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Opportunities and risks of biofertilization for leek production in urban areas: Influence on both fungal diversity and human bioaccessibility of inorganic pollutants

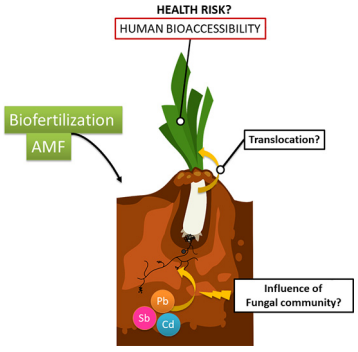
Antoine Pierart [a,*], Camille Dumat [b,c], Arthur QuyManh Maes [d], Christophe Roux [d], Nathalie Sejalon-Delmas [d,1]

[a] Ecolab, Université de Toulouse, CNRS, INPT, UPS, France
[b] CERTOP, CNRS-UT2J-UPS, France
[c] INP-ENSAT, Université de Toulouse, France
[d] LRSV, Université de Toulouse, UPS, CNRS, France

HIGHLIGHTS

- Biofertilization decreased AMF diversity associated with leek
- Changes in AMF community may have impacted TM phyto-uptake and bioaccessibility
- Sb and Pb phyto-uptake are both dependent on plant species and soil origin
- Human bioaccessibility of Sb increased with biofertilization, unlike Pb and Cd

GRAPHICAL ABSTRACT



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ABSTRACT

The influence of biofertilization with arbuscular mycorrhizal fungi (AMF) on trace metal and metalloids (TM) - Pb, Cd and Sb - uptake by leek (*Allium porrum* L.) grown in contaminated soils was investigated. The effect of biofertilization on human bioaccessibility of the TM in the plants was also examined. Leek were cultivated in one soil with geogenic TM sources and one soil with anthropogenic TM, to assess the influence of pollutant origin on soil-plant transfer. Leek were grown for six months on these contaminated soils, with and without a local AMF based biofertilizer. Fungal communities associated with leek roots were identified by high throughput sequencing (illumina Miseq®) metagenomic analysis. The TM compartmentation was studied using electron microscopy in plants tissues. In all the soils, biofertilization generated a loss of diversity favoring the AM fungal species *Rhizophagus irregularis*, which could explain the observed modification of metal transfer at the soil-AMF-plant interface. The human bioaccessibility of Sb increased in biofertilized treatments. Consequently, this latter result highlights a potential health risk of the use of this fertilization technique on contaminated soil since further field investigation is performed to better understand the mechanisms governing (1) the effect of AMF on TM bioaccessibility and (2) the evolution of AMF communities in contaminated soils.

* Corresponding author.
E-mail addresses: antoine.pierart@ensat.fr (A. Pierart), nathalie.delmas@lrsv.ups-tlse.fr (N. Sejalon-Delmas).
[1] LRSV, Laboratoire de recherche en sciences végétales, 24 chemin de Borderouge, 31,326 Castanet-Tolosan, France.

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1. Introduction

Trace metal(loid) (TM) is a generic term to gather elements naturally present at a low concentration in the environment (geochemical background). Metalloids are elements presenting properties in between those of metals and non-metals, as it is the case of antimony (Sb). Numerous human activities induce significant persistent TM contamination at the global scale by impacting the natural flows. This soil and water contamination is a major environmental issue and in particular, (*peri*)urban soils are widely polluted with cadmium (Cd) and lead (Pb) worldwide (Hu et al., 2014) and more recently with Sb (Krachler et al., 2005; Smichowski, 2008). Indeed, in the past decades, vehicle exhaust from lead gasoline combustion was a major source of Pb contamination (Robbins et al., 2010) and nowadays smelter areas are important sources of pollution (Zhuang et al., 2009). Cadmium accumulation in cultivated soils mostly comes from phosphate fertilizer usage (Schroeder and Balassa, 1963; Shahid et al., 2016). Antimony is a metalloid which normally occurs as a trace element in soils (He et al., 2012; Wilson et al., 2010) and is classified as a priority pollutant by the European Union (Filella et al., 2002) and the United States Environmental Protection Agency (USEPA, 2006). Although its concentration is highly variable worldwide, a 50% increase has been recorded in arctic snow in the last three decades, mainly from anthropogenic sources (Krachler et al., 2005). Smelting, mining and its presence in fire retardant mixtures are currently the most important sources of Sb. However, in the last decades, its presence in plant protection products manufacture and sewage sludge (Wagner et al., 2003; Edwards et al., 1995) has led to large-scale contamination of agricultural lands. In recent times, Sb has also been used in brake linings, lubricant and battery manufacturing (Fujiwara et al., 2011; Wiseman et al., 2013), leading to an increased risk near roads due to traffic dust. The recent conclusions of the ANSES (French National agency for food, environment and work safety) highlighted the need to strengthen research on these elements as their concentration in food increased between two sets of studies (2000–2004 and 2006–2010). These studies showed that in addition to an increase in Sb, Cd increased by up to 400% in the main food chain products, while Pb decreased a little but its concentrations remained of concern.

The worldwide economic crisis has driven more people to grow their own food in public, associative or kitchen gardens (Galt et al., 2014). However, these places are generally set-up in the vicinity of roads or industries or even directly in disused and not always cured industrial sites (Álvarez-Ayuso et al., 2012). In these strongly anthropogenic urban environments, soils and plants are consequently subjected to a high contamination risk through soil and amendment quality or airborne particles enriched with metal(loid)s (Leveque et al., 2013; Shahid et al., 2013).

Because food is the major exposure pathway for humans to environmental pollutants (Fries, 1995), metal(loid) transfer in the soil-plant-water system is often studied (Okkenhaug et al., 2011; Wan et al., 2013; Khan et al., 2016). Mycorrhizal symbiosis is the normal condition for approximately 85% of flowering plants (Brundrett et al., 1991). Both organisms benefit from this mutualistic exchange: arbuscular mycorrhizal fungi (AMF) transfer nutrients, mostly phosphorus, and can enhance stress tolerance (drought, pathogens…) (Li et al., 2013; Smith and Smith, 2011), while plants give back photosynthetic-based organic compounds to the fungus (Smith and Smith, 2011; Cazzato et al., 2012). The AMF are also known to be key actors in TM phytoavailability (Jarrah et al., 2014; Khan, 2005), while gardeners can already use them as bio-fertilizers (Kangwankraiphaisan et al., 2013). Pb behavior in the presence of mycorrhizal plants has been described (Jarrah et al., 2014), but has not yet been studied in leek, a widely cultivated vegetable (FranceAgriMer, 2013). Under metal(loid) stress, other leafy vegetables such as lettuce have been shown to accumulate high Pb levels, particularly in leaves (Beavington, 1975; Uzu et al., 2010; Xiong et al., 2014a). The relationship between Cd and AMF has been extensively studied for years, with either a decrease or an increase in accumulation depending on the fungal species, plant species and Cd concentration (Pierart, 2016). However, there are no reports about the human bioaccessible fraction of these TM in edible plants. As shown by Pierart et al., (2015), data about the relationship between Sb and AMF are very scattered. Different studies revealed Cd, Pb and Sb (eco)toxicity (Bech et al., 2012; Gebel et al., 1998; Kuroda et al., 1991; Pant et al., 2014; Winship, 1987). Both Pb and Sb have generally low solubility and bioavailability, while Cd is generally considered as highly mobile and bioavailable (Shahid et al., 2016). However, soil physico-chemical properties (i.e. organic matter, pH, Fe/Al oxides and hydroxides amounts) can affect these parameters (Xu et al., 2011; Diemar et al., 2009; Roper et al., 2012; Tighe et al., 2005; Xi et al., 2010; Ilgen and Trainor, 2012) as AMF can also influence them.

