



Role of arbuscular mycorrhizal fungi and bioamendments in the transfer and human bioaccessibility of Cd, Pb, and Sb contaminant in vegetables cultivated in urban areas

Antoine Pierart

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THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par : *l'Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)*

Présentée et soutenue le 26/10/2016 par :

Antoine PIERART

Rôle des champignons mycorhiziens à arbuscules et des bioamendements dans le transfert et la bioaccessibilité de Cd, Pb et Sb vers les végétaux cultivés en milieu urbain.

JURY

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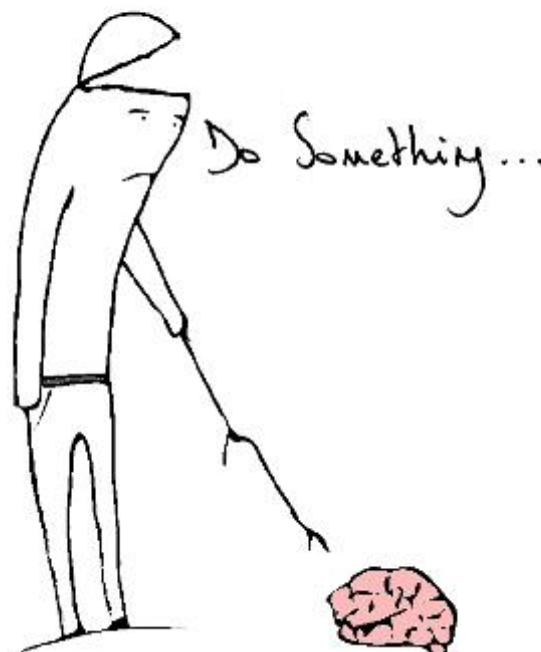
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To follow the charter of thesis from the University Paul Sabatier of Toulouse, the following thesis is written in the form of classical chapters and scientific publications (accepted, submitted or in preparation) in their edited form. For accepted and submitted articles, section, page, figure and table numbering was adapted to the manuscript. For the same reason, the abstract was written both in French and English. A summarized French version of the introduction and a full conclusion were also added.



Inspired from 9gag

If your brain just came in coma, Les orteils barbus, 2011

You can consult all the research scientific articles, posters from international congress, and research projects of my PhD with the following QR code:



“Knowledge generates ignorance”

Stuart Firestein,
The pursuit of ignorance (TEDx conference)

*“El ser humano puede ser el único animal capaz de destruirse si mismo.
Ese es el dilema que tenemos por adelante”*

Jose Muica,
Uruguay former president (2010-2015)

“Much to learn, you still have...”

Yoda,
Star Wars – attack of the clones

Oops, I did it again... *Britney Spears, 2000*

On va commencer par une petite confession. Parce que oui, dans les remerciements on dit merci, mais on se confesse aussi : Au début, je n'avais pas envie !

"Prends le temps d'y réfléchir Antoine, tu devrais faire une thèse.", m'a dit le couple de chercheurs de choc David & Marie-Hélène Macherel, un de ces (nombreux) jours pluvieux d'automne angevine pendant un stage au laboratoire IRHS il y a 5 ans.

Ma réponse, "La thèse, ça n'est pas pour moi, j'ai déjà fait bien assez de laboratoire". J'étais catégorique, plus jamais, décidé à bosser pour de bon.

Finalement, je reste dans le monde de la recherche : stage de fin d'études en laboratoire, premier emploi en station d'expérimentation ; ça s'accroche, comme ce petit caillou sur lequel on marche et qui reste coincé dans la semelle pendant des semaines. Je crois que c'est cette petite phrase, à une époque où je ne savais finalement pas vraiment quoi faire de mon avenir, qui a trottée dans ma tête et m'a conduite ici ! Peut-être que vous n'aviez pas tort finalement ? Alors, merci à vous deux, et à toute votre équipe !

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Aller plus hauuuuuuuuuuuut... *Tina Arena, 1997*
(Vous l'avez chanté dans votre tête ? maintenant oui...)

Quand on arrive à saturation, des tubes, des flacons, des échantillons et des citations, il faut un exutoire ; pour moi c'était l'escalade. Grimper, toujours plus, en salle ou en nature, surtout en nature pour tout oublier. Grace à elle, j'ai appris à connaître des gens formidables. Pour n'en citer que deux : Jojo, l'infatigable autant sur les falaises qu'en soirée ! Vincent, Je ne sais pas si je dois te remercier pour les patates ou la grimpe, on va dire les deux.

