bp) with 1,000 iterations. The parentage
747 of branches is given in the tree. Phenotypic and genotypic characterizations for each strain are
748 also shown.

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

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

757

758 **Tables**

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

764 Table 2: Cultivable colony forming units per gram dry weight soil (CFUs g^{-1}) on solid media
765 and percentage (%) of Hg-resistant cultivable bacteria to 2 and 10 $\mu\text{M HgCl}_2$ in the different
766 habitats.

767

768

769

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

4

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22

23 **Abstract**

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

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

49 **1. Introduction**

50 Soil contamination by metal(oids) and trace elements (TEs) such as Ag, As, Cd, Cr,
51 Cu, Hg, Ni, Pb, Se, or Zn is a threat to ecosystem stability and a risk to human health. In
52 2011, 127,000 sites in 33 European Union countries were confirmed to have contaminated
53 soils, and approximately 45 % of these sites (58,000) were under remediation project
54 management to reduce associated risk (Panagos et al., 2013). Data published in 2016 showed
55 that 6.24 % (137,000 km²) of European agricultural lands were unsafe for food production,
56 requiring local assessment and eventual remediation (Toth et al., 2016). Due to the frequent
57 lack of a plant cover at industrial dumps, processes such as wind erosion or leaching can led
58 to the contamination of adjacent agriculture lands leading to the decrease in soil productivity.

59 Phytomanagement-based restoration programs for contaminated soils may use woody
60 plant species that are colonized by essential microorganisms (Bell et al., 2014; Partida-
61 Martínez and Heil, 2011; Yergeau et al., 2012), especially in highly stressed and nutrient-poor
62 environments, such as TE- contaminated sites (Durand et al., 2017; Foulon et al., 2016a,
63 2016b). However, knowledge of these associated microbial communities remains scarce,
64 particularly with respect to aboveground tissues. Among the microorganisms that colonize the
65 plant rhizosphere and phyllosphere, some are known to play important roles in determining
66 plant fitness and are collectively described as plant growth-promoting bacteria (PGPB)
67 (Glick, 2014; Lindow and Brandl, 2003; Pieterse et al., 2016). PGPB directly benefit host
68 plants via hormone production (*e.g.*, indoleacetic acid, cytokinin, and zeatin) and improved
69 nutrition and indirectly by acting as biocontrol agents, inducing systemic resistance and
70 ethylene stress resistance (Bringel and Couée, 2015; Glick, 2005; Lindow and Brandl, 2003;

71 Maignien et al., 2014; Trouvelot et al., 2014; Vorholt, 2012). Belowground and aboveground
72 plant tissues provide habitats, which can be easily colonized by specific PGPB (Vessey, 2003),
73 (Vorholt, 2012). In the soil, free-living bacteria of various genera such as *Alcaligenes*,
74 *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Curtobacterium*, *Klebsiella*,
75 *Enterobacter*, *Pseudomonas*, and *Serratia* exert beneficial effects on plants (Kloepper et al.,
76 1989 ; Glick et al., 1999 ; Benizri et al., 2001) and are classified as rhizospheric PGPB. Plant-
77 associated bacteria also include endophytic bacteria, which colonize internal plant tissues.
78 They are originating from the epiphytic bacterial communities of the rhizosphere and
79 phyllosphere but may also arise from transmission through seeds or plant vegetative parts
80 (Hallmann et al., 1997). Members of the genera *Acinetobacter*, *Aminobacter*, *Arthrobacter*,
81 *Bacillus*, *Burkholderia*, *Curtobacterium*, *Devosia*, *Enterobacter*, *Gluconacetobacter*,
82 *Herbaspirillum*, *Methylobacterium*, *Microbacterium*, *Ochrobactrum*, *Paenibacillus*,
83 *Pseudomonas*, *Phyllobacterium*, *Rahnella*, *Shinella*, *Sphingomonas* and *Staphylococcus* have
84 been described as plant endophytes (Compant et al., 2009; De Meyer et al., 2015; Doty et al.,
85 2009). Other bacterial endophytes that are only localized in specific structures are referred to
86 as symbiotic PGPB such as members of the genera *Azorhizobium*, *Bradyrhizobium*, *Devosia*,
87 *Ensifer*, *Frankia*, *Mesorhizobium*, *Microvirga*, *Ochrobactrum*, *Phyllobacterium* and
88 *Rhizobium* (Glick 2014, Kloepper et al. 1989; Wang et al. 2012; De Meyer et al. 2015).
89 Aboveground, phyllospheric PGPB are subjected to fitness stresses (e.g. direct solar radiation,
90 drought), that are very different than those found belowground, suggesting a specific selection
91 pressure (Lindow and Brandl, 2003). It has been demonstrated that the survival capacity of
92 *Pseudomonas fluorescens* SBW25 in the phyllosphere is dependent on the expression of
93 habitat specific genes that are significantly more correlated to the fitness of the bacteria in the
94 phyllosphere than in the soil or the rhizosphere (Gal et al., 2003). Albeit, a full understanding
95 of these bacterial communities has not been reached (Vacher et al., 2016), several

96 phyllospheric PGPB have been already previously described such as members of
97 *Arthrobacter*, *Azotobacter*, *Beijerinckia*, *Klebsiella*, *Methylobacterium*, *Nostoc*, *Pantoea*,
98 *Pseudomonas*, *Scytonema*, *Sphingomonas* and *Stigonema* (Lindow and Brandl, 2003; Vacher
99 et al., 2016). However, gaining an understanding of the structure and composition of
100 microbial communities *in situ* is difficult due to the diversity and complexity of these
101 communities. Metabarcoding using high-throughput sequencing technologies offers a solution
102 to describe the bacterial communities associated with environmental habitats with high
103 resolution, as exemplified recently in our previous work (Durand et al., 2017; Foulon et al.,
104 2016a, 2016b). We demonstrated for instance that *Alphaproteobacteria* and *Actinobacteria*
105 dominated root bacterial communities, whereas soil samples were dominated by
106 *Alphaproteobacteria* and *Acidobacteria* (Foulon et al., 2016b). These innovative approaches
107 still need complementary culture-dependent approaches to allow for the functional
108 characterization and selection of the best-performing PGPB.

109 In this study, we combined a metabarcoding approach that employs a high-throughput
110 Illumina MiSeq platform (Durand et al., 2017; Foulon et al., 2016a, 2016b; Schmidt et al.,
111 2013; Wu et al., 2015) and a culture-dependent approach to investigate the composition and
112 assembly of bacterial communities and isolate Hg-resistant PGPB in the rhizosphere,
113 endosphere and phyllosphere of poplars at a Hg-contaminated site. We expected that we
114 would observe clear differences in the relative abundance and composition of bacterial
115 communities across poplar habitats that may improve our understanding of the microbial
116 ecology of these environments. We also aimed at better understanding the role of isolated
117 bacterial strains in Hg transfer from the atmosphere to plant tissues using PGPB inoculation
118 of the model plant *Capsicum annum*.

119 **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.

