Microbial community response to environmental changes in a technosol historically contaminated by the burning of chemical ammunitions

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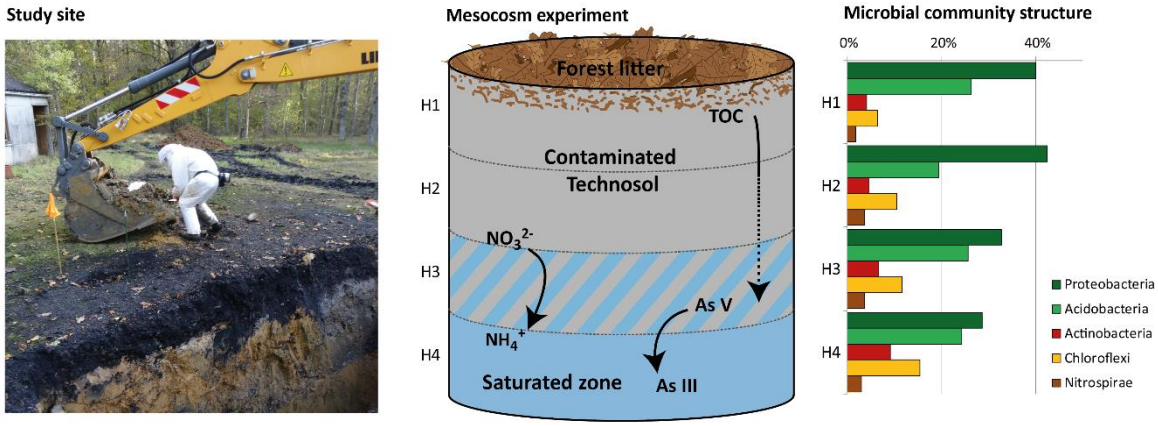
Graphical abstract



Highlights

- Microbial communities from a soil polluted by ammunition burning were described
- *Proteobacteria*, *Acidobacteria* and *Bacteriodetes* were the dominant phyla
- The main genus, *Sphingomonas*, is involved in arsenic cycle
- Organic matter supply and water saturation affected community structures

Abstract

The burning of chemical weapons in the 1926–1928 period produced polluted technosols with elevated levels of arsenic, zinc, lead and copper. During an eight-month mesocosm experiment, these soils were submitted to two controlled environmental changes, namely the alternation of dry and water-saturated conditions and the addition of fragmented organic forest litter to the surface soil. We investigate, by sequencing the gene coding 16S rRNA

and 18S rRNA, (1) the structure of the prokaryotic and eukaryotic community in this polluted technosol and (2) their response to the simulated environmental changes, in the four distinct layers of the mesocosm. In spite of the high concentrations of toxic elements, microbial diversity was found to be similar to that of non-polluted soils. The bacterial community was dominated by *Proteobacteria*, *Acidobacteria* and *Bacteroidetes*, while the fungal community was dominated by *Ascomicota*. Amongst the most abundant bacterial Operational Taxonomic Units (OTUs), including *Sphingomonas* as a major genus, some were common to soil environments in general whereas a few, such as organisms related to *Leptospirillum* and *Acidiferrobacter*, seemed to be more specific to the geochemical context. Evolution of the microbial abundance and community structures shed light on modifications induced by water saturation and the addition of forest litter to the soil surface. Co-inertia analysis suggests a relationship between the physico-chemical parameters total organic carbon, Zn, NH_4^+ and As(III) concentrations and the bacterial community structure. Both these results imply that microbial community dynamics linked to environmental changes should be considered as factors influencing the behavior of toxic elements on former ammunition burning sites.

Keywords

Microbial communities composition, contaminated technosol, mesocosm study, Illumina sequencing

1. Introduction

At the end of the First World War (1914-1918) large quantities of unfired ammunitions remained along the former front line. These weapons were either disassembled to allow recycling of reusable materials (metals and chemical compounds), destroyed by open detonation or, for the most dangerous chemical weapons, dumped at sea (Edwards and Beldowski, 2016) or destroyed by burning (Bausinger and Press, 2005; Bausinger et al., 2007). A large amount of German arsenic-containing munitions filled with diphenylchloroarsine (Clark I) and diphenylcyanoarsine (Clark II) were destroyed in destruction facilities in Belgium and in France (Bausinger and Preuss, 2005; Bausinger et al., 2007). In recent years, several of these chemical ammunition burning grounds, severely contaminated by inorganic and organic compounds, have been found along the western front. The soils of these destruction sites are polluted by arsenic (As), metals, nitroaromatic explosive compounds and organo-arsenical agents (Bausinger and Preuss, 2005; Bausinger et al., 2007).

One such site in France, known as the "Place-à-Gaz", has remained undisturbed since destruction of the shells. Between 1926 and 1928, more than 200,000 Blue Cross shells containing the Clark I and Clark II agents were destroyed on this site by thermal treatment (Hubé, 2017). The residues from this combustion formed a black layer containing slag, coal, ash and ammunition residues (Bausinger et al., 2007; Thouin et al., 2016). The layer is highly contaminated with As, zinc (Zn), copper (Cu) and lead (Pb) (which concentrations respectively reach 72.8 $g.kg^{-1}$, 90.2 $g.kg^{-1}$, 9.1 $g.kg^{-1}$ and 5.8 $g.kg^{-1}$) forming unexpected mineralogical associations (Thouin et al., 2016). Organic contamination was also observed with the detection of nitroaromatic explosives and their derivatives (Bausinger et al., 2007), dioxins, furans, polycyclic aromatic hydrocarbons (PAH) and diphenylarsinic acid (Thouin et al., 2017). The most contaminated zone of this site has no vegetation and floristic diversity is very low at the periphery. Only three species were found to have colonized this contaminated area: *Pohlia nutans*, a pioneer moss; *Cladonia fimbriata*, a lichen; and *Holcus lanatus*, a grass. These species appear to have high tolerance to metal(loïd) contamination. *Pohlia nutans* is a resistant species surviving on highly contaminated soil and accumulating high amounts of metals (Salemaa et al., 2001). *Holcus lanatus* possesses a mechanism for arsenate tolerance by suppression of the high affinity phosphate uptake system (Meharg and Macnair, 1992).

Despite the severe contamination, microorganism activity, contributing to carbon (C) and As biogeochemistry, was detected in this technosol (Thouin et al., 2016). However, the structure of the microbial community on this particular site has not yet been determined. Several studies have shown the impact of inorganic or oganic

pollution on the structure of communities and on microbial diversity (Golebiewski et al., 2014; Kuppusamy et al., 2016; Xu et al., 2017). Bourceret et al. (2016) studied the effect of combined pollution on microbial structure during revegetation of an industrial wasteland. They showed that bacterial and fungal diversity was high despite long-term contamination by PAH and metals. These results suggested that microbial community structure can adapt to and specialize in accordance with contamination over the years. Ninety years after the formation of the contaminated material of the Place-à-Gaz, it is questionable whether this contamination induced a selective pressure on microorganisms similarly to that on flora and/or if the microbial community is adapted and today has a unique structure, representative of this source of contamination.

In previous work, we showed that intrinsic organic matter (OM), mainly composed of charcoal from the contaminated material, is very high (≈ 25 %) (Thouin et al., 2016). This OM is characterized by high C/N (≈ 35) and low hydrogen index/oxygen index (HI/OI ≈ 0.1) and is therefore recalcitrant in nature (Thouin et al., 2018). Soil properties such as organic C availability affect microbial community composition (Fierer et al. 2007). Some studies have shown that addition of organic amendments modifies microbial activity and diversity (Drenovsky et al., 2004; Pérez-Piqueres et al., 2006; Abujabhah et al., 2016). In metal-contaminated soils, the addition of compost can also increase bacterial activity and diversity (Farrell et al., 2010). Due to its location in an oak forest, the Place à Gaz sols must regularly receive litter inputs that could enhance the bioavailability of OM and then modify the composition of the microbial community. The contaminated material may also be partially or completely saturated during episodes of high precipitation. Soil water content is also a major determining factor for microbial community structure (Drenovsky et al., 2004; Unger et al 2009). Randle-Boggis et al. (2017) have shown that repeated flooding altered microbial community composition in a pasture soil and observed functional shifts of nitrogen (N) and sulfur (S) transforming microorganisms.