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But of all these friends and lovers,
There is no one compares with you.
The Beatles, In my life

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AMF:	Arbuscular Mycorrhizal Fungi
%B:	Bioaccessible fraction
BF:	Bioaccumulation Factor
BARGE:	BioAccessibility Research Group of Europe
CARBOX:	Carboxylesterase
CAT:	Catalase
DEHY:	Dehydrogenase
GLUCO:	β -Glucosidase
ICP-MS:	Induced Coupled Plasma-Mass Spectrometry
ICP-OES:	Induced Coupled Plasma-Optical Emission Spectrometry
KSb:	potassium antimony tartrate trihydrate
%OM:	Total Organic Matter content
OTU:	Operational Taxonomic Unit
PCR:	Polymerase Chain Reaction
PHO_ACI:	Phosphatase Acide
PHO_ALK:	Phosphatase Alkaline
PROTEA:	Protease
SCG:	Spent Coffee Ground
SCGc:	charred Spent Coffee Ground (biochar)
SEM:	Scanning Electron Microscopy
s-μ-p:	Soil-microorganism-plant (interface)
TEM:	Transmission Electron Microscopy
TF:	Translocation Factor
TM:	(ETM in French version) General term for metalloid and heavy metal trace elements
UBM:	Unified Barge Method
UPA:	(AUP in French version) Urban and Peri-urban Agriculture
UREA:	Urease

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- Pierart A.**, Shahid M., Séjalon-Delmas N., Dumat C., 2015. Antimony bioavailability: knowledge and research perspectives for agriculture. *J. of Hazar. Mat.*, 289, 21.
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Oral communications

Pierart A., Dumat C., Séjalon-Delmas N., Plant-Fungi Association: Track to Dig for Sustainable Agriculture; Beware of Metal Pollutions in Urban Areas. 18th International Conference on Heavy Metals in the Environment, 12-15 September 2016, Ghent, Belgium.

Pierart A., Gestion raisonnée des pollutions pour une agriculture durable. Favoriser les agricultures urbaines durables Retours d'expériences et initiatives innovantes à développer, 5th november 2015, Auzeville, France.

Pierart A., Braud A., Lebeau T., Séjalon-Delmas N., Dumat C., Influence of mycorrhization and soil organic matters on lead and antimony transfers to vegetables cultivated in urban gardens: environmental and sanitary consequences. 24^e Réunion des Sciences de la Terre, 27-31 October 2014, Pau, France.

Dumat C., Wu J.T., **Pierart A.**, Sochacki L., Interdisciplinary and participatory research for sustainable management of arsenic pollution in French collective gardens: collective process of risk manufacture, 9^{ème} Journées de Recherche En Sciences Sociales. 10-11 december 2015, Nancy, France.

Mombo S., Foucault Y., Shahid M., Gaillard I., Goix S., Schreck E., **Pierart A.**, Dumat C., Metal bioaccessibility to refine human health risk assessment: case of Pb and Cd pollution in kitchen gardens. 24^e Réunion des Sciences de la Terre, 27-31 October 2014, Pau, France.

Posters

Pierart A., **Dumat C.**, Séjalon Delmas N. Influence de la mycorrhization sur la phytodisponibilité et la bioaccessibilité du plomb, du cuivre et de l'antimoine présents dans les sols. Focus sur les jardins potagers contaminés en zones urbaines. Les 12^e Rencontres de la fertilisation raisonnée et de l'analyse, 18-19 november 2015, Lyon, France.

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Pierart A., **Mombo S.**, Dumat C., Séjalon-Delmas N. *Antimony in urban gardens: mycorrhizal symbiosis and organic matter influence on vegetables accumulation and human bioaccessibility.* Congrès InterSol 24-26 Mars 2015, Paris.

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Chapter 1. SCIENTIFIC CONTEXT: HOW AND WHY STUDY

INORGANIC POLLUTION IN URBAN SOILS?

To follow the charter of thesis from the University Paul Sabatier of Toulouse, the following chapter is preceded by a summarized French version, with references to the figures and tables present in the English version. For the whole introduction, [you can skip to page 9](#).

You will find here summarized information as a frame of the following investigation work. Why do we study heavy metal at the soil-microorganism-plant interface? Which are the risks for urban and peri-urban gardeners? How can one manage a contaminated soil when the will is to use it for food production? These are some of the questions raised here.

At the end of each part, you will find a [If you want more!](#) section, with specific literature to go further in one or another field of interest as it is not exhaustive.

As antimony has been much less studied, a focus has been added for this element about its bioavailability to edible plants ([Section 1.3](#)).



Heavy metal group, Pierart, 2016

What do you want?

Heavy metal, heavy metal!

Judas Priest, Heavy Metal, Ram It Down

1.1. Introduction générale

Métaux et métalloïdes, origines, flux et dangers

Tous les éléments traces métalliques et métalloïdes (ETM) sont naturellement présents dans la croûte continentale en proportions généralement faibles mais variables (Gao et al., 1998). La seule présence d'un ETM ne suffit pas à définir une zone comme contaminée. L'origine et la quantité du métal doivent être prises en compte, tout comme sa dangerosité. Les cycles biogéochimiques des ETM sont influencés, de façon rapide (Kim et al., 2014), par les rejets dans l'environnement d'origines naturelles (activité volcanique, érosion hydrique et éolienne du sol) et anthropique depuis la révolution industrielle (Catinon et al., 2011), (Figure 1.1). Les substances chimiques (de synthèse ou d'origine naturelle) accompagnent nos sociétés humaines depuis des siècles avec leurs avantages (fabrication d'outils, de médicaments, etc.) et leurs inconvénients (intoxication au plomb, effets secondaires graves de certains médicaments, etc.).