145 *2.3. Metabarcoding analysis*

146 DNA was extracted from biological samples within 2 months after sampling. Plant
147 material (root, stem and leaf samples), was thoroughly washed with distilled water and
148 DNA was extracted from 20 mg of fresh material using an adapted cetyl trimethylammonium
149 bromide/chloroform/isoamyl alcohol protocol (Lefort and Douglas 1999; Healey et al. 2014).
150 DNA was extracted from 100 mg of sieved fresh soil, using a PowerSoil DNA Isolation Kit
151 following the manufacturer's instructions (MoBio Laboratories, Inc., Carlsbad, CA USA).
152 Extracted DNA was purified using a Power Clean® Pro DNA Clean-Up Kit (MoBio
153 Laboratories, Inc., Carlsbad, CA USA). Equimolar DNA pools were adjusted to 10 ng μl^{-1} ,
154 and PCR conditions sequencing of the bacterial 16S rRNA region was performed on an
155 Illumina MiSeq platform (Microsynth AG, Switzerland). PCR amplification of the 16S rRNA
156 gene was performed using the bacterial primers 799f (5'- AAC MGG ATT AGA TAC CCK
157 G -3') and 1115r (5'- AGG GTT GCG CTC GTT G -3'), which amplify the V5-V6 region
158 (Kembel et al., 2014). These primers target a section of the bacterial 16S rRNA region and
159 generate a small amplicon (~300 bp) that is appropriate for Illumina sequencing. Reactions
160 were performed in triplicate for each sample with the following conditions: 30 s initial
161 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,
162 with a final 10-min elongation at 72 °C.

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

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

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

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

198 *2.4. Isolation of Hg-resistant bacterial strains*

199 Bacterial strains were isolated from bulk and rhizospheric soils and from stem and leaf
200 phyllospheric habitats of poplar trees. Soil properties and Hg concentrations were detailed in
201 our previous study (Durand et al., 2017). Bulk soil samples (3.5 g) were taken far from the
202 root system under the canopy and placed in a flask containing 35 ml of phosphate buffer
203 (PBS: 8 g of NaCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄ per l, pH 7.2). Roots with
204 adhering soil (defined as the rhizospheric soil) were washed in a PBS bath in a rotatory shaker
205 (30 min, 150 rpm). Roots were separated from the soil suspension and rhizospheric soil was
206 recovered after centrifugation (14,000 g for 10 min), and 1 g of the rhizospheric precipitated
207 soil was then resuspended by shaking in 35 ml of PBS. Phyllospheric epiphytes, defined as
208 the microbes living on the surfaces of aboveground habitats, were isolated from leaves and
209 stems. Three g of leaves or 20 g of stems were thoroughly washed with distilled water and
210 suspended in 150 ml of PBS extraction solution, then placed on a rotatory shaker (150 rpm)
211 for 1 h. Isolation of endophytes from root, stem and leaf habitats was carried out after surface
212 sterilization with 2 % sodium hypochlorite for 2 min. Samples were thoroughly rinsed with
213 sterile distilled water and then crushed in 35 ml of PBS and macerated for 1 h on a rotatory
214 shaker (150 rpm). Soil and plant suspensions were diluted in 10-fold series and plated onto
215 1/10 rich medium (869) as well as onto environmental medium (284) (Eevers et al. 2015).
216 Densities of Hg- resistant bacteria were determined using the same media supplemented with
217 0, 2 or 10 µM HgCl₂ (Francois et al., 2012). The agar plates were then incubated in the dark at
218 27°C for 6 days. The number of bacteria was expressed as log (CFU) g⁻¹ dry weight (DW) of
219 sample. Hg-resistant colonies were isolated and purified by subculture on Hg-enriched 1/10

220 869 or 284 medium. A bacterial collection was established by growing each previous isolated
221 strains in 6 ml of Luria Bertani (LB) broth (Sigma-Aldrich, Steinheim, Germany) for 15 h on
222 a rotatory shaker (150 rpm) at 30°C and stored in 25 % sterile glycerol containing 75 % brain
223 heart infusion broth (Roth, Karlsruhe, Germany) at -80°C for further analyses.

224 2.5. Taxonomic identification

225 For taxonomic identification, each Hg-resistant isolate was cultured in 15 ml of LB
226 medium for 24 h on a rotatory shaker (120 rpm) at 27°C. After centrifugation, DNA was
227 extracted from the pellets using an EZNA bacterial DNA isolation kit (OMEGA Bio-tek, Inc,
228 Norcross, Georgia, USA) according to the manufacturer's instructions. The BOX-PCR
229 fingerprinting method was used to group genotypic profiles at a similarity level of 97 % as
230 previously described (Becerra-Castro et al., 2011). One strain was selected for each BOX-
231 group and R rDNA genes were amplified from 5 µl of bacterial DNA in a final reaction
232 volume of 50 µl containing 25 µl of 2x PCR master mix (Thermo Fisher Scientific, Carlsbad,
233 California, USA) and each universal primer at 0.5 µM (Eurofins Genomics, Paris, France),
234 specifically 27f (5'- AGA GTT TGA TCM TGG CTC AG -3') and 1492r (5'- GGT TAC CTT
235 GTT ACG ACT T -3') (Mark Ibekwe et al., 2007). DNA amplification was carried out in a
236 thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) under the following
237 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
238 an additional 15-min cycle at 72°C (Durand et al., 2015). All 16S rRNA PCR products were
239 sequenced by Sanger sequencing (Genewiz Beckman Coulter Genomics, United Kingdom).
240 DNA sequences were edited with BioEdit software v7.2.6. (Hall, 1999) screened against the
241 GenBank database using the BLASTn tool at the NCBI website
242 (<http://www.ncbi.nlm.nih.gov/>) and then aligned with reference sequences of bacteria
243 (LN551925, KR922297, HQ256858, KU587965, KU523561, JX827229, HQ256858,
244 HQ224627, KT825724, NR_028626, LN774179, JF496259, KR476438, JQ956529,

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

249 2.6. Functional characterization

250 Each isolate of the bacterial collection was screened for the following plant growth-
251 promoting traits: siderophore production, IAA synthesis and phosphate solubilization. The
252 siderophore production of each bacterial strain was determined using chrome azurol sulfonate
253 (CAS) agar medium (Alexander and Zuberer, 1991; Schwyn and Neilands, 1987). After 5
254 days of incubation on CAS medium at 30°C, a red-orange halo around colonies indicated the
255 production of siderophores. For each strain, the ratio between the diameter of the halo and the
256 diameter of colony was recorded. The IAA synthesis of each bacterial strain was determined
257 using Salkowski's reagent (1 ml of 0.5 M FeCl₃ in 50 ml of 35 % HClO₄) as described by
258 (Barillot, 2012). The phosphate-solubilizing capacity of bacterial strains was determined on
259 Pikovskaya medium (Mehta and Nautiyal, 2001). After 5 days of incubation at 30°C, a
260 discolored halo around colonies demonstrated phosphate solubilization. For each strain, the
261 ratio between the halo diameter and the diameter of colony was recorded. Additionally, MICs
262 (minimal inhibitory concentrations) of Hg were determined for each isolated strain. Briefly,
263 microtitration plates were prepared using 2-fold dilutions of Hg in LB liquid broth, from 0 to
264 256 µM Hg. The strain *Pseudomonas aeruginosa* PA14, which is resistant to Hg above
265 256 µM, was used as a positive control. Each well was inoculated with 2 µl of a bacterial
266 suspension, and plates were incubated for 48 h at 27 °C on a rotatory shaker table (180 rpm).
267 After incubation, the bacterial growth was evaluated by measuring the optical density at 600
268 nm (OD₆₀₀). Nitrogen fixation ability was evaluated using N-free semisolid malate medium
269 (NFb) (Baldani and Döbereiner, 1980) inoculated with the assayed strains. After 10 days,