In that context, our study aims to evaluate the role of AMF fertilization in the phytoaccumulation of Cd, Pb and Sb depending on their origin (anthropic vs geogenic). Leek (*Allium porrum* L., Poireau de Saint Victor, Ferme de Sainte Marthe®) was chosen as a leafy vegetable model plant because it is commonly grown in urban gardens for fresh and cooked consumption (Guitart et al., 2014). The TM content analyses were performed in plant organs with the adapted extraction procedure (Okkenhaug et al., 2011). Furthermore, because the fungal community could influence metal(loid) fate at the soil-plant interface (Tonin et al., 2001), we used high throughput sequencing (illumina Miseq®) focused on a commonly studied ribosomal region, the Internal transcribed spacer (ITS) (Jansa et al., 2002; Schoch et al., 2012), to analyze its composition in leek roots.

2. Material and methods

2.1. Plant cultivation

2.1.1. Study site

Two polluted soils with similar Pb concentrations, but different contamination origins were used in the study (Table 1): (i) an urban garden sandy soil from West France (Nantes, annotated NTE: 47°16′1.512″N–1°34′29.688″O) with a high geochemical Pb anomaly, a moderate Sb anomaly and a very low Cd concentration and (ii) a peri-urban sandy-clay soil (Bazoches, annotated BZC: 48°11′21.375″N–2°3′28.993″O) in which Pb, Cd and Sb were shown to come from recent anthropic contamination (50 years) (Cecchi et al., 2008; Leveque, 2014).

2.1.2. Biofertilization solution

To prepare a biofertilizer composed of local AMF strains, a trap-crop system was set-up as follows: six months before the experiment, a first set of organic leek plants was grown to increase the natural AMF inoculum from the contaminated soils. This trap culture was used because of its well-known rusticity and good mycorrhizal potential. The plants were grown under greenhouse conditions (artificial lights turned on if brightness <2800 lx, 18/20 °C day/night – 50% relative humidity), in pots filled with a mix of both soils and sterilized oildri® (1:1:6/v:v:v) to lower the TM concentration. Spores were extracted from a soil

Table 1
Bazoches (BZC) and Nantes (NTE) soil characteristics. OM = organic matter content; $CaCO_3$ = calcium carbonate content; P_2O_5 = phosphate content; CEC = cation exchange capacity.

	pH_{H20}	OM (%)	[Pb] $mg.kg^{-1}$	[Sb] $mg.kg^{-1}$	[Cd] $mg.kg^{-1}$	Clay $g.kg^{-1}$	Silt $g.kg^{-1}$	Sand $g.kg^{-1}$	$CaCO_3$ $g.kg^{-1}$	P_2O_5 $mg.kg^{-1}$	CEC $cmol^{+}.kg^{-1}$
BZC	8.1	5.9	412	15,4	3	24.0	55.3	16.7	2.0	76.7	15.85
NTE	7.0	4.4	456	2,7	0.2	14.0	28.4	57.4	0.2	218	8.71

sample, by wet sieving (45 μm), suspended in 50% glycerol (v:v) and centrifuged (2500 rpm, 5 min). Then, the spore suspension for biofertilization treatments was obtained by extracting and rinsing the supernatant.

2.1.3. Leek cultivation

For the biofertilization condition (B), 9 mL of the spore suspension were added to the soil in each 2 L pot. Leek seeds were then sown in each pot containing soil from BZC or NTE; and placed under the same greenhouse conditions as described before. A fully randomized design was adopted to distribute any eventual greenhouse climatic heterogeneity among samples. A regulated dripper line watered the pots by capillarity, filling each saucer every three days, therefore limiting spores and metal(loid) lixiviation processes.

2.2. Sampling and analysis

Aerial plant parts were cut and rinsed with deionized water to remove any soil or atmospheric particles. Root fragments were sampled, rinsed with deionized water and stored at 4 °C for mycorrhization rate measurement and DNA sequencing. Plant parts were oven dried (40 °C/5 d), weighed and then crushed with an IKA A11 grinder. Soil samples were dried (20 °C, 10 d), crushed with a ceramic mortar and 250 μm sieved.

2.2.1. Mycorrhization rate

Observation and counting of AMF colonization (%M) was carried out following the root intersect method (Giovannetti and Mosse, 1980) after root bleaching and staining (Vierheilig et al., 1998).

2.2.2. Soil characterization

Water pH was measured following the standardized protocol NF-ISO-10390 (AFNOR. ISO 10390:2005, 2005); other soil physicochemical parameters were measured by INRA (Arras, France) for NTE soil and by Cecchi (Cecchi, 2008) for BZC soil.

2.3. Metal analysis

2.3.1. Total metal content

Leaves and roots powders were digested in Digiprep® tubes following the procedure described by Foucault et al., (2013) and then filtrated on a 0.45 μm membrane before analysis with Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES – Thermo® Electron IRIS Intrepid II) or Mass Spectrometry (ICP-MS – PerkinElmer® Agilent 7500ce) in function of concentration.

2.3.2. Bioaccessible fraction

The human bioaccessible fraction of metal(loid)s was measured using the adapted Unified Barge Method (Denys et al., 2012). This test is a three step extraction protocol which simulates the chemical processes occurring in the mouth, stomach and intestinal compartments during the consumption of vegetables with freshly prepared synthetic digestive solutions (Wragg et al., 2011). We focused here on the first two steps of the extraction (mouth and stomach) as the last one is barely involved in TM extraction (Denys et al., 2012). The bioaccessible fraction was calculated as the ratio between bioaccessible TM and total TM in plant organ.

Samples were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS – PerkinElmer® Agilent_7500ce). Apple leaves (SRM-1515) were used as certified material for plant sample analysis. In our conditions, Cd, Pb and Sb recovery were respectively ~82%, ~78% and ~67%, which is in the range of previous analysis under similar acid digestion, as reported in Table 2. To study metalloid soil-plant transfer, two ratios were calculated (Pérez-Sirvent et al., 2012; Xiong et al., 2014b): the bioaccumulation Factor (BF) and the translocation factor (TF) which are respectively $[Metal]_{total\_plant}/[Metal]_{total\_soil}$ and $[Metal]_{leaf}/[Metal]_{root}$. Therefore, BF is representative of metalloid phytoaccumulation and TF shows the inside-plant transfer storage in organs.

Table 2
Metalloid recovery rate. Average ± Standard error, %.