Ces dernières années, la réglementation concernant la production, l'utilisation et les émissions dans l'environnement des substances chimiques s'est renforcée (règlement européen REACH¹ et règlement Français des ICPE² par exemple). Le « Global Harmonized System » s'est également mis en place à l'échelle de la planète afin d'assurer la même information « environnement-santé » partout dans le monde pour une substance chimique donnée. On peut donc considérer que des progrès ont été faits en termes de prise de conscience sur les risques potentiels liés à l'utilisation des substances chimiques. Malgré tout, les méthodes de mesure de plus en plus sensibles permettent de détecter certaines pollutions historiques ainsi que des nouveaux contaminants, qualifiés alors d'émergents.

Deux métaux lourds: cadmium (Cd) et plomb (Pb) et un métalloïde: l'antimoine (Sb) ont été particulièrement investigués. Le cadmium et le plomb ont été sélectionnés pour leur présence récurrente en tant que contaminants environnementaux / alimentaires, en raison de leur utilisation historique (et actuelle) dans de nombreux processus industriels. Le cadmium a augmenté de façon considérable dans notre alimentation ces 10 dernières années (+400%) tandis que les teneurs en Pb tendent à diminuer légèrement mais restent encore préoccupantes comme le montre l'évolution de leurs teneurs dans les aliments mise en évidence par l'ANSES (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail) dans deux évaluations totales de l'alimentation sur le territoire

¹ REACH - Registration, Evaluation, Authorization and restriction of CHemicals

² ICPE - Installations Classées pour la Protection de l'Environnement

Français. Les sources de Cd et Pb et leurs proportions ont été bien documentées, celles de Sb beaucoup moins ([Figure 1.2](#)). De façon générale, leur utilisation dans différents procédés industriels (batterie, retardateurs de flamme, pesticides et engrais) constitue la source majoritaire de ces éléments dans l'environnement. Dans les sols français, les teneurs en Cd et Pb ont été mesurées dans les horizons de surface par le Gis Sol (Groupement d'intérêt scientifique sol) au cours de la dernière décennie au travers du programme RMQS (Réseau de Mesure de la Qualité des Sols) ([Figure 1.3](#)). Cette étude n'a cependant pas porté sur l'antimoine. Des teneurs légales dans les aliments, les sols (dans le cadre de l'épandage de boues de stations d'épuration) et les eaux existent pour Cd et Pb (résumés dans le [Tableau 1.1](#) pour Pb). Pour Sb, en dépit de son classement comme élément à haute toxicité, il n'existe pas de norme fixant la concentration limite admise dans l'alimentation.

L'antimoine a été étudié ici en raison de risques émergents liés à son utilisation. En effet, ce dernier est de plus en plus détecté en zones urbaines et péri-urbaines ainsi que dans l'alimentation comme indiqué par l'évolution de ses teneurs entre les deux évaluations totales de l'alimentation réalisées par l'ANSES. Or, on manque encore d'information sur son comportement le long de la chaîne alimentaire. Le rôle des micro-organismes dans le transfert de ces polluants inorganiques entre le sol et les plantes est l'un des thèmes centraux de cette étude. La littérature traitant ce sujet existe pour Cd et Pb, mais les liens éventuels entre ces micro-organismes et la bioaccessibilité humaine de ces éléments n'avaient pas été étudiés. Les études et synthèses bibliographiques traitant du comportement de ces éléments à l'interface sol-plante sont nombreuses, mais les interactions avec les champignons mycorhiziens à arbuscules et les bioamendements tel que le lombricompost sont moins étudiées, particulièrement dans le cas de l'antimoine (Pierart et al., 2015, [CHAPITRE 4](#)).

Gestion raisonnée des pollutions métalliques

Alors que les surfaces des sols contaminés par des ETM persistants augmentent depuis la révolution industrielle, la réhabilitation des sols à usage résidentiel et agricole est désormais un défi environnemental et sanitaire majeur (Wong et al., 2006). Les stratégies d'assainissement sont nombreuses (Khan et al., 2004), comme résumé dans la [Figure 1.6](#). Ces techniques sont principalement divisées en deux groupes selon que la contamination ciblée soit traitée sur place (*in situ*) ou à l'extérieur de la zone, dans un lieu spécialisé (*ex situ*).

Les traitements *ex situ*, du fait de leur rapidité, sont particulièrement intéressants pour les lieux d'intérêt économique élevé (urbanisation, etc...). Cependant, ils passent par une phase

d'excavation des sols qui affecte de façon importante les écosystèmes locaux. Enfin, leur coût est généralement trop élevé pour être appliqué à de grandes surfaces.