To study the effect of environmental changes observed on the site during recent decades – mainly changes of water saturation and OM deposition – a mesocosm experiment was setup: the contaminated material was subjected, in the laboratory, to water saturation episodes and to the addition of forest litter over eight months. This previous study provided results on the processes that affect the fate of As and metals that were published in Thouin et al. (2017, 2018). Adding litter contributed to the growth of As transforming microorganisms; soil saturation enhanced arsenate-reducing activity (Thouin et al., 2018) resulting in the increase of arsenite concentration in soil solution. However, only global microbial parameters (carbon mineralization, enumeration and activity tests for As-transforming microorganisms) were considered at this stage. Here, we investigated the

structure of the microbial communities in the samples from this mesocosm experiment (Thouin et al. 2017, 2018) with the heavily contaminated technosol of the Place-à-Gaz, giving insight, for the first time, into the microbial diversity of polluted sites that are part of the complex legacy of chemical shell destruction. In a second time, the effects of two environmental parameters (namely water saturation events and natural organic matter input) on this diversity were evaluated through analyzes of the bacterial, archaeal and fungal communities in samples from the mesocosm experiment. The relationships between physico-chemical parameters and dominant bacteria were also considered.

2. Materials and methods

2.1 Mesocosm experiment and sample collection

The studied technosol was collected at the Place-à-Gaz site (Spincourt forest, 20 km from Verdun, France) and was characterized by Bausinger et al. (2007) and Thouin et al. (2016). As concentrations ranged from 102 to 150 $g.kg^{-1}$, Zn concentrations from 70 to 107 $g.kg^{-1}$, Cu concentrations from 13 to 21 $g.kg^{-1}$, and Pb concentrations from 3 to 6 g.kg-1 (Thouin et al., 2016). A mesocosm study, previously described in Thouin et al., 2017 was conducted in a 1 m diameter column filled with 610 kg of this polluted material that had a high organic content (25.9 %), mainly in the form of non-biodegradable coal ash (Thouin et al., 2016, 2017). The experimental soil cylinder was divided into four distinct layers (H1, H2, H3 and H4), from H1 (surface), to H4 (bottom). After three months of stabilization, the soil was subjected to dry/wet periods of about one month each (during eight months). Rainfall was simulated by a sprinkler system. The dry periods were characterized by a saturation of the bottom of the mesocosm limited to the H4 level, and by the addition of around 12 L of mineral water once a week. The mineral composition of this water is close to that of rainwater (Mont Roucous: pH = 6.38, electrical conductivity = 160 $\mu S.cm^{-1}$, Cl^- = 2 $mg.L^{-1}$, NO_3^- = 1.5 $mg.L^{-1}$, SO_4^{2-} = 1.5 $mg.L^{-1}$, Na^+ = 2.5 $mg.L^{-1}$, K^+ = 0.3 $mg.L^{-1}$, Ca^{2+} = 1 $mg.L^{-1}$, Mg^{2+} = 0.5 $mg.L^{-1}$; Thouin et al. 2017). The wet periods were characterized by elevation of the water table in order to saturate both H3 and H4, and by the addition of 6 L of water every two days. At the midpoint of the experiment (i.e. after four months), 24 kg of fragmented organic forest litter sampled at the Place-à-Gaz site were added to the top of the surface soil (level H1). The forest litter added was characterized by high organic carbon (30.5 %), nitrogen (1.8 %) concentrations, and high HI/OI (Thouin et al., 2017). Coring was performed in the mesocosm using 5 cm diameter stainless steel pipes at the beginning of the experiment (T0), just prior to the addition of fragmented forest litter (4 months, T4), and at the end of the experiment (8 months, T8). Each core was separated into four samples, H1 0–12.5 cm, H2 12.5–

28 cm, H3 28–42 cm, and H4 42–75 cm. Sub-samples were dried and dedicated to soil characterization presented in Thouin et al. (2018) and 2 g were immediately placed in sterile tubes, and stored at -20 °C until DNA extraction.

2.2 DNA extraction

Immediately after sampling, total soil DNA were extracted in triplicate from 0.5 g of wet weight of each sample, using the FastDNA® SPIN Kit for soil (MP Biomedicals, Illkirch, France) and the FastPrerp®-24 Classic Instrument used per the manufacturer's instructions. The three replicates of each soil sample were pooled and further purified with the Geneclean Turbo Kit (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions.

2.3 Real-time quantitative PCR of 16S, *aioA* and 18S rRNA genes.

Real-time quantitative polymerase chain reaction (qPCR) was performed to assess the fungal and bacterial abundance, and more specifically the abundance of arsenite-oxidizing bacteria. Primer sets FR1 (5′-AICCATTCAATCGGTAIT-3′) / FF390 (5′-CGATAACGAACGAGACCT-3′) (Chemidlin Prévost-Boure et al., 2011), 341F (5'-CCTACGGGAGGCAGCAG-3') / 515R (5'-ATTACCGCGGCTGCTGGCA-3') (López-Gutiérrez et al., 2004), and m4-1F (5'-CCGGCGGGGGCTTYGARRAYARNT-3') / m2-1R (5′-CGATAACGAACGAGACCT-3′) (Quéméneur at al., 2010; Quéméneur, 2008), were used, targeting the 18S rRNA gene of *Fungi*, the 16S rRNA gene of *Bacteria* and the *aioA* gene encoding the catalytic subunit of arsenite-oxidase, were used. Amplification reactions were carried out for each gene, using 2 ng of soil DNA, 1X iQSYBR Green SuperMix (Bio-Rad), each primer at 0.5 µM (18S rRNA gene), 0.4 µM (16S rRNA gene) or 0.3 µM (*aioA* gene). 100 µg of T4gp32 (MP Biomedicals) was also added for qPCR of 16S rRNA and *aioA* genes. Programs for qPCR were run in a CFX Connect (BioRad) and consisted of an initial denaturation at 95 °C for 3 min, 40 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s (18S rRNA gene), 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s (16S rRNA gene) or 40 cycles of 95 °C for 10 s, 54 °C for 20 s, 72 °C for 10 s (*aioA* gene), and a data acquisition step at 80 °C for 10 s at each cycle. At the end, a melting curve analysis was performed by measuring the SYBR Green I signal intensities during a 0.5 °C temperature increment every 10 s, from 65 °C to 95 °C. A six-point serial decimal dilution of a linearized plasmid carrying the target gene (18S rRNA, 16S rRNA or *aioA*) was used to generate a linear calibration curve of threshold cycle versus a number of gene copies ranging from 10^2 to 10^7. All measurements were run in duplicates. Data were expressed as gene copy numbers per gram of dry weight soil.

2.4. Next generation sequencing

A portion of the 16S rRNA gene (V4-V5 region) was amplified using the barcoded, universal primer set (515WF/918WR) (Wang et al. 2009). A portion of the 18S rRNA gene was amplified using the universal 18S primer set (574WF/952WR) (Hadziavdic et al, 2014). PCR reactions were performed using the AccuStart II PCR ToughMix kit, followed by cleaning (HighPrep PCR beads, Mokascience). Pooled triplicates were submitted for sequencing on Illumina MiSeq instrument at GeT-PlaGe (Auzeville, France). Sequences were processed by INRA Transfert (Narbonne, France) using Mothur (version 1.36.1) according to MiSeq SOP pipeline (Schloss et al, 2009).