Element	Recovery rate, %	
	Apple leave (SRM-1515)	Recovery rate (IAEA–336) (Agnan, 2013)
Pb	78 ± 0.6	81 ± 9
Sb	67 ± 2.4	66 ± 20
Cd	82 ± 2.5	84 ± 8

It is possible to estimate whether or not a health risk exists by comparing the Tolerable Daily Intake, TDI ($\mu g_{TM} \cdot kg_{BW} \cdot d^{-1}$) for each element and the ratio DI/62 (with DI the Daily Intake in $\mu g_{TM} \cdot d^{-1}$ and 62 being the average human body weight in kg) for a fixed DC ($\mu g_{vegetable} \cdot d^{-1}$) (Eq. (1)).

$$DI = [pollut]_{veg} \times DC \quad (1)$$

2.4. Metalloid localization

Scanning Electron Microscopy coupled with X-Ray analysis in low vacuum (ESEM Quanta 250 FEG; with Platine and cryo PP3000T module - Quorum; X-ray analysis Edax - APEX 2i; CMEAB, Toulouse, France) was used to evaluate Pb and Sb localization in plant cells and fungal structures in fresh leeks root fragments: samples were fixed with tissue-Tek (Tissue-Tek® O.C.T. Compound, Sakura® Finetek) on a microscope rack and frozen in pasty nitrogen (−210 °C) under vacuum conditions (<1 Torr). Before observation, samples were placed into the microscope low-vacuum preparation room to be cryo-fractured (to reveal inside intact structures). The final steps then consisted of (1) sublimation (10 min) to vaporize the water and (2) platinum covering (5–10 nm).

2.5. Fungal community identification

2.5.1. DNA extraction and sequencing

Leek root fragments were frozen in liquid nitrogen and crushed with a ball-point crusher before fungal DNA extraction using the PLANT KAPA 3G® Kit (Kapa Biosystems). To identify the fungal community in the biofertilizer solution, microbial DNA was extracted from the spore suspension using the DNeasy® Plant Mini kit (Quiagen) with pre-steps: (1) spore isolation; (2) Water take-out through deposition of the spore suspension on a sterile membrane and vacuum depression; (3) Spore crushing in Fastprep 2 mL tubes containing silica fragments and ceramic balls by vortexing (15 s each) the tubes five times after freezing in liquid nitrogen. DNA extracts were diluted 10× in DNA-free water to limit PCR inhibition.

2.5.2. Primer choice

A fungal amplicons library was obtained with ITS3tagmix4-ITS4ngs primer pair on the ITS2 region (Internal Transcribed Spacer) as these primers gave good matching for Glomeromycota (Tedersoo et al., 2015) (CATCGATGAAGAACGTAG - TCCTSCGCTTATTGATATGC), but also matched with plants. The primer pair was barcoded with a unique (sample-specific) error-correcting 10–12 bases barcode for Illumina MiSeq.

2.5.3. Amplification cycle

All amplifications were performed in twofold 25 μL reactions, with 2.5 μL of sample DNA extracts and 22.5 μL of a PCR mix composed of: 1 μL of each primer (10 $pmol \cdot \mu L^{-1}$), 12.5 μL of KAPA 3G Buffer mix containing DNTPs, 0.4 μL of Taq DNA polymerase (2.5 U) and 7,6 μL of DNA-free H_2O. The amplification cycles were carried out following the KAPA 3G protocol. Three independent PCR amplicons for each sample were combined to construct PCR libraries.

2.5.4. Sequencing mix preparation

Samples were processed at the GET-PlaGe platform (INRA, Auzeville, France) for: (1) DNA purification, (2) second PCR with specific Illumina Miseq primers containing indexes, (3) equimolar (based on DNA quantity) pool preparation and (4) Illumina MiSeq sequencing. Sequences are available on Genbank (INSDC) from number MG670787 to MG671929. Raw data are available on SRA (Sequence Read Archive).

2.5.5. Sequence analysis

The quality of MiSeq sequences was analyzed (pairing, primer trimming, chimera removal) with an optimized protocol based on MOTHUR softwares (Schloss et al., 2009). The 97% OTU clustering (Operational Taxonomic Unit) was carried out with an enhanced protocol based on UPARSE (Edgar, 2013). Sequences were trimmed on ITS2 with HMMscan (Bengtsson-Palme et al., 2013). Taxonomy and statistical analysis were obtained with a personal pipeline named EGRETTA. The pipeline uses a custom enhanced database which contains fungal sequences (Glomeromycota, Ascomycota…) and contaminants (Streptophyta, Nematoda…) mostly from the INSDC (International Nucleotide Sequence Database Collaboration), and Miseq sequencing from collections. Alpha-diversity metrics were estimated by specific richness ($Chao_1$) and the Specific entropy (H′, $Shannon_{10}$ index) index calculation. Fungal community composition were obtained by Weighted Unifrac matrix calculation (Lozupone et al., 2011).

2.6. Statistical analysis

After verification of data normality and log-transformation when necessary, general linear models were applied on dataset with a significance level of 5% using the SAS-JMP 11 Software. Significant groups were determined using a post-hoc Student test. As the experiment was carried out on a small number of replicates ($n = 5$), a non-parametric range-based statistical test (Kruskal-Wallis one-way ANOVA) was also calculated with SPSSv23 (IBM Statistics®) to strengthen confidence in GLM significance.

3. Results

Leek plants were cultivated on TM contaminated soils. Half of the plants received no fertilization (control; CTR) while in the other half, local AMF were added as biofertilizer to assess their effect on the soil-plant transfer of TM and their human bioaccessibility in edible organs. Plants were harvested after 25 weeks of cultivation to compare Cd, Sb and Pb accumulation in edible parts. Leek roots were used to study the fungal community and metal compartmentation on considered samples.

3.1. Biofertilization effects on plant growth and accumulation

3.1.1. Effect of biofertilization on plant growth

The aerial biomass and mycorrhizal rate for the leek plants are reported in Table 3. Biofertilization had no general influence on these two parameters. In addition, TM stress did not cause any visible phytotoxic symptoms.

3.1.2. Cadmium, lead and antimony phyto-uptake

3.1.2.1. Plant content and organ accumulation. After harvesting and sample preparation, metalloid concentrations were measured in plant organs as reported in Fig. 1. For Cd (Fig. 1.A. and 1.D), biofertilization led to significantly contrasted results in roots, with a decrease in BZC soil but an increase in NTE soil. However, the biofertilization treatment did not have an effect on either the Cd translocation factor or Cd accumulation in leaves.

Biofertilization did not significantly affect Pb accumulation (Fig. 1.B. and 1.E) at the plant scale in either soil. However, this treatment did significantly increase Pb in leaves in BZC soil. This result was associated with a change in the TF from root to leaves (TF_{Pb}). Indeed, even if TF_{Pb} was generally low in all treatments (Table 3), the response varied between soils with an increasing trend in TF_{Pb} in BZC biofertilized soils was observed, while the same treatment had no effect in NTE.

Regarding Sb phytoaccumulation in leek (Fig. 1.C), biofertilization decreased Sb in leaves, with significant results only in BZC soil (*t*-test, P value = 0.0059). Sb transfer from root to leaves was almost complete in the two tested soils, as no measurable Sb was found in root organs in either soil, while measurable concentration of Sb were detected in leaves.

3.1.2.2. Bioaccumulation of TM from soil to plant. The Bioaccumulation Factor (BF) results are shown in Table 3. These represent the plant's ability to accumulate TM from soils. Under our conditions, biofertilization did not affect this ratio for Pb and Sb. However, it significantly increased the BF_{Cd} in NTE biofertilized soil but not in BZC soil.

3.1.3. Human health risk assessment

Human bioaccessibility was estimated for the edible part of the plants to assess the health risk of eating these crops (Fig. 1-F). Our results showed that a significant fraction of Cd in leek leaves is gastric bioaccessible (85%), while Pb was generally significantly less bioaccessible (~60%). Biofertilization had no effect on these two fractions. Antimony bioaccessibility was lower than the latter, ranging between 15 and 33%. The biofertilization treatment significantly increased the Sb bioaccessible fraction in leeks cultivated in NTE soil (+13%). In BZC soil, an increase (+5.5%) also appeared to occur, however the results were not significant.