Les solutions *in situ*, comme la phytoremédiation ou la phytostabilisation, sont en principe moins chères et certainement meilleures d'un point de vue environnemental. Cependant, elles restent moins efficaces pour les taux élevés de contamination. L'utilisation de produits chimiques donne des résultats rapides, mais impacte aussi les écosystèmes, tandis que les solutions biologiques perturbent beaucoup moins l'environnement, mais requièrent un temps de dépollution beaucoup plus long.

Enfin, pour de nombreux sites, une approche complémentaire est généralement développée sur la base d'un examen préalable des zones en fonction du degré de pollution. Dans les zones d'agriculture urbaine et péri-urbaine, la volonté principale des utilisateurs est de continuer à cultiver leurs terres, élément clé à considérer lors du choix du plan de gestion à mettre en place (Dumat et al., 2015). Par conséquent, la recherche et le développement de solutions (1) non-destructives (2) économiquement viables et (3) si possible, permettant l'accès au site en cours de traitement, fait partie des objectifs de la présente étude.

Agriculture urbaine et biofertilisants/bioamendements

L'agriculture urbaine et péri-urbaine (AUP) est l'acte de produire des aliments à l'intérieur ou à proximité immédiate des villes. Toutefois, l'AUP couvre un objectif plus large et son développement révèle également un rôle crucial dans le développement écologique, social et individuel (McClintock, 2010). Pour résumer brièvement, l'agriculture urbaine a commencé au 19^{ème} siècle avec la révolution industrielle et l'exode rural qu'elle engendra (Boulianne, 2001). En effet, à cette époque les villes ont prévu des espaces arables pour les travailleurs, leur permettant de cultiver leur propre nourriture avec l'objectif de maintenir et de développer la puissance de travail et la productivité. Puis, au cours des deux guerres mondiales, et entre ces périodes, leur rôle était plus nettement alimentaire. Le boom économique qui a eu lieu au début des années 70 a transformé les habitudes de consommation en raison du développement de l'agriculture industrialisée telle qu'on la connaît aujourd'hui. Dès lors, l'agriculture urbaine est devenue davantage récréative et paysagère, et a été partiellement délaissée par les citoyens. Cette période a perduré jusqu'à la dernière décennie, qui a marqué une rupture dans la perception de l'agriculture urbaine: actuellement, elle semble susciter l'intérêt en raison de la crise économique et plus profondément du rejet des modèles de développement économiques et urbains. L'agriculture urbaine est aujourd'hui un thème complexe et diversifié, un mot-clé regroupant de nombreux types de jardiniers et autant de lieux aux objectifs différents (production, lien social, loisirs, ...). Aujourd'hui, les projets d'agriculture urbaine et la littérature associée germent au moins aussi vite que les cultures elles-mêmes.

En France par exemple, l'agence nationale de la recherche a financé un programme de recherche « villes durables » de trois ans appelé JASSUR (ANR-12-VBDU-0011), comme acronyme de Jardins ASSociatifs URbains. L'objectif principal était d'y étudier les services fournis par les jardins urbains dans le développement durable des villes et également d'analyser les pollutions éventuelles ainsi que leur gestion. Le présent travail s'est déroulé dans le cadre de ce projet national et a été financé par l'ANR JASSUR.

Les zones urbaines et péri-urbaines sont connues pour être des lieux souvent contaminés en raison de nombreuses activités anthropiques historiques ou plus récentes (Werkenthin et al., 2014). Par ailleurs, les citoyens sont généralement mal informés sur les potentiels risques de cultiver leur propre nourriture liés à la qualité des milieux (sols, eaux, air) et/ou la qualité des supports de cultures, amendements et fertilisants utilisés (Dumat et al., 2015). En effet, à l'échelle globale la qualité de l'air, de l'eau, du sol et de divers amendements organiques diminue en raison d'une augmentation significative des teneurs en contaminants organiques et inorganiques (Borrego et al., 2006).

Les sols urbains sont soumis à une telle pression économique que les espaces dédiés aux projets d'agriculture urbaine sont souvent implantés sur d'anciennes friches industrielles, des sols très anthropisés, ou des lieux à proximité des industries et des axes de communication où aucun projet immobilier ne pourrait naître. Par conséquent, la question de la qualité du sol, de l'eau et de l'air est essentielle à traiter de façon collective et transparente afin que les projets d'AUP jouent pleinement leurs rôles de vecteurs de santé et bien-être des populations et de réduction des inégalités écologiques.

Par ailleurs, les agriculteurs et jardiniers urbains par leurs pratiques peuvent également améliorer ou dégrader la qualité de leurs productions et de leurs parcelles. En effet, la qualité et les quantités des divers fertilisants, amendements et traitements utilisés soulèvent des questions comme par exemple des problèmes de sur-fertilisation ou d'emploi de pesticides aux conséquences toxiques (pour lesquels l'agriculture bio n'est pas épargnée, du fait de l'écotoxicité du cuivre présent dans certaines substances autorisées par exemple).