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

272 Using the previous extracted bacterial DNA, the presence of the genes *merA* and *nifH*
273 in the genome of each strain was assayed. PCR amplification of the *merA* gene was performed
274 using 3 µl of bacterial DNA in a final volume of 20 µl that also contained 10 µl of 2x PCR
275 master mix (Thermo Fisher Scientific, Carlsbad, California, USA) and each primer at 0.5 µM,
276 specifically merA2F (5'- CCT GCG TCA ACG TCG GCT G -3') and merA2R (5'- GCG
277 ATC AGG CAG CGG TCG AA -3') (Poulain et al., 2015). DNA amplification was carried
278 out in a thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) using the
279 following conditions: 10 min at 95°C, followed by 35 cycles of 30 sec at 95 °C, 15 sec at 63
280 °C, and 30 sec at 72°C, with an additional 5-min incubation at 72 °C. PCR amplification of
281 the *nifH* gene was performed in a final volume of 25 µl containing 5 µl of bacterial DNA,
282 12.5 µl of 2x PCR master mix, and each primer at 0.5 µM including nifHF (5'- TAC GGN
283 AAR GGS GGN ATC GGC AA -3') and nifHI (5'- AGC ATG TCY TCS AGY TCN TCC A
284 -3') (Laguerre et al., 2001). DNA amplification was carried out in a thermocycler under the
285 following conditions: 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 53.5
286 °C, and 2 min at 72 °C, with an additional 15-min incubation at 72 °C.

287 2.7. Pot experiment with Hg-resistant bacteria and pepper

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

295 plant growing in a 0.5 liter pot in a growth chamber (photoperiod, 16 h; temperature, 18 °C
296 (night) and 22 °C (day); relative humidity, 60-80 %; photosynthetic photon flux density, 250
297 $\text{mmol m}^{-2} \text{s}^{-1}$). In total, 16 *Capsicum annuum* plants were used for each of the following
298 treatments: non-inoculated and Hg-unexposed (ni-); inoculated and Hg-unexposed Hg (i-);
299 uninoculated and Hg-exposed (ni+); and inoculated and Hg-exposed (i+). Hg-unexposed
300 plants (ni- and i-) were grown in the same growth chamber, while Hg-exposed plants (ni+ and
301 i+) were grown in a separate growth chamber. Hg-enriched soil ($6.38 \text{ mg kg}^{-1} \text{ Hg}$) from the
302 industrial tailings described above was added to the Hg-contaminated growth chamber to
303 create an Hg-enriched atmosphere as previously described (Assad et al., 2016). The soil was
304 not in direct contact with the plants in any way but provided an atmospheric enrichment with
305 Hg, similar to that observed in the natural environment. Atmospheric Hg in the contaminated
306 and uncontaminated growth chambers was measured at 21.0 and 3.48 ng m^{-3} , respectively.
307 Using the previously isolated bacteria stored at -80 °C, two bacterial consortia were produced
308 to inoculate both the belowground and aboveground compartments of *Capsicum annuum*.

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

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

327 *2.8. Hg analysis in the substrate and biomass*

328 Hg was measured in the soil and plant samples using an AMA-254 (Advanced
329 Mercury Analyzer) cold vapor atomic absorption (CV-AAS) Hg analyzer (Altec Co., Czech
330 Republic) under the standard conditions recommended by the manufacturer (120 sec drying,
331 150 sec heating, and 45 sec cooling). The validity of the analytical method was assessed using
332 certified reference material (CRM), specifically Oriental Basma Tobacco Leaves (INCT-
333 OBTL-5) with a certified Hg content of $20.9 \pm 1.3 \text{ ng g}^{-1} \text{ DM}$ (Samczyński et al., 2012).
334 Quality controls were regularly performed as described elsewhere (Maillard et al., 2016).

335 *2.9. General statistical analysis*

336 The Shapiro test and the Bartlett test were used to check the normality and
337 homoscedasticity of the data, respectively. Our data verifying normality and homoscedasticity
338 were assessed using analysis of variance (ANOVA). In other cases the data were examined
339 using the Kruskal-Wallis test. Statistical analyses were performed with R software, version
340 3.3.2 (Team R, 2014).

341 **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 \geq stem, revealing that aboveground habitats were
365 significantly less rich than belowground habitats. Comparing within belowground habitats,

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

372 The Venn diagram highlights shared and unique OTUs for each habitat (Fig. S2). The
373 sums of the total observed bacterial OTUs in the four sample types in the poplar tree plots
374 were 475, 337, 94 and 77 for soil, root, stem and leaf samples, respectively (Fig. S2). Overall,
375 9 OTUs (1.4 %) were shared by all compartments, and only 4.2 % of OTUs were found in
376 both belowground and aboveground habitats. Moreover, aboveground habitats shared 50.8 %
377 of their OTUs, while belowground habitats shared 43.9 % of their OTUs. Soil samples
378 contained the highest proportions of unique OTUs (47.3 %), followed by leaf (31.2 %), root
379 (25.2 %) and stem (18.1 %) samples.

380 Through environmental 16S rRNA sequencing, bacterial communities from the four
381 habitats (soil, root, stem and leaf) were categorized into 30 phyla and 80 classes, the most
382 abundant of which are shown in Fig. 2. *Actinobacteria* (28 %) and *Alphaproteobacteria* (26
383 %) dominated the entire dataset. The largest proportion of *Actinobacteria* was found in the
384 root habitat (41.7 %) compared to the other compartments (Kruskal-Wallis $X^2 = 45.8$; $P < 1.3$
385 $\times 10^{-11}$), and the aboveground samples contained more *Alphaproteobacteria* (35.7 %)
386 compared to belowground samples (Kruskal-Wallis $X^2 = 59.7$; $P < 1.1 \times 10^{-14}$). Altogether,
387 belowground habitats were enriched for *Acidobacteria* (Kruskal-Wallis $X^2 = 68.9$; $P < 2.2 \times$
388 10^{-16}), *Chloroflexi* (Kruskal-Wallis $X^2 = 66.9$; $P < 2.9 \times 10^{-16}$), *Betaproteobacteria* (Kruskal-
389 Wallis $X^2 = 42.7$; $P < 6.3 \times 10^{-11}$), *Deltaproteobacteria* (Kruskal-Wallis $X^2 = 66.7$; $P < 3.2 \times$
390 10^{-16}) and *Gammaproteobacteria* (Kruskal-Wallis $X^2 = 66.6$; $P < 3.2 \times 10^{-16}$), whereas