3.1.4. TM compartmentalization in plants

Scanning electron microscopy observations of root tissues were performed to study TM compartmentalization and transfer within the plant in relation to the culture conditions. As shown in Fig. 2-A, TM were detected in both fungal arbuscules within cells (Hu et al., 2014) and colonized cells (Krachler et al., 2005). When measurements were performed in cells from the central cylinder (Fig. 2-B), Pb and Cd were still detected but at a lower intensity, while Sb levels were not above the background (Smichowski, 2008). This decrease observed for Pb after the Casparian, an endodermal hydrophobic suberin layer, strip suggests that this element is efficiently blocked by this physical barrier, which is consistent with the low TF_{Pb} from roots to leaves mentioned above (Section 3.1.1).

Table 3
BZC and NTE soil physicochemical data (pH, % OM) and plant parameters (mycorrhizal rate-%MYC, biomass, translocation and bioaccumulation factors TF and BF respectively). CTR: control, B: biofertilized. Statistical analyses were performed within each treatment. Comparison in columns by soil, different letters indicate significant groups at $\alpha = 5\%$. No letters indicate no significance.

		% MYC	pH	% OM	Leaf $_{DW}$, g	Root $_{DW}$, g	TF Pb	BF Pb	TF Sb	BF Sb	TF Cd	BF Cd
NTE	CTR	39.8 ± 5.1***b***	7.1 ± 0.4	4.4 ± 0.1	1.8 ± 0.5	0.6 ± 0.2	0.02 ± 0.004	0.05 ± 0.02	Max[a]	0.17 ± 0.06	0.15 ± 0.01	0.9 ± 0.2***b***
	B	53.6 ± 5.9***a***	7.9 ± 0.1	3.9 ± 0	1.6 ± 0.5	0.3 ± 0.1	0.02 ± 0.02	0.06 ± 0.02	Max	0.13 ± 0.04	0.14 ± 0.05	1.5 ± 0.1***a***
BZC	CTR	42.7 ± 1.2	7.9 ± 0.02	5.9 ± 0.02	1.8 ± 0.3	0.9 ± 0.3	0.025 ± 0.004	0.041 ± 0.002	Max	0.024 ± 0.003	0.3 ± 0.1	3.1 ± 0.2
	B	53.3 ± 10.2	7.8 ± 0.02	6 ± 0.05	2.2 ± 0.6	1.3 ± 0.3	0.038 ± 0.01	0.045 ± 0.01	Max	0.017 ± 0.002	0.5 ± 0.1	2.7 ± 0.3

[a] Max means no Sb found in roots, implying a subsequent complete root-to-leave transfer.

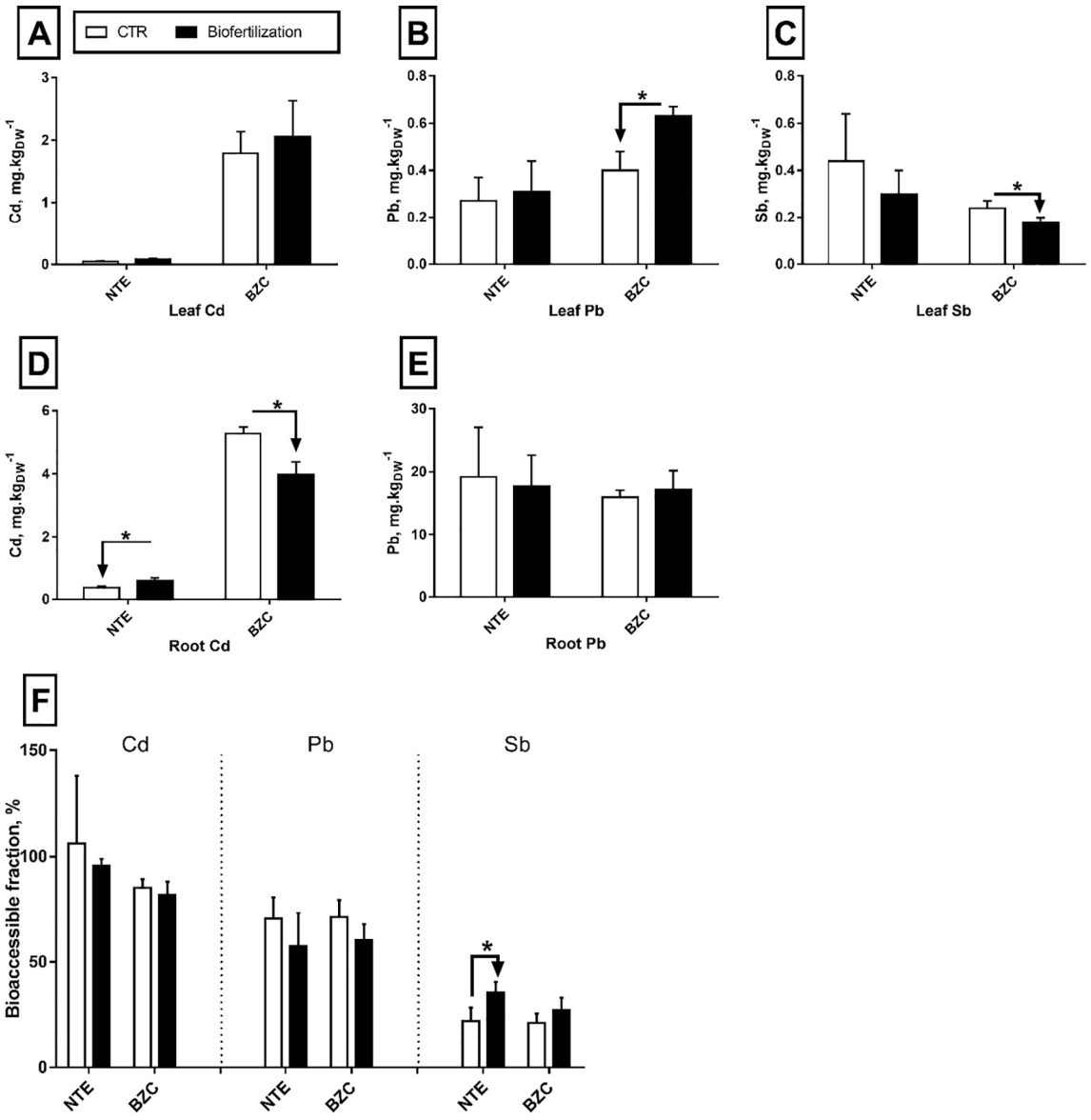

Fig. 1. TM accumulation ($mg.kg_{DW}^{-1}$) in leek leaves [A-Cd, B-Pb, C-Sb] and roots [D-Cd, E-Pb] in natural (CTR) and biofertilized soil (Biofertilization). The bioaccessible fraction of each metalloid [F] is expressed as the ratio between the bioaccessible TM concentration and the total TM concentration. Sb was under the detection limit in leek roots. Significant differences are highlighted with * (*t*-test, $\alpha = 5$).

3.2. Influence of the biofertilization on the fungal community

3.2.1. AMF community richness

High throughput sequencing and metagenomics were used to identify fungal communities present in the biofertilization suspension (BS). Among 2072 OTUs identified, 615 corresponded to Glomeromycota (Table 4). They were mainly composed of a pool of Glomerales species (85.2%) including mostly the genera Rhizophagus (25.4%), Funneliformis (19.6%), Claroideoglomus (10.7%), and unidentified Glomeraceae taxa (14.3%).