Barcodes, primers, and sequences showing homopolymers of more than 8bp were discarded. Sequences showing 100% homology were grouped in unique sequences, then in OTUs (operational taxonomic unit, based on 97% homology). Sequences were then assigned to match a sequence in Greengenes (August 2013 release of gg_13_8_99, containing 202,421 bacterial and archaeal sequences) and PR2 databases for prokaryotes and eukaryotes respectively, to identify the genus level. A total of 319,516 prokaryote sequences and 94,806 eukaryote sequences were obtained in 8 and 2 samples respectively.

2.4 Statistical analyses

All statistical analyses were carried out in statistical environment R, version 3.0 (R Development Core Team, 2015) using the ade4 (Dray and Dufour, 2007) and vegan (Oksanen et al., 2007) packages. To analyze the differences in the overall bacterial diversity across the samples, dissimilarity matrices based on Bray-Curtis distances were produced from the OTUs table and Principal Coordinates Analyses (PCoA) were generated. Venn diagram, conducted on the presence/absence matrice of the bacterial OTUs, was used to illustrate the community overlaps between the samples levels at T0 and T8. To evaluate a possible relationship between environmental parameters and bacterial composition during the experiment, bath data sets were analyzed by co-inertia analysis (COIA: Dray et al., 2003). Environmental parameter data are from Thouin et al. (2017, 2018) and only the 15 dominant bacterial OTUs present in the overall samples were used in alaysis.The RV-coefficient, representing the strength of the co-variation, was calculated and statistically tested using the Monte Carlo randomization test with 999 random permutations.

3. Results

3.1 Bacterial and fungal abundance

Abundance of total bacteria, arsenite-oxidizing bacteria and fungi were estimated in the four levels of the mesocosm (Table 1) at the beginning (T0) and at the end of the experiment (T8). Bacterial abundance (mean value of all samples of $8.38\times10^{8} \pm 3.11\times10^{7}$ 16S rRNA gene copies g dw soil^{-1}) was about ten times higher than fungal abundance (mean value of all samples of $7.68\times10^{7} \pm 2.57\times10^{6}$ 18S rRNA gene copies g dw soil^{-1}). At T0, the total bacterial abundance was lower in the H4 level, but at T8 bacterial abundance had increased at all levels and no difference was observed between sampling levels. Fungal abundance also increased over time in the surface level H1, with the value reaching $2.87\times10^{8} \pm 1.92\times10^{5}$ 18S rRNA gene copies g dw soil^{-1}.

Table 1: Abundance of bacterial, arsenite-oxidizing bacterial and fungal communities (respectively16S, *aioA*, 18S rRNA genes copy numbers) at the four levels of sampling at the beginning (T0) and at the end of the experiment (T8). Standard deviation (Std. dev.) was calculated from qPCR replicates (n=2). Non saturated H1 0–12.5 cm; non saturated H2 12.5–28 cm; transiently saturated H3 28–42 cm; and saturated H4 42–75 cm. T0 beginning of experiment; T8 after 8 months.

Sample	16S rRNA gene		*aio*A gene		*aioA*/16S	18S rRNA gene	
	copies. g dw soil^{-1}	Std. dev.	copies. g dw soil^{-1}	Std. dev.		copies. g dw soil^{-1}	Std. dev.
H1-T0	4.14E+08	1.42E+08	3.44E+08	1.80E+07	0.83	2.68E+07	4.03E+06
H2-T0	2.72E+08	3.34E+07	1.60E+08	5.57E+06	0.58	7.26E+06	1.48E+06
H3-T0	5.38E+08	1.11E+07	2.49E+08	1.51E+07	0.46	2.36E+07	3.48E+06
H4-T0	6.37E+07	3.87E+06	5.58E+07	2.05E+07	0.88	1.80E+06	3.85E+05
H1-T8	2.07E+09	5.00E+07	1.27E+09	7.67E+07	0.61	2.87E+08	3.48E+05
H2-T8	1.17E+09	6.87E+07	5.94E+08	2.10E+07	0.51	1.14E+08	2.10E+07
H3-T8	1.20E+09	1.67E+08	3.71E+08	1.02E+08	0.31	-	
H4-T8	9.79E+08	2.24E+07	1.39E+08	2.97E+07	0.14	-	

Abundance of the *aioA* gene, encoding for the bacterial arsenite-oxidase catalytic subunit, increased in all levels between T0 and T8. However, the proportion of *aio*A genes, decreased from T0 to T8. This decrease was more pronounced in the deeper levels H3 and H4 than in levels H1 and H2, which were never water saturated.

3.2 Community species richness and diversity

After treatment steps, 84% and 77% of 16S and 18S rRNA gene reads, respectively, were kept for further analysis. The rarefaction curves reach a plateau for all samples showing that the diversity of communities was well sampled (SM.1). Based on complete data, the bacterial communities were composed of 741 to 1,069 OTUs per soil sample (Tab. 2). The bacterial richness sampled increased between T0 and T8 in the levels H1 (798 to 1,069 OTUs) and H3 (917 to 1,003 OTUs). Nearly all the sequences were assigned to *Bacteria*, with only a few sequences being attributed to *Archaea*. Thirteen archaeal OTUs were found amongst all samples. The number of archaeal OTUs ranged between 4 and 7 per soil sample (Tab. 2). At level H1, the number of eukaryotic OTUs increased from 80 at T0 to 324 at T8.

The bacterial diversity was homogeneous, varying between 4.1 and 5.2 for the Shannon index and between 5.0 and 5.7 for the Simpson index. At T8, there were higher bacterial diversities in the H1 and H3 levels than in H2 and H4 (Tab. 2). The archaeal diversity was low (1.1–2.0 H' and 0.3–1.4 D') and decreased in H1 between T0 and T8. Eukaryotic diversity increased in H1 between T0 and T8, this increase resulted from the increased diversity of fungal communities.

Table 2: Sequencing data characteristics, and richness and diversity indices. Non saturated H1 0–12.5 cm; non saturated H2 12.5–28 cm; transiently saturated H3 28–42 cm; and saturated H4 42–75 cm. T0 beginning of experiment; T8 after 8 months.

Sample	Sequencing results		Diversity estimates	
	Reads	OTUs[a]	Shannon (H')	Simpson (D')
Bacteria				
H1-T0	35,787	798	4.7	5.2
H2-T0	28,244	741	4.6	5.1
H3-T0	32,145	917	5.1	5.3
H4-T0	27,282	789	5.0	5.2
H1-T8	30,058	1069	5.5	5.7
H2-T8	27,885	778	4.1	5.0
H3-T8	36,246	1003	5.2	5.5
H4-T8	33,482	786	4.9	5.0
Archaea				
H1-T0	279	7	2.0	1.4
H2-T0	308	6	1.4	0.8
H3-T0	427	6	1.5	0.8
H4-T0	463	6	1.2	0.4

H1-T8	874	7	1.1	0.3
H2-T8	449	5	1.2	0.4
H3-T8	737	4	1.2	0.5
H4-T8	429	7	1.4	0.7
Eukaryota				
H1-T0	42,642	80	2.6	2.2
H1-T8	52,164	324	3.7	3.7

[a]Species level, 97% similarity threshold used to define OTUs.