391 aboveground habitats were enriched for *Deinococcus-Thermus* (Kruskal-Wallis $X^2 = 68.0$; $P <$
392 2.2×10^{-16}) and *Bacteroidetes* (Kruskal-Wallis $X^2 = 59.1$; $P < 1.5 \times 10^{-14}$). Although soil and
393 root habitats were closely related to each other compared to aboveground habitats, both root
394 and soil samples demonstrated also specificity in terms of the composition of their bacterial
395 communities. The soil habitat was distinguished from the root habitat by a higher proportion
396 of *Chloroflexi* (Kruskal-Wallis $X^2 = 66.6$; $P < 3.2 \times 10^{-16}$) and *Gemmatimonadetes* (Kruskal-
397 Wallis $X^2 = 34.6$; $P < 4.1 \times 10^{-9}$) and lower *Actinobacteria* (Kruskal-Wallis $X^2 = 33.1$; $P < 8.6$
398 $\times 10^{-9}$). Additionally, soil was the only habitat containing bacteria from the phylum
399 *Nitrospirae*. Bacterial communities inhabiting aboveground habitats were similar in both stem
400 and leaf samples, although *Firmicutes* was only found in stems (8.5 %). A heatmap
401 constructed with the most abundant OTUs in each of the 4 habitats (Fig. 3, 59 OTUs in total)
402 indicated a net clustering between bacterial communities in belowground and aboveground
403 habitats (Fig. 3). Moreover, the “multipatt” function of the “indicspecies” package identified
404 the most specific OTUs for soil, root and phyllosphere habitats (Table S3). Among these 59
405 OTUs, only 4 were shared by all four habitats, exemplified by OTU00025 assigned to
406 *Pseudomonas* and OTU00023 assigned to *Comamonadaceae*, with both OTUs comprising
407 between 0.5 and 1.5 % of the relative abundance of each habitat. A total of 34 OTUs were
408 specific to belowground samples, and 16 were specific to aboveground samples. Five OTUs,
409 all from the phylum *Firmicutes*, were essentially found in the stem habitat, exemplified by
410 OTU00083 assigned to *Paenibacillus amylocticus*. Among the 16 OTUs shared by stem and
411 leaf communities, the most abundant were species from the genera *Hymenobacter*,
412 *Deinococcus*, *Sphingomonas*, *Kineococcus* and *Methylobacterium*. Among the 34 OTUs
413 shared in belowground habitats, several were more specific to the root habitat, such as
414 *Streptomyces* (OTU00003) and *Micromonosporaceae* (OTU00005 and OTU00014). Indeed,
415 *Streptomyces* was most specific to the root community, accounting for 24.4 % of the relative

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

418 3.2. Mercury resistance and functional traits of bacteria isolated from belowground and 419 aboveground habitats

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

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

440 *Sphingomonas*, *Frigoribacterium*, *Curobacterium*, *Micrococcus*, *Paenibacillus* and
441 *Staphylococcus* (Fig. 4). For those 56 identified strains, the Hg resistance of bacteria from
442 belowground habitats was higher than that of bacteria from aboveground habitats (Table S4).
443 Indeed, the strains found in aboveground habitats presented MIC values between 4 and 64 μM
444 HgCl_2 (Fig. 4), whereas the strains from belowground habitats exhibited MIC values between
445 16 and 254 μM HgCl_2 . In bacteria from various habitats, the presence of the *merA* gene did
446 not consistently correlate with their Hg MICs (Table S4).

447 Regarding the study of PGP traits, surprisingly, the *nifH* gene was observed in all 13
448 aboveground *Pseudomonas graminis* strains (Fig. 4 and Table S4), and an experiment in
449 semi-solid nitrogen-free medium confirmed the ability of these strains to fix N_2 . In both
450 aboveground and belowground habitats, all strains demonstrated IAA production, with
451 variations in the amount of IAA produced (mg l^{-1}) between strains. The lowest IAA producers
452 were AT38 (*Paenibacillus amylolyticus*) in the phyllosphere (with 0.9 mg l^{-1} IAA) and FT43
453 (*Pseudomonas fluorescens*) in the belowground samples (with 2.1 mg l^{-1} IAA), while the best
454 IAA producers in the phyllosphere were AT8 (*Pseudomonas graminis*), with 12 mg l^{-1} IAA,
455 and FT97 (*Bacillus subtilis*), with 12.5 mg l^{-1} IAA (Table S4). In belowground habitats, all
456 strains with the exception of ST81 (*Bacillus licheniformis*) showed siderophore activity,
457 reported as the ratio of the diameter of the orange-colored zone compared to the diameter of
458 the colony; ratios ranged from 1.8 to 4.8. For the belowground bacterial consortium, we
459 selected strains with ratios higher than 3.0. In aboveground samples, 28 of the 34 colonies
460 demonstrated siderophore production, with ratios ranged from 1.1 to 3.0. Phosphate
461 solubilization ability (ratio > 1) was more frequently detected in aboveground bacteria (31 of
462 34 colonies) than in belowground bacteria (6 of 22 colonies) (Table S4). Finally, the strains
463 with the best profiles in terms of Hg resistance and PGP potential were selected for
464 inoculation (IAA > 4 mg l^{-1} and siderophore ratio > 2.4, plus *nifH* detection, high Hg

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

468 *3.3. Influence of the Hg-resistant bacteria PGPB consortia on plant growth and Hg* 469 *accumulation in pepper*

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

486

487 **4. Discussion**

488 The bacterial communities of the 4 habitats (soil, root, stem and leaf) associated with
489 poplars growing at an Hg-contaminated site were characterized using metabarcoding tools and
490 traditional isolation-based techniques. In the present study, analyses of metabarcoding data
491 made no distinction between epiphytic and endophytic microbial communities of the plant
492 habitats. Previous studies showed that only a limited proportion of the microbiome was
493 specific of the epiphytic or endophytic habitats (Coleman-Derr et al., 2015). We sampled
494 communities under trees during the growing season (from leaf emergence to leaf fall).
495 Notably, sampling was carried out in mid-season (July-August), and we did not attempt to
496 study the seasonal dynamics of our habitats. According to previous studies, the mid-season
497 community in the phyllospheric habitat is more diverse and contains more unique species than
498 the early (May-Jun) or late-season (September-October) communities (Redford and Fierer,
499 2009).