Miseq analysis performed on leek roots generated 22,000 OTUs. After taxonomic assignation and extraction of contaminant OTUs (mostly plants) 228 Glomeromycota OTUs remained. BZC_B and NTE_CTR were mostly characterized by the presence of Diversispora *sp*., *F. mosseae* and *R. clarus*. Biofertilized conditions contained more *R. irregularis* OTUs with the highest number in NTE_B (87%). NTE conditions showed the presence of *F. mosseae* and Claroideoglomus *sp*.

The Chao1 (S, Specific richness) and the Shannon10 (H′, Specific Entropy) indices both showed the same trend (Fig. 3) that biofertilization led to a loss of diversity in NTE roots (35 versus 54) but not in BZC (53 versus 33). Moreover, their comparison (by using the formula: $H'/\log_{10}(S)$) showed that BZC_B and NTE_CTR have a lower evenness than BZC_CTR and NTE_B. Even if they both correspond to a higher number of species, some core AMF remains dominant in the pool.

The precise taxonomic attribution allowed confirming statistical analysis. Leek roots communities were composed in average of 46% of *Rhizophagus irregularis* (= *Rhizoglomus irregulare*, *Ri*); 5% of *Funneliformis mosseae* (*Fm*), 3.5% of Claroideoglomus species, 1.5% of Diversispora species and 14.5% of unidentified Glomeraceae taxa. Glomeraceae are in a broad majority with an average of 29.75 OTU for 1.25 Claroideoglomeraceae OTU and 0.5 Diversisporaceae OTU.

3.2.2. Comparison of AMF communities composition

BZC_B and NTE_CTR were mostly characterized by the presence of Diversispora species and Fm (Table 4). Biofertilized conditions

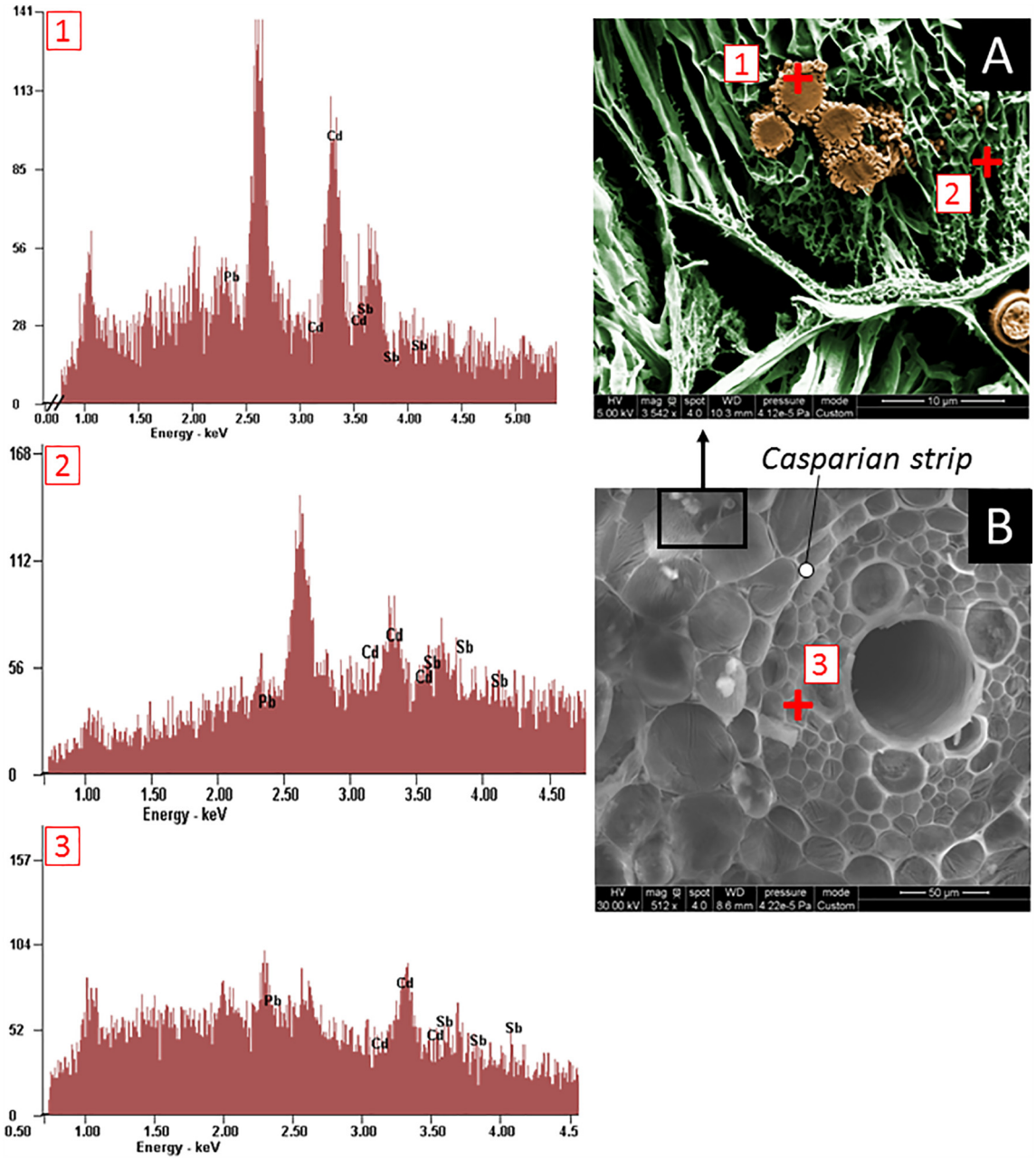

Fig. 2. Scanning Electron microscopy coupled with X-ray analysis of mycorrhizal leek roots. Pb and Sb measurement in (Hu et al., 2014) arbuscule, (Krachler et al., 2005) infected cells and (Smichowski, 2008) the central cylinder, beyond the Casparian strip. A, colored picture of a mycorrhizal cell (green), with the presence of fungal structures (orange): arbuscule and hyphae. B, cross section of leek root showing the central cylinder and nearby cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

contained more *Ri* OTUs with the highest number in NTE_B (87%). NTE conditions showed the presence of Claroideoglomus species. However, Principal Coordinate Analysis (ACoP) and hierarchical clustering based on WUnifrac distances between AMF communities showed that the biofertilized conditions were closer to each other and to the BS than the controls (Fig. 4A and B). Also, BZC conditions were closer to the BS than NTE. They were well clustered together. We also found that fungal communities are grouped considering their origin (Fig. 4B). This indicates that the fungal diversity is mainly driven by soil origin, but also that the addition of BS shifted these communities.

Ambispora, Archaeospora, Scutellospora and Paraglomus species only characterized the BS and were not found in leek roots. Those matrices gave phylogenetic distances, which are more decisive than taxonomic analyses notwithstanding that 55% of the variation was explained by Coordinates 1 and 2.