3.3 Bacterial community structure

Taxonomic assignment of OTUs allowed us to identify 44 bacterial phyla, 4 archaeal phyla and 7 fungal phyla. The bacterial community was mainly composed of *Proteobacteria* (37.2±3.3%), *Acidobacteria* (21.7±1.5%), *Chloroflexi* (10.0±1.0%), *Bacteroidetes* (9.4±1.9%) and *Actinobacteria* (6.5±0.6%) (Fig. 1). *Proteobacteria* were dominated by *Alpha-* (22.0±1.4%), *Beta-* (6.9±0.6%) and *Gammaproteobacteria* (5.2±0.7%). The archaeal community was composed almost solely of *Thaumarchaeota* (97.9±1.3%) but *Euryarchaeota* were detected in the upper level (SM. 2). The *Eukaryota* was mainly composed of *Archaeplastida* and *Metazoa* in H1T0. The fungal community represented 16% of the *Eukaryota* in H1-T0 and increased to 61% in H1-T8. At T0 the fungal community was mainly composed of *Ascomycota* (66%), *Basidiomycota* (17%) and *Chytridiomycetes* (10%). At T8, the fungal community had changed and the main communities were *Chryptomicotina* (33%), *Ascomycota* (32%), *Chytridiomycetes* (19%) and *Mucoromycota* (11%).

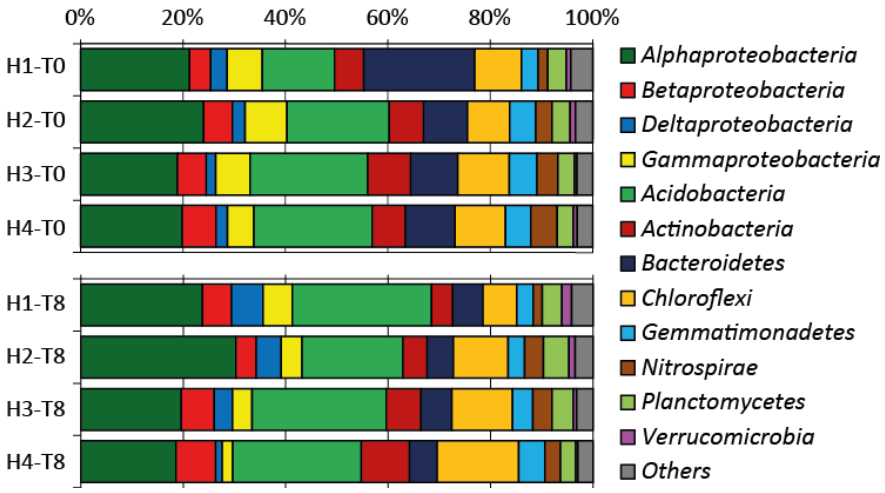


Figure 1: Percentage of the major bacterial phyla in the eight samples. Non saturated H1 0–12.5 cm; non saturated H2 12.5–28 cm; transiently saturated H3 28–42 cm; and saturated H4 42–75 cm. T0 beginning of experiment; T8 after 8 months.

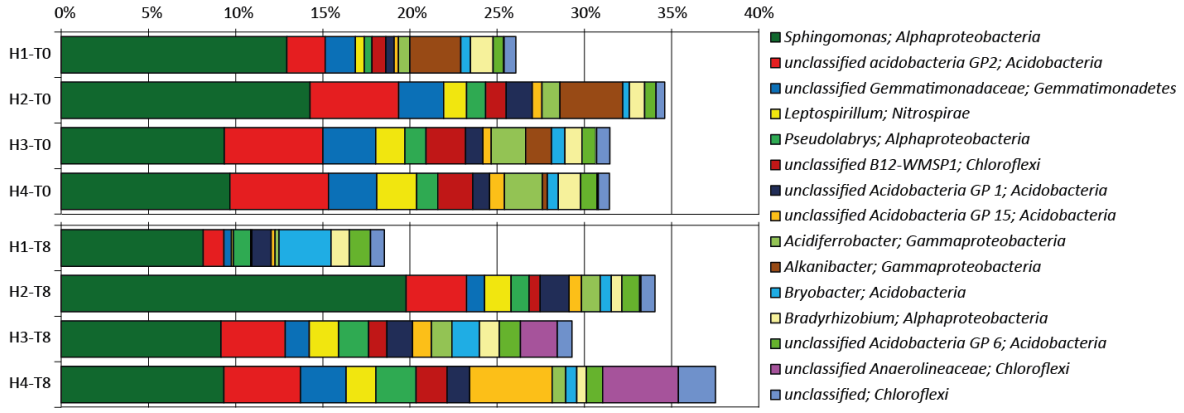


Figure 2 : Relative abundance of the 15 dominant bacterial OTUs in the eight samples. Non saturated H1 0–12.5 cm; non saturated H2 12.5–28 cm; transiently saturated H3 28–42 cm; and saturated H4 42–75 cm. T0 beginning of experiment; T8 after 8 months.

The most abundant bacterial genus in all soil samples was *Sphingomonas* (8.1–19.8% *Alphaproteobacteria*) comprising heterotrophic species (Fig. 2, SM.3). At T0, the bacterial community was also dominated by two OTUs affiliated to an unclassified member of *Acidobacteria* subdivision 2 and the *Gemmatimonadaceae* family (Fig. 2). Two acidophilic chemo-autotrophic and FeII-oxidizing bacteria, i.e. *Leptospirillum* and *Acidiferrobacter*, were included in the 15 most abundant OTUs. Eight OTUs were unclassified at genus rank, and their relative abundance varied from 33.1% in H1-T0 to 50.7% in H4-T8. *Archaea* were principally composed of *Candidatus nitrosotalea* (57.3–94.4%; *Thaumarchaeota*) and *Candidatus nitrososphaera* (1.6–10.0%; *Thaumarchaeota*) (SM.2). There was a difference in the fungal community at the genus rank in the H1 level between the beginning and the end of the experiment (SM.4). H1-T0 was dominated by *Pyrenochaeta* (22%; *Ascomycota*), *Coniochaeta* (21%; *Ascomycota*), *Wallemia* (12%; *Basidiomycota*) and *Chytridiomycetes*_X (10%; *Chytridiomycota*) genera. H1-T8 was mainly composed of *Cryptomycotina*_XX (21%; *Cryptomycotina*); *Helotiales* (21%; *Ascomycota*) *Chytridiomycetes*_X (18%; *Chytridiomycota)*, LKM11-*Rozella* (13%; *Cryptomycotina*) and *Mortierella* (11%; *Mucoromycota*) genera.

3.4 Modification of the bacterial community structure

The bacterial phylum structure changed between the beginning and the end of the experiment: the proportion of *Acidobacteria*, *Actinobacteria*, *Betaproteobacteria*, *Cloroflexi* and *Gemmatimonadetes, Nitrospirae*) increased, while that of *Deltaproteobacteria*, *Gammaproteobacteria* and *Verrucomicrobia*

decreased with depth (Fig. 1). Relative abundance of *Gammaproteobacteria* and *Bacteroidetes* decreased and that of *Chloroflexi* increased in all levels of the mesocosm during the experiment.

Changes were also observed in the abundance of the 15 dominant OTUs. Several OTUs increased in proportion with depth: *Leptospirillum* (*Nitrospirae*), *Pseudolabrys* (*Alphaprotebacteria*), two unclassified *Acidobacteria* from GP2 and GP15, while the proportion of *Bryobacter* and unclassified GP1 (*Acidobacteria*) decreased with depth (Fig. 2). *Alkanibacter* (*Gammaproteobacteria*), which was more abundant at levels H1 and H2 at T0, disappeared at T8. By contrast, an unclassified *Anaerolineaceae* (*Chloroflexi*) appeared in the deepest levels H3 and H4 at T8.