500 Illumina-based sequencing of bacterial communities from plant habitats, such as roots,
501 stems and leaves can be problematic due to unwanted chloroplast DNA amplification. A
502 similar study investigating oak phyllospheric bacterial communities in oak trees using 16S
503 rRNA amplification of the V6 region with Illumina technologies, revealed higher proportions
504 of oak chloroplast sequences (up to 92.1 % from the total sequences) than bacterial sequences
505 (Jakuschkin et al., 2016) when using a previously described amplification protocol (Gloor et
506 al., 2010). Using a different amplification protocol described by Kembel et al. (2014) with
507 specific primers (799F-1115R, V5-V6 region) and optimized hybridization conditions (64°C),
508 we still observed chloroplast DNA amplification for the stem and leaf habitats. However, the
509 majority of amplified sequences were of bacterial origin, and the total pool of sequences
510 (2,661,227 effective sequences) was consistent with high Good's coverage and satisfactory
511 taxonomical assignment. In our study, Illumina MiSeq sequencing allowed us to obtain a

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

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

525 The soil and root habitats shared approximately half of their OTUs, which was lower
526 than the proportions previously observed for *Arabidopsis thaliana* (> 64 % for the soil, rhizosphere
527 and root communities) (Dombrowski et al., 2016). However, evidence of a lower alpha
528 diversity in the roots compared to the soil was highlighted in our results. The dataset obtained
529 for root and soil samples in the present work corroborates our previous studies, which was
530 carried out at the same site on native tree species (poplar and willow), revealing the
531 dominance of *Proteobacteria* and *Actinobacteria* (Zappelini et al., 2015). *Proteobacteria* and
532 *Actinobacteria* also dominate in root bacterial communities from other polluted (Foulon et al.,
533 2016b) or non polluted (Beckers et al., 2016) soils, although these authors used 454
534 pyrosequencing in their study. The dominance of the phylum *Chloroflexi* in soil samples from
535 the present study may be explained by the adaptability of members of this phylum to
536 oligotrophic environments (Barton et al., 2014). Moreover, this phylum has been found to be

537 dominant within the *Alyssum murale* rhizosphere growing on ultramafic soils (Lopez et al.,
538 2017) and also geothermal soils, low-temperature meadow soils, sea and lake sediments, and
539 hydrothermally active sediments (Yamada and Sekiguchi, 2009). These findings suggest that
540 this phylum may be particularly abundant in extreme environments.

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

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

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

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

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

590 We further assessed densities of bacteria isolated from various habitats of the poplar
591 tree plantation when Hg was added as a stressor. The bacterial communities from the
592 phyllospheric habitats were altogether less resistant than the communities hosted in the
593 belowground habitats, potentially due to the direct contact between soil bacterial communities
594 and higher amounts of Hg in the soil. The rhizospheric soil hosted a bacterial community that
595 was richer than that of the bulk soil, which was expected. Indeed, previous works have shown
596 that planted soils host significantly richer bacterial communities compared to unplanted soils
597 (Durand et al., 2015). This is explained by the presence of plant exudates in the soil
598 surrounding the roots, which play a role in bacterial growth (Baudoin et al., 2003). However,
599 bacteria isolated from the bulk soil were more resistant to Hg than bacteria isolated from the
600 rhizospheric soil. We isolated Hg-resistant strains harboring PGP traits, such as isolates of
601 *Bacillus subtilis* and *Pseudomonas* sp. from each habitat. The species *Bacillus subtilis* is
602 known for its antifungal properties, siderophores, IAA production and biocontrol effects on
603 *Fusarium wilt* (Zaidi et al. 2006; Chebotar' et al. 2009). This species was previously tested as
604 a bioinoculant to improve Ni bioaccumulation by *Brassica juncea* and promoted the growth of
605 pepper plants (Yu et al., 2011). Many plant-associated *Pseudomonas* species directly and
606 indirectly promote plant growth, a phenomenon that has previously been reviewed (Preston,
607 2004). These species compete with and suppress pathogenic microorganisms to promote plant
608 growth.

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

612 communities/be correlated with these results. In our study, several *Pseudomonas sp.*
613 (including *P. graminis*) were isolated from the phyllosphere of poplar in the Hg-enriched
614 growth medium. The presence of this genus was also observed using the metabarcoding
615 technique essentially with the OTU00025 (Fig. 3 and Table S3), which were mostly detected
616 in the leaf (0.96 %), the root (0.66 %) and soil (0.73 %) habitats. *Pseudomonas* are ubiquitous
617 bacteria that survive on plant surfaces and inside plant tissues in both aboveground and
618 belowground habitats (Schreiber et al., 2004). Specifically, *Pseudomonas graminis* is found in
619 phyllospheric habitats (Behrendt et al., 1999) and was previously isolated from poplar (Doty
620 et al., 2009). Moreover, this strain was also defined as an endophyte but unable to grow in N-
621 free media (Hutner, 1972). However, we observed the presence of the *nifH* gene in all
622 *Pseudomonas graminis* strains, and we confirmed that these strains were able to reduce
623 atmospheric N₂ in nitrogen-free medium. A previous study showed that a *Pseudomonas*
624 *graminis* strain isolated from poplar and re-inoculated on sterile *Populus trichocarpa* as part
625 of a microbial consortium promoted root production, improved total biomass production and
626 increased leaf area (Knoth et al., 2014). In addition, inoculation resulted in a 25 % increase in
627 the total nitrogen content. The presence of high Hg resistance and the potential to fix
628 atmospheric N₂ such as the *Pseudomonas graminis* strains identified in this study might be
629 valuable traits to further investigate in a future study and may therefore be key
630 microorganisms for the restoration of disturbed environments. We were unable to isolate
631 endophytic Hg resistant bacteria from any of the three habitats studied; suggesting the internal
632 plant tissues may not host resistant bacteria.

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

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

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

648

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656

657 **Figure legends**

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

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

666 Figure 3. A heatmap comparing the abundance of bacterial OTUs among the 20 most
667 abundant OTUs in at least one of the four poplar habitats. The dendrogram represents linkage
668 clustering using Euclidean distance measures. OTU delineation was based on a cutoff of < 97
669 % sequence similarity. Assignments between brackets indicate the lower taxonomic level
670 associated with the OTU using the Greengenes database, k: kingdom, p: phylum, o: order, c:
671 class, f: family, s: genus and species.

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

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

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

685

686 **Tables**

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

692 Table 2: Cultivable colony forming units per gram dry weight soil (CFUs g^{-1}) on solid media
693 and percentage (%) of Hg-resistant cultivable bacteria to 2 and 10 $\mu\text{M HgCl}_2$ in the different
694 habitats.