4. Discussion

4.1. Changes in trace metal(loid) phyto-uptake and compartmentalization

4.1.1. Antimony

Previous studies focused on Sb accumulation in leafy vegetables such as leek from contaminated soils are scattered (Kouimtzis et al., 1992). It has been shown that its accumulation in edible plants is highly variable and some vegetables such as spinach can be hyperaccumulators (Pierart et al., 2015). Kouimtzis et al., (1992) showed that compared to lettuce, leek appeared not to be a hyperaccumulator (~0.2 mg·kg_{DW}^{-1}), which is consistent with our results, although our range of concentrations was higher but it could be explained by our different experimental conditions. Indeed, the experimental period (5 months) and the pot cultivation, where the root

Table 4
Relative abundance of AMF taxa in the biofertilization solution (BS) and leek roots. Effects of BS on OTU appearance and disappearance, highlighted in green or red respectively.

Fungal taxa	OTU abundance (%)				
	CP	BZC B	BZC CTR	NTE B	NTE CTR
Ambispora sp.	**2.2**	**0**	**0**	**0**	**0**
Ambispora fennica	1.3				
Ambispora gerdemannii	0.9				
Archaeospora sp.	**0.9**	**0**	**0**	**0**	**0**
Claroideoglomus sp.	**10.7**	**0**	**0**	**4.3**	**9.8**
Claroideoglomus claroideum	1.8				
Claroideoglomus luteum					7.3
Claroideoglomus walkeri	8.9			4.3	
Diversispora sp.	**10.3**	**2.8**	**0**	**0**	**2.4**
Diversispora celata	0.4				
Diversispora eburnea	2.7				
Diversispora insculpta	0.9				2.4
Scutellospora sp.	**1.3**	**0**	**0**	**0**	**0**
Scutellospora coralloidea	1.3				
Dominikia sp.	**3.6**	**0**	**0**	**0**	**0**
Dominikia duoreactiva	0.9				
Funneliformis sp.	**19.6**	**5.6**	**0**	**0**	**14.6**
Funneliformis mosseae	19.2	5.6			14.6
Rhizophagus sp.	**25.4**	**66.7**	**50**	**87**	**63.4**
Rhizophagus clarus					7.3
Rhizophagus intraradices	12.1	8.3	11.5		
Rhizophagus irregularis	10.3	50.0	34.6	56.5	43.9
Septoglomus sp.	**4.5**	**5.6**	**0**	**0**	**0**
Septoglomus viscosum	4.5	5.6			
Paraglomus sp.	**0.4**	**0**	**0**	**0**	**0**
Glomus sp.	**7.1**	**2.8**	**11.5**	**8.7**	**7.3**
Glomus cubense	0.9				
Unidentified genus	**14.3**	**16.7**	**38.5**	**0**	**2.4**
Glomeraceae sp	14.3	16.7	38.5		2.4

Bold corresponds to the gender level of identification while non-bold refers to the species level.

system grew in the same Pb/Sb-rich soil all throughout the experiment, are in contrast to natural conditions where it could grow deeper in less contaminated soil horizons (as anthropic contamination usually shows a decreasing pattern of TM from the surface to deeper soil). Regarding these accumulations, emerging Sb contamination should be carefully monitored in (peri)urban areas where numerous new urban agriculture projects are being considered nowadays.

4.1.2. Lead

Leek accumulated Pb mainly in root tissues, and showed a low TF_{Pb} to leaves (0.02). Michalska and Asp, (2001) found a similar pattern of root accumulation in another leafy vegetable (lettuce) cultivated under hydroponic conditions. In addition, these authors highlighted that Pb phytoaccumulation variations could occur at the cultivar scale, suggesting the same possibility for leek. In their conditions, TF_{Pb} was very low (0.005 to 0.2 depending on the cultivar) compared with our results where 0.2 was the average TF_{Pb} in both soils. Such variation could be explained by the plant species studied or the experimental conditions (hydropony vs natural soil), suggesting the importance of strengthening these studies with field experiments.

4.1.3. Cadmium

Leafy vegetables easily accumulate Cd from the environment (Zorrig and El Khouni, 2013). Its phytoavailability appears to be relatively high compared with other currently observed metals in urban areas such as lead (Beesley et al., 2010) and our results confirmed previous studies.

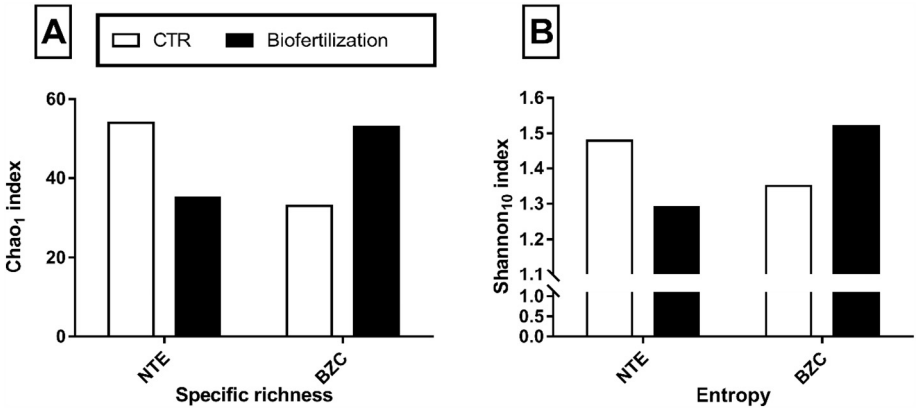

Fig. 3. Specific richness (A) and Specific Entropy (B) of AMF community in control and biofertilized roots. Measured by $Chao_1$ and $Shannon_{10}$ indices, respectively.

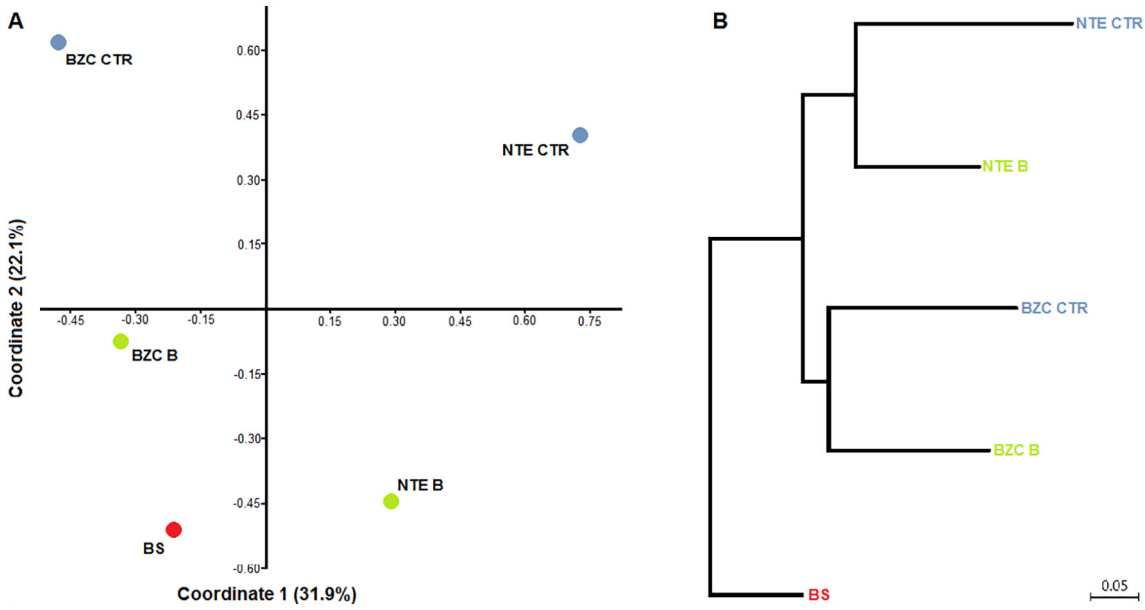

Fig. 4. Principal coordinate analysis (A) and NJ hierarchical clustering (B) with WUnifrac distances of AM community. Red = Biofertilization solution (BS), Green = Biofertilized conditions (B) and Blue = Control conditions (CTR). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In addition, the results of Cd compartmentation are in agreement with the fact that Cd is known to be internalized from the plant apoplast into the symplast, most likely through Zn, Fe or Ca transporters.