Amongst the 100 dominant OTUs detected in all samples, 41 were unclassified (Fig. 3). No general tendency specific to bacterial groups was evidenced and, moreover, some genera varied between T0 and T8 and/or with depth. The proportions of *Arthrobacter, Mucilaginibacter, Flavisolibacter, Variibacter, Alkanibacter* or *Undibacterium* decreased in all levels of the mesocosm. The proportions of *Gaiella*, *Geothrix*, *Lacibacter*, *Sediminibacterium*, *Pseudolabrys*, *Opitutus* and *Polaromonas* increased only in the bottom saturated (either permanently or transiently) level of the mesocosm. Conversely, the proportions of *Gemmatimonas*, *Variibacter* and *Acidiferrobacter* tended to decrease in this saturated zone of the mesocosm. In the upper non-saturated zone of the mesocosm, the genera *Holophaga* and *Rhizomicrobium* increased in proportion, whereas the proportion of *Blastocatella*, *Parafilimonas*, *Chryseolinea*, *Cytophaga*, *Varilibacter* and *Pierellula* decreased in this non-saturated part of the system.

The phenotypes of the identified genera in the group of 100 dominant OTUs (SM.5) revealed that aerobic chemo-organotrophic organisms were preponderant in all compartments of the mesocosm. However, chemo-autotrophic genera *Leptospirillum* and *Acidiferrobacter* were still included in the group of the 15 dominant genera. Two other characteristics were frequently found in species of the dominant genera: the tolerance to acidity (found in 5 out of 7 classified genera in the group of 15 dominant) and the ability to degrade complex organic molecules.

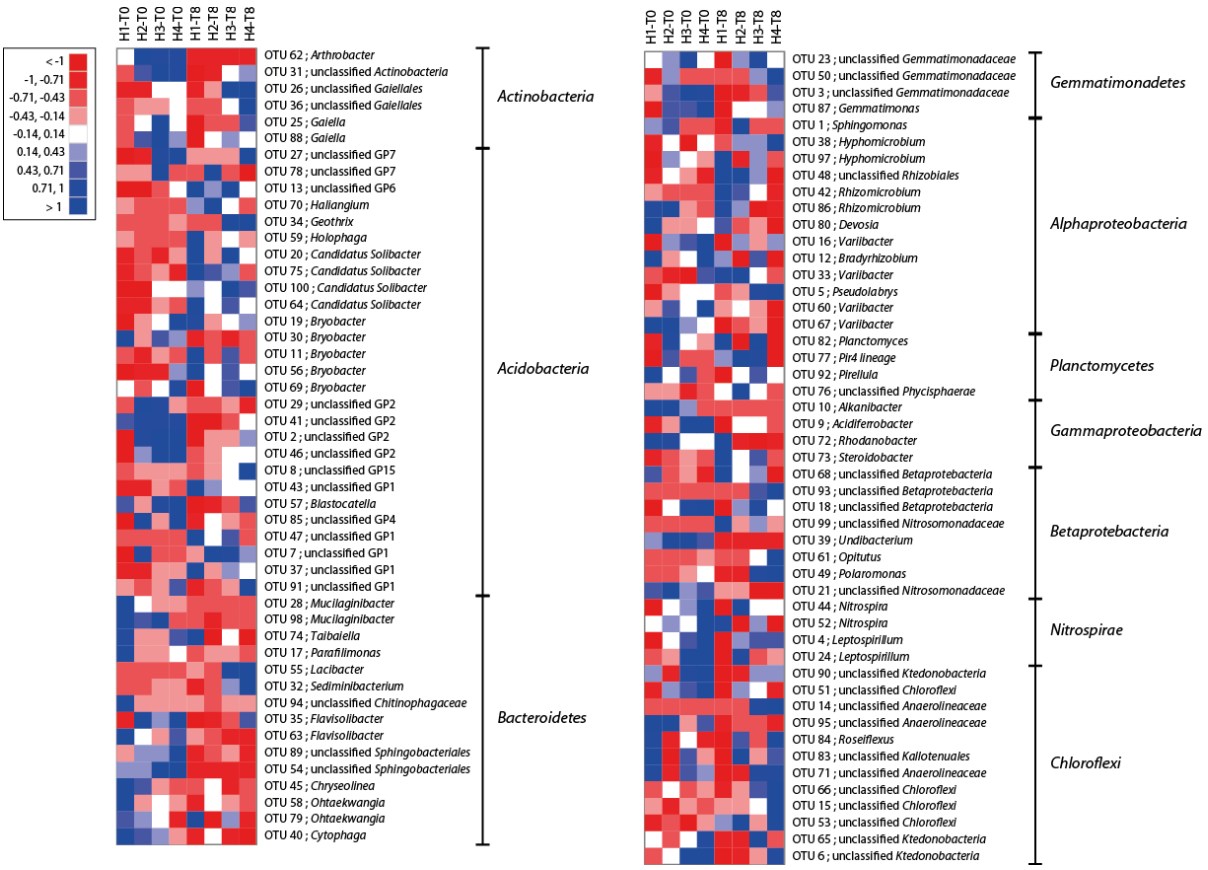


Figure 3: Relative abundance of 100 dominant OTUs in each soil sample. OTUs order is defined by phylogenetic neighbor-joining from partial 16S rRNA gene sequences. Non saturated H1 0–12.5 cm; non saturated H2 12.5–28 cm; transiently saturated H3 28–42 cm; and saturated H4 42–75 cm. T0 beginning of experiment; T8 after 8 months.

The bacterial community overlaps were depicted as Venn diagrams (Fig. 4). The results showed a global decrease between T0 and T8 in the quantity of OTUs common to all levels of the mesocosm and a simultaneous increase in the number of OTUs that are specific to a single level; this was more pronounced in the surface level H1 than in the other zones of the system.

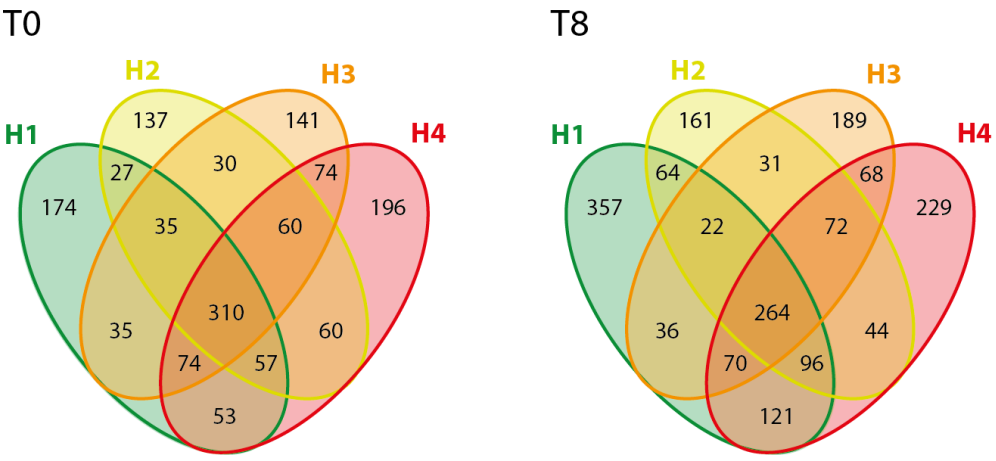


Figure 4: Venn diagrams of shares or unique OTUs for different sample levels at T0 and T8. Non saturated H1 0–12.5 cm; non saturated H2 12.5–28 cm; transiently saturated H3 28–42 cm; and saturated H4 42–75 cm. T0 beginning of experiment; T8 after 8 months.

Principal coordinates analysis (PCoA) of soil samples based on Bray-Curtis distances of bacterial communities (Fig. 5) distributed the diversity structures according to two axes. The F1 axis (37.2% of the variability) relates to the temporal variability, as all the T0 communities are gathered on the right side of the figure. The F2 axis (24.1% of the variability) is associated with depth in the soil column, from the surface (H1) to the bottom (H4) of the mesocosm. This analysis shows that a progressive vertical evolution of the structures of the bacterial communities was already established at the beginning of the experiment, i.e. at the end of the stabilization period. Over the 8 months of the experiment, bacterial communities changed at all levels and the divergence between the bacterial communities of levels H2, H3 and H4 was amplified. An evolution of the bacterial community was also observed in the surface level H1. However, the bacterial community in this zone of the mesocosm was markedly different from those in the deepest levels both at T0 and T8.