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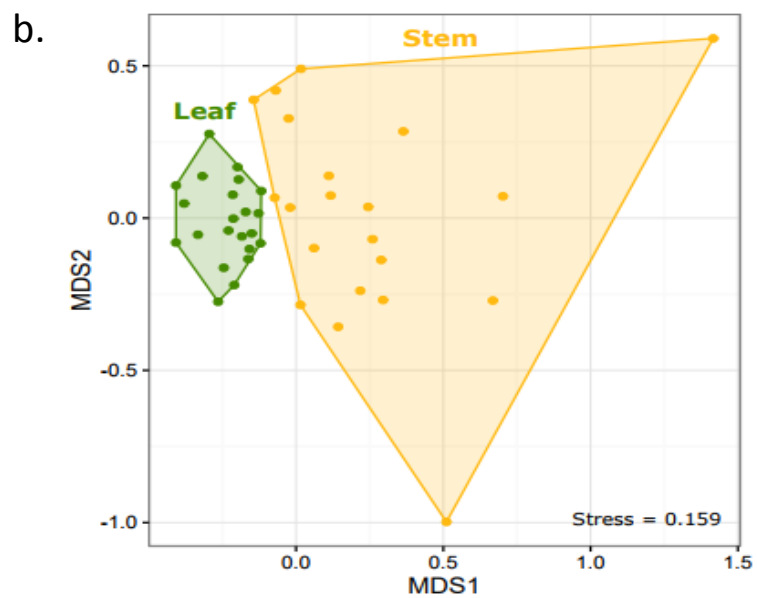
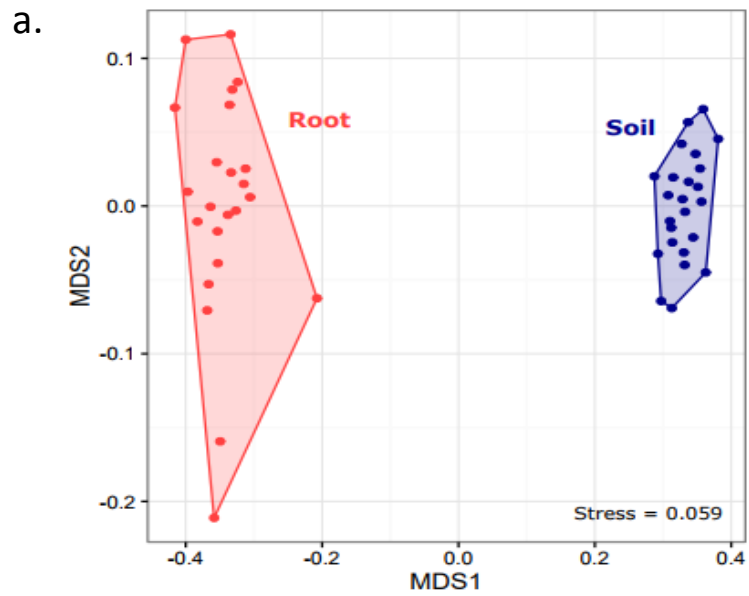