4.1.3.1. Influence of biofertilization on the fungal community and TM accumulation. The role of AMF biofertilization in TM phytoaccumulation in (*peri*)urban areas was investigated here for the first time in relation to human bioaccessibility using the Unified BARGE method.

Along with plants, microorganisms from the rhizosphere can exudate organic ligands and affect soil pH (Hinsinger et al., 2003; Wang et al., 2006) and redox soil potential, leading to a change in TM speciation and mobility (Lin et al., 2004). However, the mechanisms of metal stress alleviation are numerous and plant/fungal dependent (Hassan et al., 2013; Gu et al., 2013; Wei et al., 2016). Indeed, AMF fungi are known to play a major role in metal speciation and transfer at the soil-plant interface (Amir et al., 2014; Sharma and Sharma, 2013).

Our results for Cd accumulation are consistent with data obtained in Cd spiking studies (Aloui, 2009). In this thesis, the author found that mycorrhized plants accumulated more Cd than non-mycorrhized plants, through the activation of stress alleviation metabolic pathways. Furthermore, an isolated metal-tolerant AMF (*Funneliformis mosseae*) was shown to passively adsorb up to 0.5 $mg_{Cd} \cdot mg^{-1}{}_{soil}$ (Joner et al., 2000). This study also highlighted other Cd stress alleviation mechanisms such as synthesis of organic acids (malic, oxalic, and citric) which lowered the mycorrhizospheric pH, leading to either an increased mobility or a precipitation of Cd (Garg and Bhandari, 2013). However, contrasting results were also previously obtained with mycorrhized tobacco plants (Janoušková et al., 2005), which supports the consensus that fungal uptake is strain dependent (Liu et al., 2014). These differences in AMF-related TM accumulation could be due to: (i) the presence of glomalin, a largely studied fungal protein exudate from AMF, which can bind high amounts of TM and other pollutants in soil(González-Chávez et al., 2004) changing TM speciation and behavior, or (ii) a difference in the fungal and bacterial community as the fate of heavy metals at the soil-plant interface was previously shown to vary with the fungal species (Hassan et al., 2013; Zhang et al., 2015).

Commercial biofertilizers are generally monospecific, with an efficient fungal strain selected for its mycorrhization rate and P solubilization (leading *in fine* to potential increased biomass production). However, as shown by our biofertilization solution, a loss of diversity generally occurs even by using a solution with multiple strains as a fertilizer, favoring *Rhizophagus* species (*irregularis* and others). A similar loss in diversity occurred in soil treated with organic manure (Pierart, 2016; Chen et al., 2016). In contrast, a study focused on the effect of compost on soil microbial diversity did not observe a loss in diversity of AMF fungi in soil when they used spore counting and morphology to identify the fungi (Viti et al., 2010). The difference between these results and ours could come from the different approach (morphological identification versus next generation sequencing), but it could also show that spores diversity in soils and expressed AMF diversity in roots could shift under different time scales. Our analyses showed that studied TM is concentrated differently in BZC and NTE soils. In short term, it probably changes the different AMF strains biochemical response. But it's important to consider that the diversity of the initial community in leek roots before the biofertilization is the result of an ecological succession process. Furthermore, those soils have different history; since NTE is geogenically contaminated while BZC soil is the result of a recent anthropogenic pollution:

Ecological successions have already been theorized for plant communities (Lepart and Escarre, 1983) facing biotic/abiotic disturbances and stresses. “Intermediate” disturbances play a part in the increasing of community diversity by maintaining it in a “nonequilibrium state”, while rare and intense disturbances may induce a radical collapse of diversity (Connell, 1978; Wilkinson, 1999). Communities' reaction to this biotic and abiotic environment depends on their biology, and different TMs are known to produce very specific responses leading to the formation of continental islands (Jaffré et al., 1995).

The NTE_CTR condition presents the highest diversity and the closest community to the BS, with dominant ubiquitous fungi and numerous uncommon species. It might have fulfilled a succession process leaded for ages by TM pressure. The BZC_CTR presents an undiversified community where identified fungi were mainly ubiquitous, usually considered as pioneer, with very fast cycling species such as Ri. Besides TM effects on AMF, the succession process could be too recent or avoided by agricultural practices at BZC. Those communities' diversity and composition set their resilience against biofertilization, which could be considered as an anthropogenic biotic perturbation. It was shown that Diversisporaceae and Claroideoglomeraceae species have a longer life cycle, produce low biomass inside roots and sporulate much less than

Rhizophagus species which can penalized themselves during a competition (Maherali and Klironomos, 2007). Rhizophagus species are very good competitors because (i) their mycorrhizal rate is usually high and fast, (ii) their sporulation is tremendous compared to other species, and (iii) they can anastomose and efficiently re-spread from decaying roots (Smith and Read, 2010). They are very resistant to several typical agricultural disturbances like ploughing and plant exportation from fields (Peyret-Guzzon et al., 2016) but less specialized in dealing with heavy metals as their effect greatly vary depending on the TM considered (Pierart, 2016). The sudden introduction of many competitors in the NTE highly-specialized community could lead to a collapse of its biodiversity by eliminating very rare non-glomeraceae individuals. *Ri* could have taken advantage of the disturbance to increase dramatically its development. At BZC, the biofertilization could act like a biotic intermediate disturbance and new species are added to the existing community. Accordingly, the B community is closer to the BS than control and diversity is higher. It can be assumed that the final community would better deal with TM contamination with raising ecosystem-services quantity. However, it can't be assert that those strains would be able to persist in time.

AMF secondary succession has been under-explored for different type of disturbances like tillage, fire and flood (Piotrowski and Rillig, 2008). These results highlight the need to dig further the theme of fungal community evolution after a biofertilization as the final community is unlikely predictable, so are its biochemical functions.

A recent long term field study (8 years) based on cloning and genome sequencing showed an increase in AMF diversity associated with plants when organic amendments were applied (del Montiel-Rozas et al., 2016) suggesting that (1) long term exposure could alleviate the loss of diversity observed in the short term; (2) the diversity observed in pot experiments is biased and does not represent the whole fungal diversity (Sýkorová et al., 2007) and (3) the diversity shift could be mainly driven by both the characteristics of the product applied to the soil and the succession path of the local community. Regardless, the diversity loss observed here could explain the changes in TM accumulation and bioaccessibility:

Rhizophagus irregularis has been shown to enhance metal (Cd) transfer from low level contaminated soil to plant (Hassan et al., 2013). However, the disappearance of *F. mosseae* in NTE soil when biofertilized did not lead to an increase in Pb in plants, but a decreasing trend for both Pb and Sb. This could highlight that TM uptake or binding by fungi is metal dependent. For Sb, Wei et al., (2016) showed that inoculation with *F. mosseae* led to an increased phytoaccumulation, which was also suggested in a former study performed under hydroponic conditions (Pierart et al., 2017).