Afin de recycler les déchets organiques et de développer des pratiques culturales biologiques, l'utilisation de fertilisants et amendements organiques 'faits maison' ou non tels que les microorganismes ou le compost est très courante en agriculture urbaine. Cependant, peu de littérature a été publiée concernant leur influence sur la mobilité des ETM à l'interface sol-plante, ce qui est l'objectif principal de la présente thèse, en particulier dans le cas de l'antimoine (polluant émergent) mais aussi du plomb et du cadmium (très couramment observés en zones urbaines dans le monde).

Les champignons mycorhiziens à arbuscules (AMF) sont des microorganismes symbiotiques de plus de 94% des plantes à fleurs (Brundrett, 2009, [Figure 1.8](#)). Ils sont naturellement présents dans les sols et sont d'orée et déjà vendus comme biofertilisants du fait de leur capacité à solubiliser le phosphore, élément majeur de la croissance des plantes. Cependant, plusieurs études tendent à montrer leur rôle dans le transfert des métaux lourds des sols vers les plantes.

Le compost peut être produit à partir de très nombreux résidus organiques et selon des procédés variables, ce qui modifie ses caractéristiques biophysicochimiques. Or, selon Moreno Flores (2007), l'utilisation de lombricompost en agriculture urbaine se développe, alors que ces matières organiques sont connues pour séquestrer/relarguer les métaux lourds en fonction de l'acidité du sol, du fait des liaisons ioniques en jeu (Gerritse and van Driel, 1984; Sauvé et al., 2000). Or, Les AMF sont connus pour affecter le pH du sol rhizosphérique (Hu et al., 2013), ce qui pourrait affecter la mobilité des ETM liés à la matière organique. [Ces interactions sont donc au cœur de la présente étude.](#)

Enfin, le biochar ([Figure 1.10](#)) est actuellement très étudié pour ses nombreuses qualités physicochimiques en tant qu'amendement organique (Lehmann and Joseph, 2015). Dans le cas des sols contaminés en ETM, son utilisation a également révélé un bon potentiel de fixation de ces polluants (Paz-Ferreiro et al., 2014), dont l'efficacité varie principalement selon le processus de préparation (température, matériaux initiaux) (Uchimiya et al., 2011). C'est pourquoi nous nous sommes intéressés au rôle d'un biochar préparé à base de marc de café dans le transfert de l'antimoine vers les plantes comestibles.

Gestion du risque sanitaire

La teneur totale en contaminants dans les sols cultivés et les plantes n'est pas nécessairement un bon indicateur de leur toxicité. En effet, au cours de la digestion seule une fraction (dite bioaccessible) de ces contaminants est extraite et mise en solution dans le tractus digestif (Pascaud et al., 2014; Xiong et al., 2014a, 2014b). Cette fraction bioaccessible est ensuite de nouveau filtrée par la barrière intestinale, qui limite le passage de ces éléments vers le système sanguin ([Figure 1.11](#)).

Pour étudier ces phénomènes en laboratoire (sans avoir à pratiquer des analyses sur des êtres vivants), des solutions d'extraction de synthèse ont été développées, (Denys et al., 2009) afin de simuler les processus de digestion, ainsi que les temps et températures physiologiques adaptés. Cette méthode a été standardisée et validée *in vivo* par le groupe de recherche européen sur la bioaccessibilité (BARGE) sous le nom d'Unified BARGE Method (UBM) (Foucault et al., 2013).

Qu'étudie-t-on et comment ?

Les objectifs généraux de cette thèse étaient les suivants :

- Évaluer la phytodisponibilité et la bioaccessibilité des ETM dont l'origine de la contamination diffère (géogénique vs anthropique), sur les plantes comestibles.
- Étudier l'impact des champignons mycorhiziens à arbuscules et de la matière organique sur le transfert des ETM du sol vers les plantes (transfert, translocation et compartimentation).
- Évaluer l'influence de la communauté microbienne sur le devenir des ETM à l'interface sol-microorganisme-plante.
- Étudier l'influence de ces paramètres physico-chimiques et biologiques sur la bioaccessibilité humaine des ETM dans les sols et les organes comestibles des plantes, afin d'en évaluer le risque sanitaire.

La complexité des mécanismes impliqués dans le devenir des métalloïdes à l'interface sol-microorganisme-plante (s-μ-p) met en évidence la nécessité de mieux comprendre les rôles de chacun à travers une approche multidisciplinaire et multi-échelles basée sur la complémentarité des approches de biogéochimie, agronomie, génomique et (éco)toxicologie.

Ce travail fait lien avec l'approche de (1) Leveque, (2014) sur l'écotoxicologie et la biosurveillance des sols contaminés par des ETM et (2) Xiong, (2015) sur le devenir de la pollution atmosphérique en ETM à l'interface air-plante.