Table 1

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

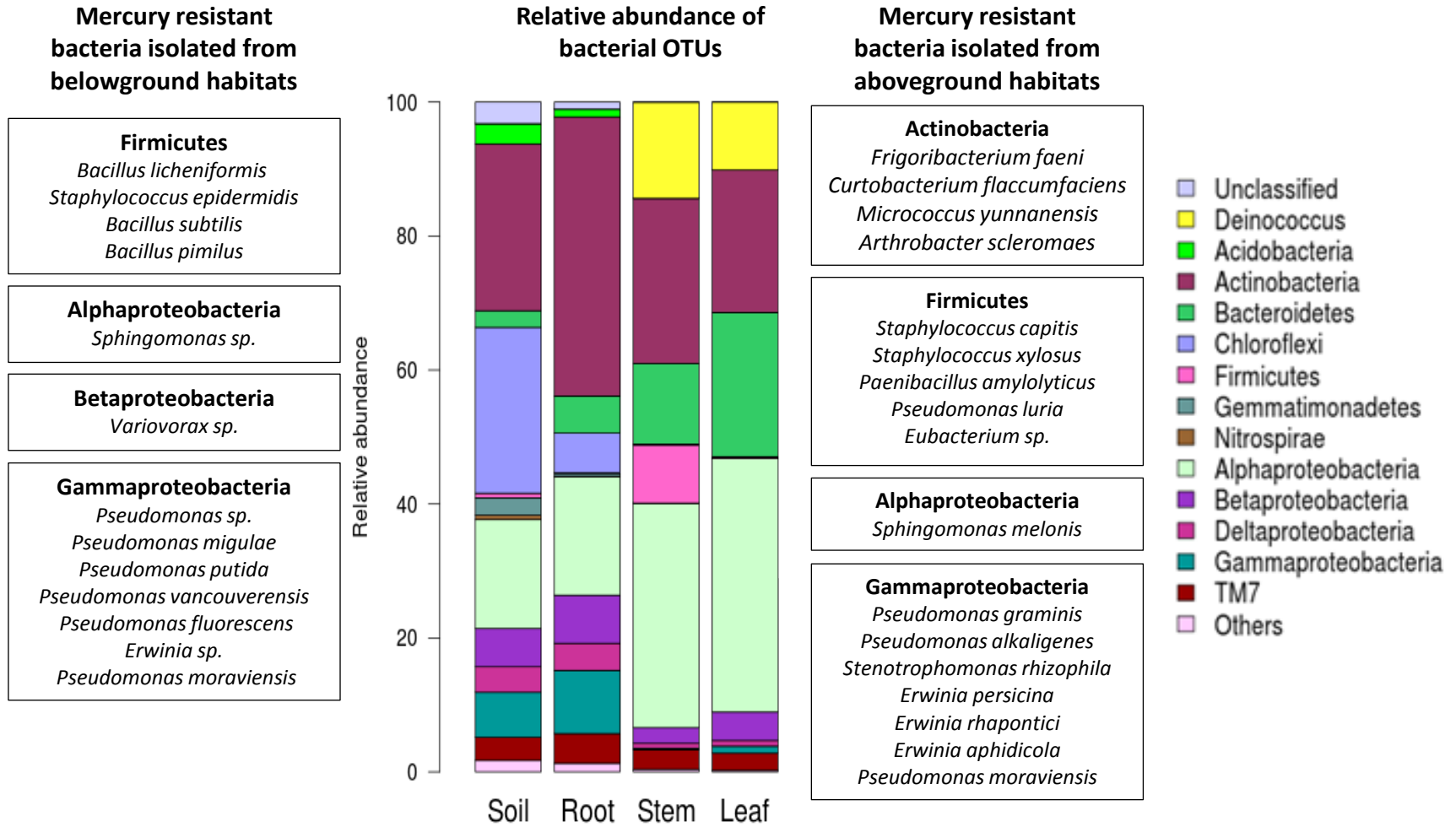


Figure 3

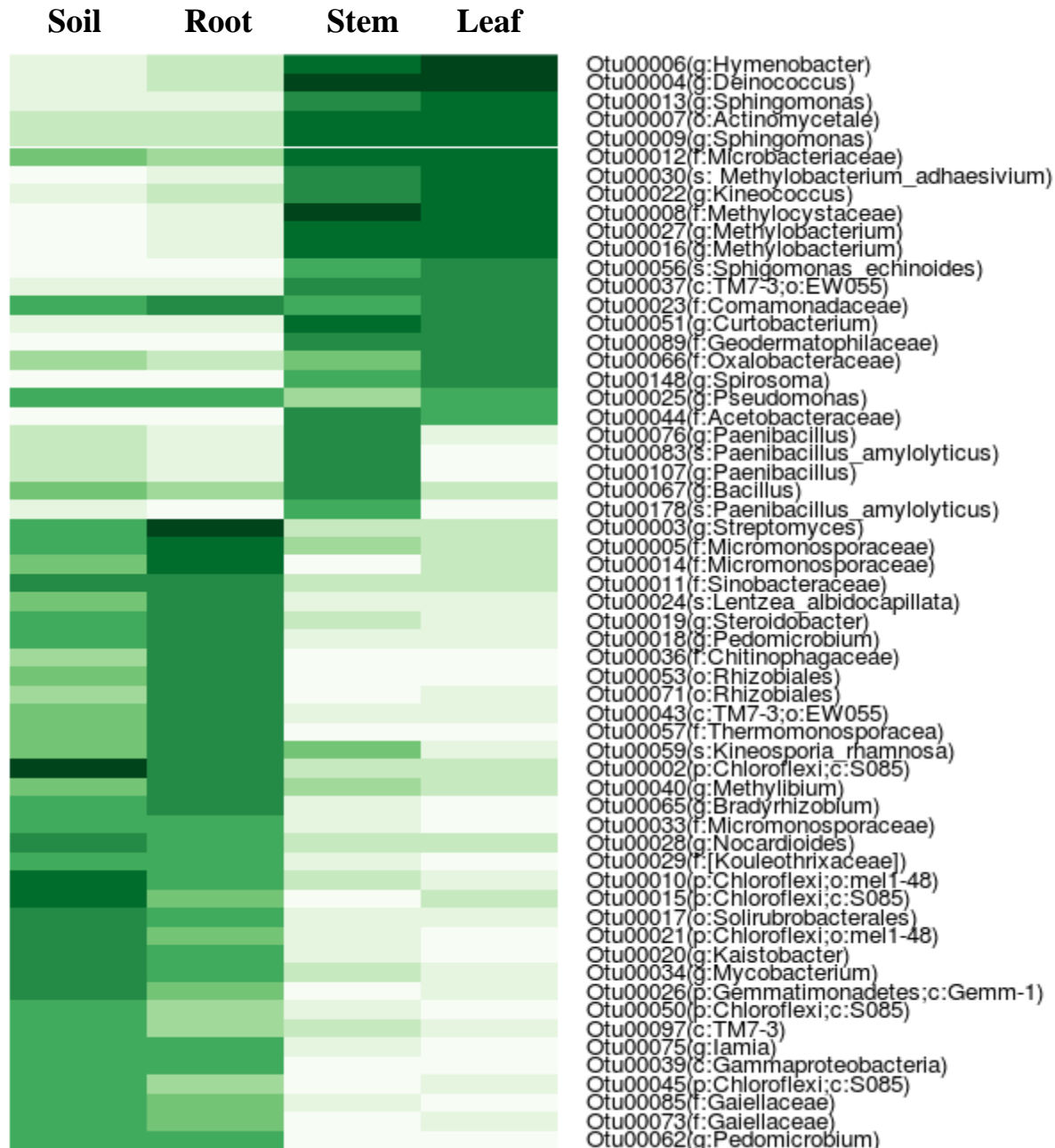
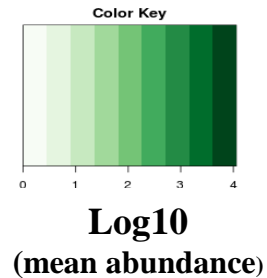
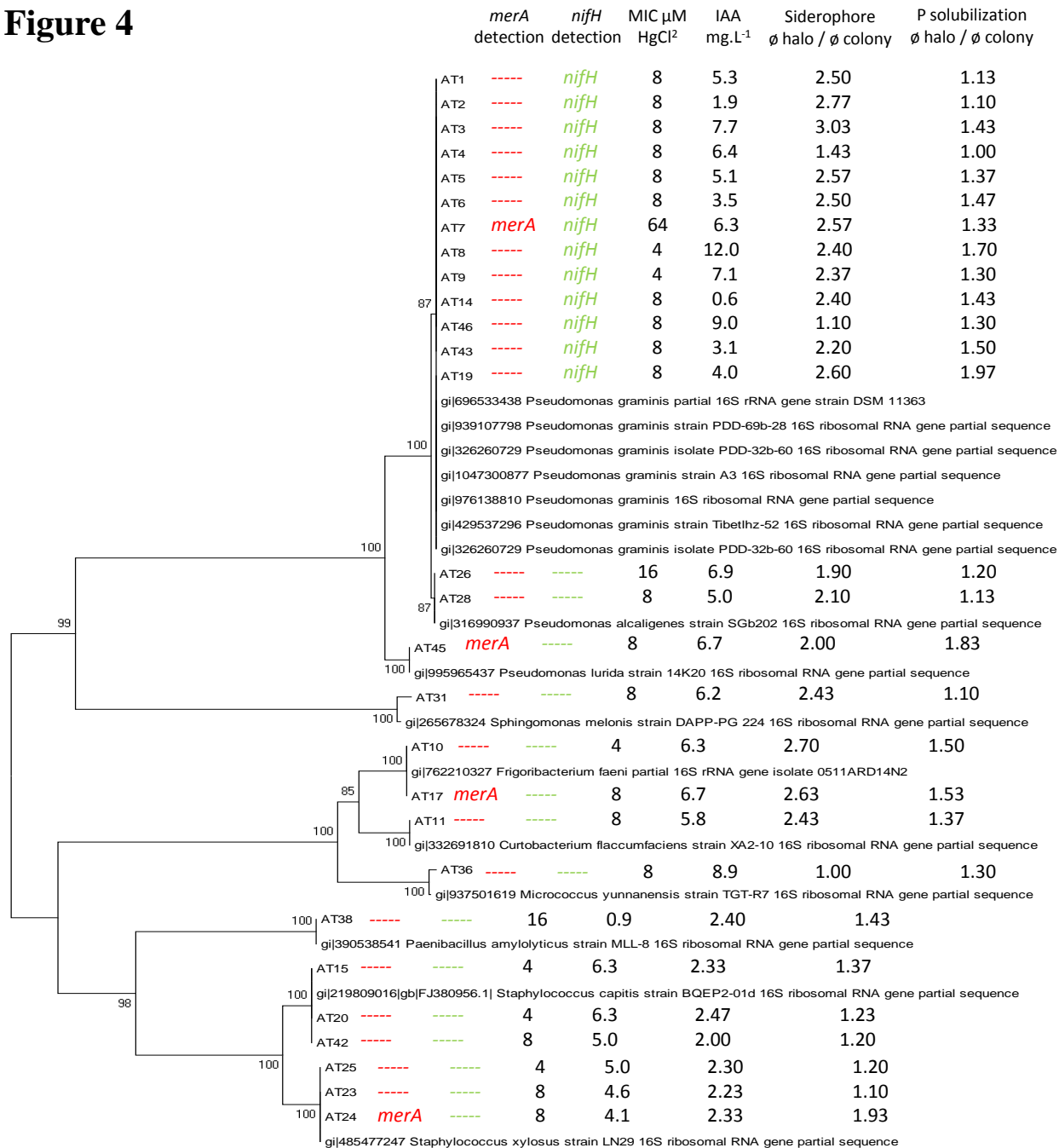