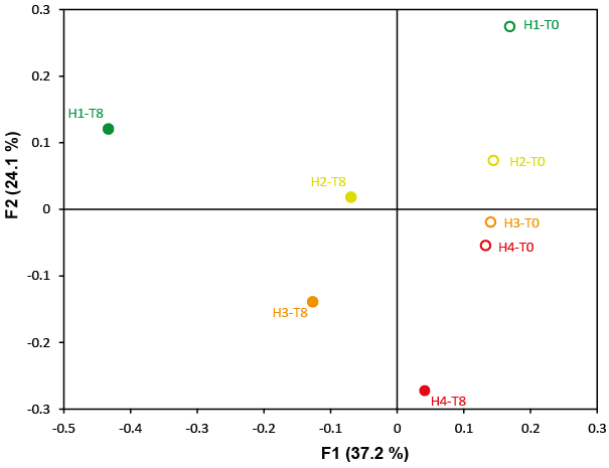


Figure 5: PCoA of soil samples based on Bray-Curtis distances of bacterial communities. Non saturated H1 0–12.5 cm; non saturated H2 12.5–28 cm; transiently saturated H3 28–42 cm; and saturated H4 42–75 cm. T0 beginning of experiment; T8 after 8 months.

The relationship between the 15 dominant bacterial OTUs and the environmental variable was evaluated using COIA (Fig. 6). The first two axes explained 85% of the total co-variation between the bacterial communities and the environmental parameters and the results showed a significant correlation (P=0.004) (Fig. 6.a). A shorter arrow in H4 level than in other levels at T0 and T8 indicates stronger relationship between environmental parameters and composition of dominant bacteria.

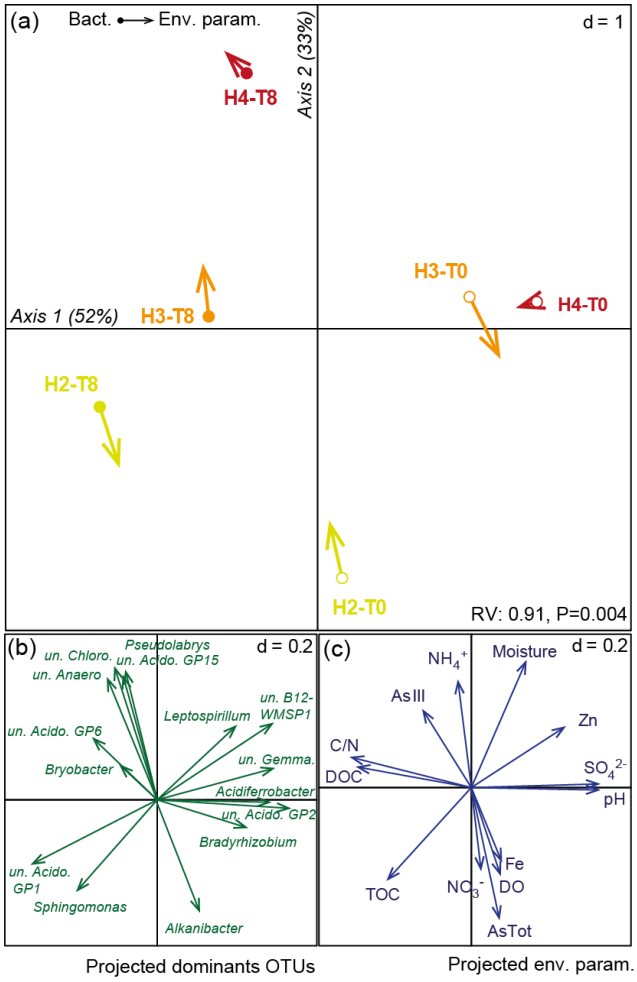


Figure 6: Co-inertia analysis (COIA) of the relationship between the 15 dominant bacterial OTUs and environmental parameters at different sample levels at T0 and T8. (a) Mutual ordination of the plots as a function of dominant bacterial OTUs and environmental parameters. Arrow length is proportional to the difference between the ordination of the dominant bacteria and environmental data. The position of arrow tails is determined by the ordinations of the bacterial data, that of the arrowheads by the environmental data. (b) and (c) are projections of dominant OTUs and environmental parameters. Environmental parameters are described in Thouin et al., 2017, 2018. H1 sampling level was not included in COIA due to missing data. Non saturated H1 0–12.5 cm; non saturated H2 12.5–28 cm; transiently saturated H3 28–42 cm; and saturated H4 42–75 cm. T0 beginning of experiment; T8 after 8 months.

The COIA ordination plot (Fig.6) suggests a positive effect of organic C and low moisture (aerobic conditions) on the abundance of the dominant heterotrophic OTU *Sphingomonas*. The dominant OTUs *Leptospirillum* and *Acidiferrobacter* seem to be anti-correlated with organic C and positively correlated with Zn concentration. The reduced chemical species As(III) and NH_4^+ were associated with high levels of unclassified *Chloroflexi* and unclassified *Anaerolineaceae*, and with the aerobic heterotrophic genus *Pseudolabrys*.

4. Discussion

4.1 Diversity of the microbial community of the "Place-à-Gaz" multi-contaminated soil

The Place-à-Gaz site presents complex and intense mixed contamination by metals, metalloids and organic pollutants inherited from the destruction of chemical weapons (Bausinger *et al*., 2007, Thouin *et al*., 2016). The soil is moderately acidic (pH 5.5–6) and has a high organic C concentration arising from the abundance of charcoal. The lack of plant growth on this site for more than 90 years may result either from a low availability of nutrients and/or from global toxicity. Only three tolerant plant species were able to grow on this technosol, and the most polluted central zone remains bare. There was a need to describe the influence of the particular selection pressure parameters that had inhibited the development of vegetation cover on the composition and diversity of the microbial community of this specific environment.

Microbial diversity

Soil pH, C content and C/N ratio are often found to be the main factors influencing the structures of soil microbial communities on sites contaminated by metals (Chodak et al, 2013; Eo and Park, 2016). Lauber et al. (2009) have shown that the overall phylogenetic diversity of bacterial communities correlates with soil pH, with peak diversity in soils being found at near-neutral pH values. However, other authors have shown that microbial communities are influenced by both classical soil characteristics and toxic pollutant concentrations (Frossard et al., 2017). Recently, Wu et al. (2017) showed that mercury concentration was the key parameter influencing the microbial diversity of soils affected by the destruction of electronic wastes.

Richness and diversity indices of the "Place-à-gaz" soil mesocosm microbial communities were of the same order as those found in other heavy-metal contaminated soils (Golebiewski et al., 2014). However, they were lower than those found in a coking plant wasteland affected by multiple forms of contamination for nearly a century (Bourceret et al., 2016). The authors explained the high microbial diversity on this site by a long-term adaptation of the bacterial communities to the pollution. The lower diversity observed on the Place-à-Gaz site, where the time scale for adaptation is similar, may be linked to the higher intensity of contamination (Zn and Cu concentrations were 10 times greater), to a moderate acidic pH (5.5-6, while close to neutral on the coking plant site), and to the lack vegetation.