Pour étudier le transfert des ETM à l'interface s-μ-p ainsi que l'impact des micro-organismes et de la matière organique, nous avons développé plusieurs expériences organisées autour d'un gradient de complexité (Figure 1.12). En effet, les systèmes les plus simples produisent des résultats plus fondamentaux, sans beaucoup d'interactions entre paramètres, mais leur réalité sur le terrain nécessite d'être vérifiée. En outre, les essais sur sol réels (et non substrats) tentent d'aborder plus précisément la réalité du terrain, avec toutes les interactions qui peuvent s'y produire. Cependant, ces résultats sont généralement plus complexes à interpréter, même si le degré de confiance accordé est considéré comme supérieur.

Le présent manuscrit est organisé de la façon suivante :

Le [CHAPITRE 1](#) est une introduction contextuelle, recueil d'informations essentielles sur le thème scientifique traité dans cette thèse. Un manque de littérature sur la biodisponibilité de Sb dans les plantes comestibles et le rôle des AMF dans son transfert a été mis en évidence ; par conséquent, un focus traite ce sujet au travers d'un article publié, intitulé "Antimony bioavailability: knowledge and research perspectives for sustainable agriculture" en seconde partie de ce premier chapitre. Ensuite, l'ensemble des méthodes et outils utilisés sont détaillés au [CHAPITRE 2](#). Le manuscrit étant organisé comme une séquence cohérente de publications, certaines méthodes apparaîtront également dans les chapitres suivants, mais sous une forme condensée. Les principaux résultats sur le devenir du cadmium à l'interface s- μ -p sous différents traitements (bioaugmentation avec AMF, lombricompost) sur deux sols péri-urbains aux caractéristiques et histoires contrastées sont décrits au [CHAPITRE 3](#). Celui-ci est présenté sous forme d'un article soumis, intitulé "How do soil biofertilizers and organic amendments influence Cd phytoavailability and human bioaccessibility?". La même expérience a également été utilisée pour étudier le devenir de Sb et Pb à l'interface s- μ -p, avec une approche plus détaillée de microscopie et d'analyse des communautés fongiques ([CHAPITRE 4](#), un article en phase finale de rédaction avant soumission intitulé "Pb and Sb accumulation influenced by arbuscular mycorrhizal fungi community and organic matter in a crop rotation *Lactuca sativa* L. \rightarrow *Allium porrum* L."). La complexité des systèmes a rendu l'interprétation délicate. Par conséquent, une approche expérimentale simplifiée traitant du rôle des AMF dans le transfert de Sb vers des plantes comestibles en hydroponie a été développée. Les résultats sont développés dans le [CHAPITRE 5](#). Jusqu'à présent, l'impact de Sb sur l'activité enzymatique du sol (exsudats racinaires, microbiens et activité microbienne interne) en relation avec l'addition de biochar n'a pas été décrit à ce jour. C'est pourquoi nous avons abordé ces mécanismes dans le [CHAPITRE 6](#), au travers de l'étude de l'effet du biochar de marc de café sur le transfert sol-plante de Sb. Tous ces résultats et questionnements sont ensuite discutés et mis en perspectives dans le [CHAPITRE 7](#) avant une conclusion finale ([CHAPITRE 8](#)).

1.2. Metal(loid) soil contamination and pollution

1.2.1. Terminology and definition, what are we talking about?

All of the trace metals and metalloids (TM) occur naturally in the continental crust in small but variable proportions (Gao et al., 1998). The only presence of an element isn't sufficient to classify these areas as 'contaminated'. The origin and quantity of the metal need to be taken into account to define whether or not it is a contaminant. According to the FAO, there is a difference to be made between contamination and pollution. Indeed, "Pollution (is) the introduction by man, directly or indirectly, of substances or energy into the (...) environment (...) resulting in such deleterious effects as harm to living resources, hazards to human health. Contamination, on the other hand, is the presence of elevated concentrations of substances in the environment above the natural background level for the area and for the organism"³. For instance, a positive geochemical anomaly means naturally high amount of metal in a soil, such as for urban gardens in Nantes (France) which are enriched with lead from the mother rock.

However, this semantic distinction is rarely done because terms are inverted and the substance origin isn't always easy to trace.

1.2.2. Why Cadmium, Lead and Antimony need to be studied in urban soils?

Among the whole periodic table, we focused on two heavy metal: cadmium (Cd) and lead (Pb) and one metalloid: antimony (Sb). These elements were studied because their concentration in food was shown to increase between two recent studies performed by the ANSES in France (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail). Indeed, it showed that Cd increased up to 400%, Pb decreased a little but its concentrations remained of concern and Sb content began to increase due to its emerging use. Indeed, the latter is being more and more detected in urban and peri-urban areas while a critical lack of information on its behavior along food chain exists. The role of microorganisms in the transfer of these inorganic pollutants between soil and plants is one of the central themes of our study. Literature dealing with this topic exist for Cd and Pb, but nothing about the eventual links between these microorganisms and TM human bioaccessibility. Moreover, very few data has been published for Sb, which is why a part of this thesis was focused on a review of Sb biogeochemistry and bioavailability (CHAPTER 1 – Section 1.3) (Pierart et al., 2015).