This could suggest that among the Glomeraceae and *Rhizophagus* sp. which increased with biofertilization treatment, one (or more) might be good candidate for phyto-stabilization of metals in soil. Unfortunately, we were unable to precisely identify these species due to a lack of data in ITS AMF databases (Schoch et al., 2014).

In addition, Next generation sequencing techniques are generating a revolution in community analysis thanks to the depth of analysis and the quantity of sequences amplified. Although it was previously shown that ribosomal ITS regions are variable among the fungal kingdom (Simon and Weiß, 2008; Nilsson et al., 2008), this region remains a good DNA barcode (Schoch et al., 2012). It is estimated that the diversity of AMF could reach 1600 species (Öpik et al., 2013; Sudová et al., 2015; Pagano et al., 2016), underlying the necessity to develop studies to isolate and describe new species of these organisms, as they can be used as biofertilizers both in agriculture for yield improvement and contaminated areas (Kangwankraiphaisan et al., 2013) for phytoextraction or phytostabilization. The main limitation of these approaches comes from the lack of precise databases. Indeed, while these techniques reveal an extreme diversity as shown by the number of OTU identified, most of the sequences were only identified at the family or genus level (Table 4) while their functional diversity when faced with TM contamination is at least species specific (Chen et al., 2007; Hassan et al., 2013).

4.2. Health risk assessment

Urban areas have been intensively studied during the last decade both out of economic need and due to the growing interest of such places for growing food with a low ecological footprint (Martellozzo et al., 2014). The evaluation of health risk is of major concern in these high population areas, as food is considered as the main source of TM ingestion (Mansour et al., 2009). The Unified Barge Method is used to estimate the human bioaccessible fraction of TM in food through the use of bio-chemical synthetic solutions mimicking human digestion. However, as bioaccessibility tests represent the fraction of metal digested and solubilized in the lumen, it might be possible that the intestinal epithelium also blocks some of these compounds, leading to a lower concentration in blood than in the digestive tract.

The elevated bioaccessible fraction in plants cultivated in BZC soil is consistent, and in the same range as previous measurements on samples from the same study site (Xiong et al., 2014a).

Our findings cannot be used to infer a general rule concerning the consumption of leafy vegetables cultivated in contaminated soils. However, we demonstrated that leek cultivation should be avoided in TM contaminated soils.

AMF biofertilization did not affect the human Cd bioaccessibility in leek, but was found to be generally high (80–100%), which is consistent with previous studies (Xiong et al., 2014b). For example, another experiment performed near the BZC soil area revealed that two leafy vegetables (lettuce and leek) cultivated in urban gardens contained up to 0.7 $mg \cdot kg^{-1}_{DW}$ Cd, with about 80% bioaccessibility (Mombo et al., 2016).

Lead bioaccessibility was also not affected by AMF biofertilization. Its value was consistent with previous studies which found that approximately 60% of Pb was bioaccessible in vegetables from a field study in China (Xiong et al., 2016).

In the present study we found for the first time that AMF biofertilization can have a significant impact on Sb bioaccessibility in leek, growing in a geogenic contaminated soil. These results strengthen similar patterns observed in hydroponic mycorrhized lettuces spiked with various Sb chemical species (Pierart et al., 2017).

Additionally, due to the lack of certified material for the BARGE method, we do not have a reference for the recovery rate. Thus, comparison of data obtained here with the published literature was not possible. The development of these certified materials for bioaccessibility extraction methods would allow the focus to be put on bioaccessible concentrations, with the final aim of defining more representative legal thresholds for metals in edible rather than total concentration per kg dry weight, as is currently the case.

The Daily Intake (DI, Table 5) was calculated using Eq. (1). The Kantar Wordpanel survey performed in 2011 (FranceAgriMer, 2013) estimates a daily consumption of leek of about 3.6 g per person. Accordingly, no health risk was detected in our conditions, as all the DI were far below the TDI_{62}.

Furthermore, the variability in DI values indicate that edibility of leek is highly dependent on soil type/TM origin, but is generally very low (i.e. low toxicity). Hence, in a case of recreational production, the home-grown fraction could be low, diluting out even further the potential local pollution in the whole diet.

More generally, phytoaccumulation also appears to be site-specific (Hough et al., 2004), but also season and fertilizer dependent (Tyksiński and Kurdubska, 2005; Florijn et al., 1992). Hence, according to our results, together with current knowledge on TM accumulation in leafy vegetables (Xiong et al., 2014b; Michalska and Asp, 2001; Tyksiński and Kurdubska, 2005; Zhang et al., 2013), the precaution principle suggests to avoid these crops (from a health point of view) in the case of (*peri*)urban areas facing significant pollution. However, a good

Table 5
Average TM Daily Intake (DI) in Bazoches (BZC) and Nantes (NTE) soils. CTR: control, B: biofertilization.

	Soil	Treatment	Cd	Pb	Sb
TDI, $\mu g_{TM} \cdot kg_{BW}^{-1} \cdot d^{-1}$			0.36	0.15	6
TDI_{62}, $\mu g_{TM} \cdot d^{-1}$	(TDI for a 62 kg person)		**22.3**	**9.3**	**372**
DI, $\mu g_{TM} \cdot d^{-1}$	NTE	CTR	0.02	0.10	0.16
		B	0.03	0.11	0.11
	BZC	CTR	0.64	0.14	0.09
		B	0.74	0.23	0.06

selection of low-accumulator cultivars could reduce the health risks when grown on low or medium contaminated soils, as was previously shown for cabbage (Liu et al., 2010). When the contamination is too high, hyper-accumulators (Escarré et al., 2000) could also be provided to remediate the soil before planting leafy vegetables, or special growing conditions could be proposed such as pots, greenhouses or hydroponics to avoid human exposure.

5. Conclusions and perspectives

Our results indicate that TM species and origin (anthropic or geogenic) strongly influenced TM phytoaccumulation as we observed differences in results obtained for each TM and soil.

Biofertilization efficiency appeared to be both TM and soil dependent, making results hard to interpret and to generalize as the overall context strongly influences the mechanisms involved. In addition, the developed fungal community in the BS and the changes in fungal communities in soil when applied would need to be carefully monitored.

Indeed, our results underline the eventual risk of using these biofertilizers when soils are polluted with heavy metals, particularly in urban gardens (Janoušková et al., 2006). Under natural conditions the soil flora and fauna would be much more complex, with the presence of major constituents of the mycorrhizosphere and the drilosphere such as bacteria and earthworms, which could also influence TM mobility, nutrient solubilization processes and plant development. For example, a recent study focusing on the combined or separate role of AMF and earthworms reported that the latter might also affect Cd speciation, even more than AMF (Aghababaei et al., 2014). Consequently, a field study or at least a study in more complex systems could be interesting to confirm the present data.

Finally, our study focused on soil to plant transfer of inorganic contaminants and showed that Pb and Sb bioaccumulation and translocation factors were low for leek (but were high for Cd). However, contaminated particles from anthropic sources are also major sources of TM phytoaccumulation for leafy vegetables, through direct deposition on edible organs(Xiong et al., 2014a). Thus, growth of these crops is not advised in polluted urban areas, even less if their bioaccessibility can increase when plants are mycorrhized, as shown for Sb. Altogether, the present study increased our knowledge of TM health risks, especially in the context of biofertilization, and points out that field studies are needed to ensure increased safety in urban agriculture.

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