Microbial community structure

The main bacterial phyla were *Proteobacteria*, *Acidobacteria*, *Bacteroidetes* and *Chloroflexi*. These phyla have already been found to be the major ones in both contaminated and non-contaminated soils (Zhalnina et al., 2015; Wu et al., 2017). They comprised common representatives from copiotrophic groups (*Proteobacteria*, *Actinobacteria*), microorganisms with mainly oligotrophic characteristics (*Acidobacteria*, *Chloroflexi*, *Verrucomicrobia*). However, the absence of the *Firmicutes* phylum distinguishes the "Place-à-Gaz" bacterial communities from those of previously studied environments, and particularly from a non-polluted soil with the same pH as that of our site (Zhalnina et al., 2015). *Clamydiae* is another phylum generally found in the dominant groups of soil bacterial communities (Zhalnina et al., 2015; Wu et al., 2017) but absent from the "Place-à-Gaz" site.

Proteobacteria are usually the dominant phylum in soils contaminated by heavy metals (Golebiewski et al., 2014; Bourceret et al., 2016). Contrary to previous studies, *Alphaproteobacteria* were present in a higher proportion than the other sub-divisions. They are usually in higher proportion in non-contaminated soils, while *Beta*- and *Gamma*- subdivisions are favored in hydrocarbon contaminated soils (Labbé et al., 2007). In spite of the pollution of the soil by PAHs, dioxins, furans and other complex organic molecules, the structure of *Proteobacteria* communities did not seem to be controlled by the organic contamination of the site.

The proportion of *Chloroflexi* reached 10 to 20% of the communities, in the same range as in the Zn and Pb-contaminated soil studied by Xu et al. (2017). *Chloroflexi* are usually main bacterial phyla in soils, but in lower proportion (less than 1%) on non-polluted sites (Zhalnina et al., 2015). The *Verrucomicrobia* phylum, representing only a few percent of the "Place-à-gaz" soil community, includes organisms that are tolerant to Zn and Pb (Xu et al., 2017).

The 15 most abundant bacterial OTUs included a high proportion of genera usually found in polluted or extremely acidic mining environments. The main genus, *Sphingomonas*, comprises some heterotrophic species commonly found on contaminated sites, including sites polluted by metals (Beattie et al., 2018). They are able to degrade aromatic compounds (Fredrickson et al., 1995 ; Zylstra and Kim, 1997) and carry functional genes, such as the *ars* genes, linked to As resistance (Macur et al., 2001 ; Escalante et al., 2009) and the *aioA* genes linked to As oxidation (Kinegam et al., 2008). Other major heterotrophic genera, such as *Pseudolabrys* and *Bryobacter*, may contribute to the degradation of organic molecules initially present or provided with the organic litter during the mesocosm experiment. The *Bryobacter* genus, comprising acid-tolerant species (Kulichevskaya et al., 2010), had already been found in association with *Sphingomonas* and *Bradyrhizobium* (Hong et al., 2015). A large decrease in the abundance of *Alkanibacter* – one of the major heterotrophic genera initially present in the

mesocosm, and whose members use alkanes as energy and C source (Friedrich and Lipski, 2008 ; Wu et al., 2017) – occurred between T0 and T8 at all levels of the mesocosm. This phenomenon may be linked to the sensitivity of the members of *Alkanibacter* to any change in environmental conditions, as species of this genus have been shown to be very difficult to cultivate and do not grow in classical rich media. They may have suffered from competition with more versatile heterotrophs when organic litter introduced fresh organic compounds into the mesocosm.

Amongst the major bacterial genera two of them, *Leptospirillum* and *Acidiferrobacter*, belong to the group of acidophilic organisms frequently associated with the iron (Fe) cycle on mining sites. The strictly aerobic organisms belonging to the *Leptospirillum* genus are characterized by their high affinity with ferrous iron, which they can oxidize even at very low concentrations (Norris et al., 1987). This characteristic, associated with a high resistance to metals and As (Li et al., 2010; Casas-Flores et al., 2015), could explain the presence of organisms belonging to the *Leptospirillum* genus in our system. Indeed, labile amorphous Fe oxide minerals were suspected of playing an important role in the dynamics of inorganic pollutants (Thouin et al., 2016), whereas dissolved Fe concentrations remained very low in the soil interstitial water of the mesocosm (Thouin et al. 2017). The second major chemolithotrophic acidophile genus, *Acidiferrobacter*, comprises facultative anaerobes that can use either molecular oxygen or ferric Fe as terminal electron acceptor, while both ferrous Fe and S can play the role of electron donor (Hallberg et al., 2011). However, as the technosol contains only 0.1% total S but nearly 8% total Fe, *Acidiferrobacter* may contribute more to Fe oxidation than to Fe reduction coupled to S oxidation, this last hypothesis being supported by the fact that the abundance of this genus tended to decrease in the water-saturated level of the mesocosm. Both *Leptospirillum* and *Acidiferrobacter* genera share with *Bradyrhizobium*, the other dominant genus, the potential ability to fix N_2 and thus contribute to the N cycle in the mesocosm (Thouin et al., 2017). Some *Bradyrhizobium* strains also carry the *aio*A gene (Sultana et al., 2012), while being potentially tolerant to Zn and Pb (Xu et al., 2017).

Candidatus solibacter and *Nitrospira* were the genera found most frequently on an electronic waste site polluted by metals (Wu et al., 2017). The species of these genera are resistant to heavy-metal toxicity (e.g. Cd, Cu, and Pb) and are predominant in metal-contaminated environments (Li et al., 2015; Nitzsche et al., 2015). Other studies have demonstrated that *Geothrix* bacteria can degrade toxic organic compounds such as polychlorinated biphenyls and polybrominated diphenyl ethers (Song et al., 2015), which normally co-exist with metals at electronic waste recycling sites (Robinson, 2009).

When comparing the identified genera most present in the "Place-à-gaz" technosol (Fig. 3) with the genera found to be most abundant in a non-polluted soil (Zhalnina et al., 2015), six common microorganisms stand out, corresponding to roughly 16% of the genera: *Bradyrhizobium* (dominant in both soils), *Sphingomonas*, *Opitutus*, *Candidatus Solibacter*, *Steroidobacter* and *Nitrospira*. Thus, these genera, although often found in polluted soils, are also amongst the common major micro-organisms found in non-polluted soils. Conversely, the presence of acidophilic genera involved in the Fe cycle among dominant OTUs may be a more specific feature of the "Place-à-Gaz" type of soil with elevated levels of metals and As concentrations.

Amongst the *Archaeae*, the *Thaumarchaea* lineage is composed of ammonia-oxidizing strains (Spang *et al.*, 2010). It has been demonstrated in soil microcosms that the toxic metal(loïd)s Cu, Zn, and As considerably reduce the abundance, composition, and activity of ammonia-oxidizing archaea (AOA; Mertens et al., 2009; Subrahmanyam et al., 2014). Furthermore, it seems that AOA were less impacted by PAH than by pH (Wu et al., 2016). At the phylum level, fungal communities included *Ascomycota*, *Basidomycota* and *Chytridiomycetes*. Similar fungal phylum diversity has been observed in multi-contaminated soils (Bourceret et al., 2016) but also in non-polluted soils (Buée et al., 2009).

4.2 Impact of soil saturation and organic matter on soil microorganisms during the mesocosm experiment

While the community structure at the phylum level was not deeply modified by the environmental changes imposed on the mesocosm during the 8 months experiments, significant changes were observed at the bacterial OTUs level. The Venn diagram showed specialization of bacterial communities at each level, and PCoA analysis showed the effects of water saturation and addition of organic matter.

Saturation, Flooding

Randle-Boggis et al. (2017) have shown that increasing flooding frequency alters microbial communities and functions of soils under laboratory conditions: flooding induced a decrease of *Actinobacteria* proportion, and an increase of the proportion of *Gemmatimonadetes* and *Acidobacteria*.