³ [http://www.fao.org/docrep/x5624e/x5624e04.htm#1.1 contamination or pollution](http://www.fao.org/docrep/x5624e/x5624e04.htm#1.1%20contamination%20or%20pollution)

The biogeochemical cycles of TM generate releases in the environment which can be both natural (volcanic activity, hydric and aeolian soil erosion) and anthropic (industrial activities), the latter being generally the most important (Catinon et al., 2011) and the fastest dispersion pathway (Kim et al., 2014) (Figure 1.1).

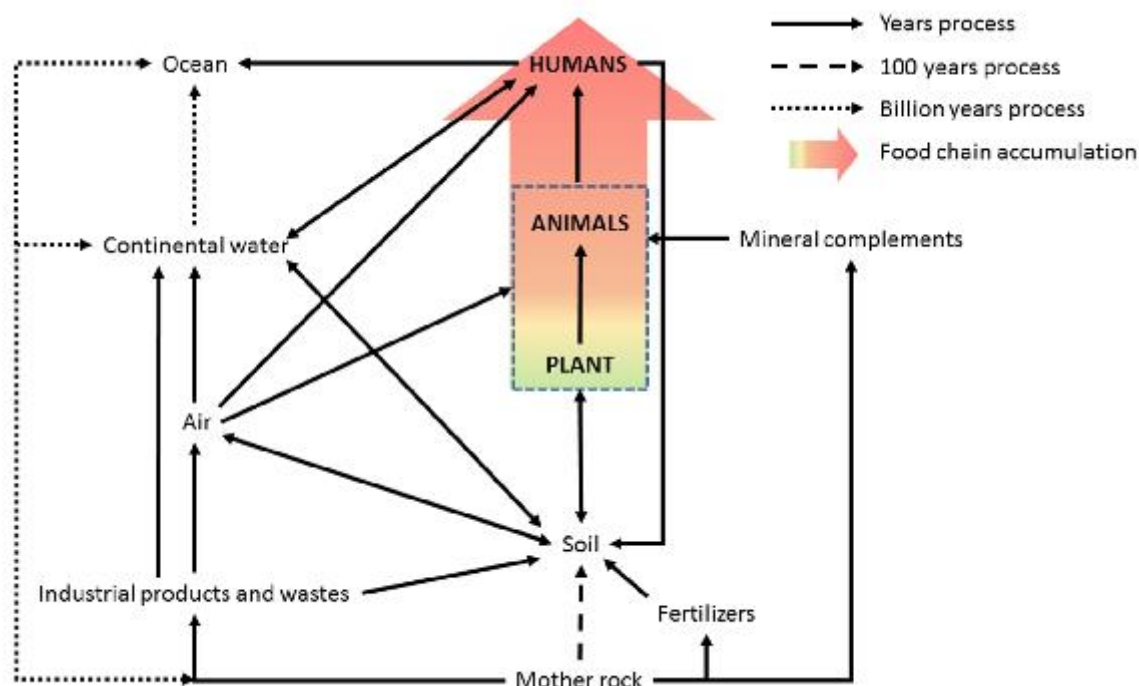
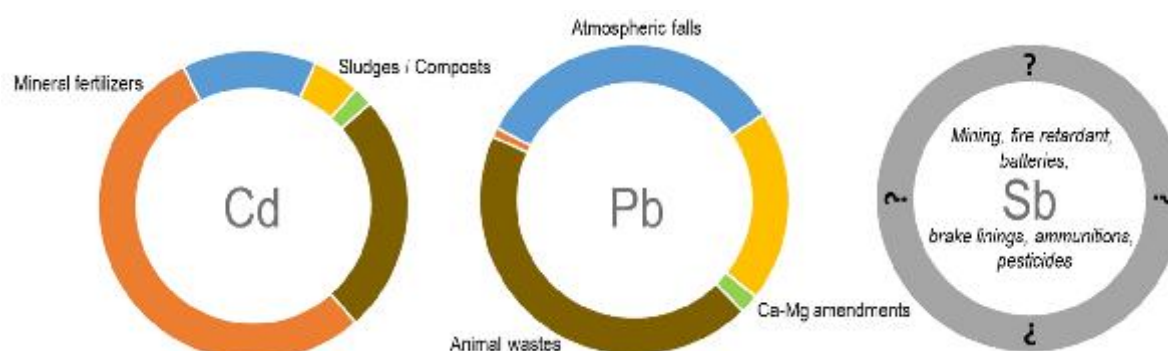


Figure 1.1: Biogeochemical cycle of TM, adapted from Tremel & Feix, 2005

Sources of Cd and Pb have been estimated in France. But, as such detailed information does not exist for Sb (Figure 1.2), it is estimated that its use in fire retardant, battery manufacture, brake linings and mining processes are the major Sb sources. However, Sb can also reach agricultural soils through lead arsenate pesticides application, since it is present as a contaminant in these metal ores (Wagner et al., 2003).