Table 2

Habitats		Medium 1/10 869			Medium 284	
		0 μM Hg Cl ₂	2 μM Hg Cl ₂	10 μM Hg Cl ₂	0 μM Hg Cl ₂	10 μM Hg Cl ₂
Aboveground	leaf episphere	3.59 10 ⁵ (100%)	9.28 10 ⁴ (25.86%)	2.43 10 ⁴ (6.77%)	2.14 10 ⁵ (100%)	2.85 10 ⁴ (13.31%)
	stem episphere	5.38 10 ⁴ (100%)	1.21 10 ⁴ (22.45%)	3.56 10 ³ (6.62%)	0	0
	leaf endosphere	2.24 10 ⁴ (100%)	ND	0	0	0
	stem endosphere	1.71 10 ⁶ (100%)	ND	0	0	0
Belowground	root endosphere	4.77 10 ⁶ (100%)	ND	9.54 10 ² (0.02%)	1.45 10 ⁶ (100%)	0
	rhizospheric soil	6.28 10 ⁸ (100%)	ND	9.84 10 ⁷ (10.89%)	2.01 10 ⁸ (100%)	4.02 10 ⁶ (2.00%)
	bulk soil	1.34 10 ⁷ (100%)	ND	9.02 10 ⁶ (67.33%)	8.20 10 ⁶ (100%)	3.18 10 ⁶ (38.78%)

Figure 4



0.020

Figure 5

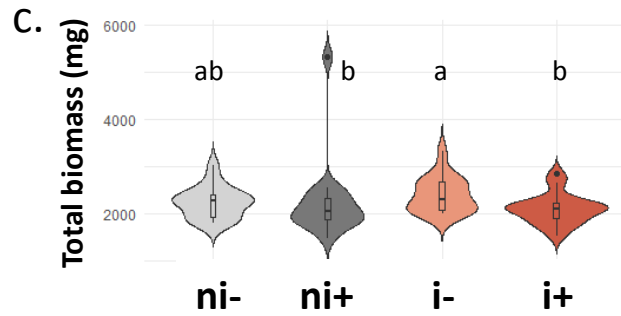
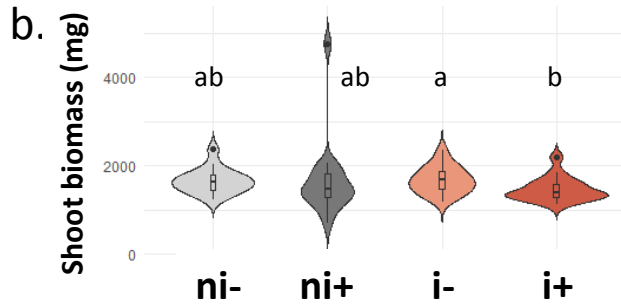
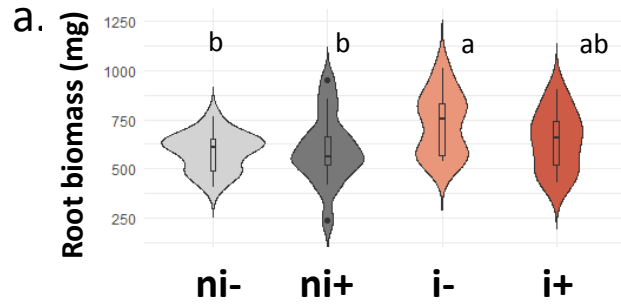
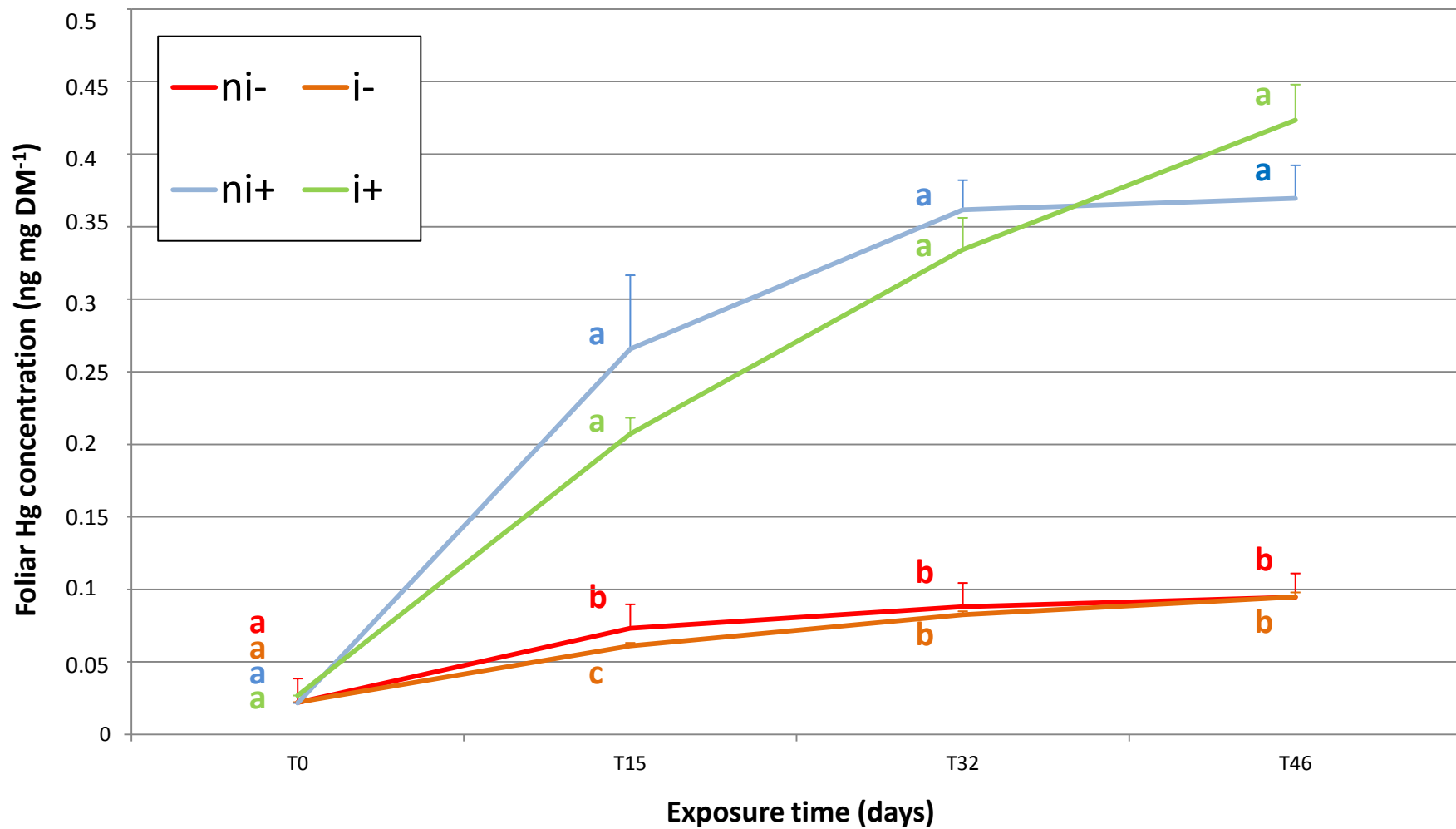


Figure 6



Supplementary material for on-line publication only

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