In our experiment, from amongst the six known genera whose proportions increased in the saturated levels, two were anaerobic: *Geothrix*, able to reduce Fe(III) (Coates et al., 1999), and *Opitutus*, that can reduce nitrate into nitrite (Chin et al., 2001). Such evolution is coherent with the evolution of the geochemistry of water and solids in the mesocosm. In fact, reduction of nitrate was correlated with the

saturation conditions (Thouin et al., 2017). In parallel, dissolution of the amorphous Fe oxide phase covering soil particles was observed in the permanently saturated H4 level (Thouin et al., 2018). Moreover, the dissolved Fe was assumed to be immediately re-precipitated, since the dissolved Fe concentration remained low. The information provided by the structure of the bacterial community seems to strengthen the hypothesis of anaerobic micro-environments being established in the saturated levels. In fact, both aerobic and anaerobic organisms were detected, and/or proliferated in the permanently saturated level. These could contribute to rapid transformation and recycling of chemical species such as Fe that could be reduced by *Geothrix* then re-oxidized by *Leptospirillum*, thereby inducing rapid dissolution and re-precipitation of Fe oxides. No incidence of dissolved oxygen depletion was found in the water sampled from the saturated zone (Thouin et al., 2017). The hypothesis of anaerobic micro-environments interacting with oxygen-containing water in saturated zones is supported by the increase in abundance of both aerobic (*Pseudolabrys*, Kämpfer et al., 2006) and anaerobic (*Opitutus*, Chin et al., 2001) genera. Moreover, OTUs of unclassified *Anaerolineaceae* were amongst the major organisms whose abundance clearly increased in the saturated levels and that probably exhibit anaerobic metabolisms: *Anaerolineaceae* are commonly found in oil reservoirs and oil-contaminated soils and may play a key role in the anaerobic degradation of long-chain alkanes (Liang et al., 2016). Soil saturation also impacted the structure of the bacterial communities with regard to As(III) oxidation: both 16S and *aio*A gene abundances increased in all levels of the mesocosm from T0 to T8. However, the *aio*A/16S gene ratio decreased, and this decline was particularly significant at the H4 level, which was permanently saturated. This result may be linked to the development of strictly anaerobic bacteria growing in micro-environments where As(III) oxidation cannot occur because of the absence of suitable electron acceptors. The saturation also induced a strong decrease in fungal abundance, as 18S rRNA gene concentration was too low to be quantifiable in the H3 and H4 levels at the end of the experiment.

Effect of organic matter

The addition of fragmented forest litter to the soil surface induced a clear shift in bacterial and fungal diversity, considered at the OTU level, in the upper level of the mesocosm. This phenomenon was probably directly linked to the movement of microorganisms from the litter to the soil during watering. For bacteria, the shift was mainly observed at the soil surface layer: it seemed that the selective pressure exerted by high pollutant concentrations, and the different edaphic parameters (soil saturation, available OM) prevented the development of microorganisms from the litter in the deeper zones of the mesocosm. Thouin et al. (2017) observed a peak of

CO_2 production in the mesocosm after the addition of fragmented litter able to supply heterotrophic microorganisms with fresh organic molecules. In fact, the bacterial abundance increased in all levels of the mesocosm between T0 and T8, and the proportion of many heterotrophic genera – including some of the most represented OTUs such as *Bryobacter*, *Pseudolabrys*, and *Sphingomonas* (SM.5) – increased in some or all levels of the mesocosm during the experiment. It is well known that supply of fresh organic matter to soils enhances activity and growth of previously starving microbial populations, thus inducing strong changes in the size and structure of microbial communities (Fontaines et al., 2003). Concerning the fungal diversity and composition more specifically, their evolution in the surface layer of the mesocosm was probably related to the addition of fragmented litter. Fungi play a key role in forest litter decomposition thanks to their ability to synthetize extracellular enzymes that are necessary to degrade organic polymers such as lignocellulose. A range of diverse fungi were shown to contribute to the successive steps of organic matter decomposition of the leaf litter (Vorikova and Baldrian, 2013). The contribution of litter therefore probably contributed to the large increase in abundance of fungal OTUs at the soil surface. Fungal enzymes may have contributed to the transformation of some of the complex organic pollutants inherited from the chemical weapons or the by-products of their burning, as previously observed (Hofmann et al., 2001). Furthermore, their decomposition activity has probably produced simple organic molecules, promoting the heterotrophic bacteria, particularly at the surface of the mesocosm.

Relationship between physico-chemical parameters and dominant bacteria

A close relationship between physico-chemical parameters and dominant bacteria was observed during the experiment (Fig. 6). The particular structure of the bacterial community of this soil was strongly linked to environmental parameters as characterized by the high concentrations of inorganic contaminants and DOC, and by the variations in conditions induced by soil saturation. The availability of organic molecules seemed to be a major factor determining bacterial community structure, as shown by the correlation between the TOC and the abundance of the dominant heterotrophic genus *Sphingomonas*. Conversely, the autotrophic dominant OTUs *Leptospirillum* and *Acidiferrobacter* – commonly found in mining environments (Garcia-Moyano et al., 2008; Hallberg et al., 2011) rich in metals and metalloids – seemed to correlate negatively with organic C and positively with Zn concentration. In parallel, the COIA suggested that the relationship between bacterial communities and environmental parameters was stronger in the saturated level than at other levels. The high moisture levels and chemical species present in reduced environments (As(III), NH_4^+) were, probably, positively

correlated with both anaerobic organisms (*Anaerolineaceae*) and aerobic bacteria (*Pseudolabrys*). The redox potential of sampled interstitial water never reached negative values and dissolved oxygen was always detected, even in the permanently saturated level (Thouin et al., 2017). These observations together with the geochemical and biological analyses are in agreement with the presence of anaerobic micro-environments in the saturated soil. The development of anoxic microsites allowing anaerobic microbial reactions in soils has been evidenced elsewhere (Silver et al., 1999). Here, such micro-heterogeneities may allow the simultaneous microbial cycling of As, Fe, N and C involving both aerobic/anaerobic and autotrophic/heterotrophic metabolisms.

5. Conclusion

The study of the soil microbial communities of the "Place-à-gaz" site where As-containing chemical weapons were burned revealed that extremely high levels of As, Zn, Pb and Cu concentrations were consistent with diverse bacterial and fungal assemblages, whereas no higher plant species had been able to grow in this zone since the polluting event. Dominant B*acteria* communities included genera usually found in non-polluted soils together with genera more commonly inhabiting extreme mining environments. The mesocosm experiment allowed the observation of significant shifts in bacterial and fungal communities when submitted to environmental changes such as litter or flooding, which could occur on-site on a time scale of a few months. The deposition of forest litter could strongly modify the microbial communities of the surface soil because of (1) direct spreading of organisms present in the litter, and (2) production of organic molecules resulting from its decomposition and diffusing into the soil. Furthermore, saturation of the soil by rain water may induce evolution of the bacterial diversity and of the bio-geochemistry of nutrients and pollutants (C, N, Fe, As) through the development of anaerobic micro-environments. These results highlight the importance of researches on the evolution of microbial communities and their response to environmental changes in order to better evaluate the fate of pollutants on sites affected by the destruction or storage of chemical weapons.

Acknowledgements

The authors wish to thank Daniel Hube (BRGM) who introduced the environmental issue and provided the polluted material. This work was co-funded by the Région Centre Val-de-Loire and BRGM (convention 00087485) and the Labex Voltaire (ANR-10-LABX-100-01). For the equipment we gratefully acknowledge the financial support provided to the PRIME-PIVOTS platform by the Région Centre – Val de Loire (ARD 2020 program and CPER 2015 -2020) and the French Ministry of Higher Education and Research (CPER 2015 -2020

and public service subsidy to BRGM). This operation is co-funded by European Union. Europe is committed to the Center-Val de Loire region with the European Regional Development Fund.

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