Repères. Sols et environnement, Chiffres Clés, 2015

Facing these contaminations, worldwide regulation is highly heterogeneous, with loose regulation in some countries while in Europe for example, the release in the environment of major TM contaminant is being controlled (Xiong, 2015). Since 2006, the European REACH regulation (Registration, Evaluation, Authorization and restriction of CHemicals) classified some of them (Cd, Pb, Ni, As, Hg) as Substances of Very High Concern requiring a life cycle monitoring by industrials and communication towards citizens (EC 1907/2006⁴).

1.2.3. Cadmium: a highly mobile carcinogenic element

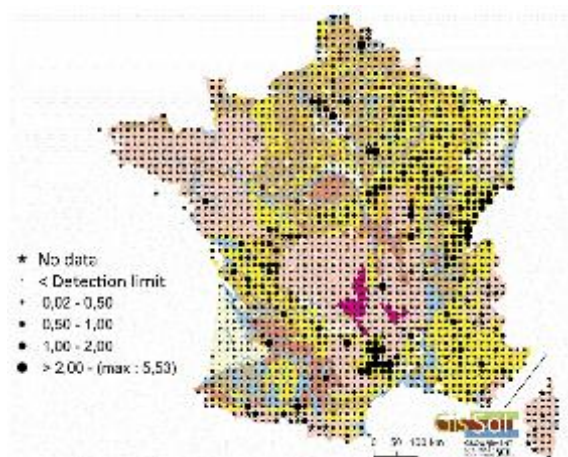


Figure 1.3: Cadmium content in French soils. mg.kg^{-1} at 0-30cm depth, between 2000 and 2009. Adapted from foot note 5

An extensive review of Cd behavior and toxicity at the soil-plant interface has been published by Shahid et al (Shahid et al., 2016); here are some summarized data showing the needs to study it: cadmium is naturally present in small quantity in the continental crust, being the 65th most abundant element within the periodic table. Its industrial exploitation always comes as a by-product, mostly in Zinc refining process. Its concentration in natural soil ranges between 0.1 and 1.1 mg.kg^{-1} soil (Shahid et al., 2016).

In European agricultural soils, it has been estimated to range between 0.06 and 0.6 ppm (Salminen et al., 2005). However, in the case of soil developed above sedimentary rocks or subjected to recurrent Cd-phosphate fertilizers (Schroeder and Balassa, 1963) and sewage sludge amendment, Cd content can easily reach more than 15 mg.kg^{-1} . In French soils, Cd content has been monitored on surface horizons by the Gis Sol organism⁵ during the last decade through the RMQS⁶ program with a concentration range of 0.02 to 5.5 mg.kg^{-1} (Figure 1.3).

Cd causes strong deleterious effects on living organisms such as kidney disorder and increased probability of bone fractures (Järup, 2003). Since 1993, the CIRC (French International Centre for Cancer Investigation) classified Cd as a group 1 harmful element (carcinogenic for human being). Cd hazard is of major concern because of its relatively high mobility in soil and water systems (Beesley et al., 2010). Indeed, its phytoavailability is high

⁴ <http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02006R1907-20140410>

⁵ <http://www.statistiques.developpement-durable.gouv.fr/lessentiel/ar/272/1122/contamination-sols-elements-traces.html>

⁶ Réseau de Mesure de la Qualité des Sols – connected to European ENVASSO (Environmental Assessment of Soil for Monitoring)

with a relatively weak bondage to clays, but strong affinity to OM (Prokop et al., 2003). For non-smoking people, the major source of Cd exposure has been estimated to be food diet (90%) (EFSA, 2009). For example, horticultural crops such as lettuce can present a high Cd accumulation in edible parts when cultivated both in contaminated soil and in spiking experiment (Peris et al., 2007; Zorrig and El Khouni, 2013). Cd is also found in cereals but in lower concentrations (Denaix et al., 2010). Cd concentration has been limited in drinking water to $5 \mu\text{g.L}^{-1}$ (INERIS, 2007), in agricultural soil (where sewage sludge are willing to be spread) to 2 mg.kg^{-1} and in leafy and stem/root vegetables to 0.2 and 0.1 mg.kg^{-1} fresh weight by the European directive EC1881/2006 because of its toxicity. But, this threshold might be lowered in the upcoming years according to recent discussions from the European commission (Nguyen et al., 2013).

1.2.4. Lead: a major contaminant widely present in urban soils



Figure 1.4: Lead content in soil. In mg.kg^{-1} at 0-30cm depth, between 2000 and 2009. Adapted from: see foot note 4

Lead (Pb) is a post-transition metal belonging to the carbon group for which four stable isotopes are found in the environment. Although Pb can occur naturally in the environment through alteration of a rich mother rock, its presence in the environment is predominantly due to anthropogenic activities such as mining, smelting, battery manufacture and past (but intense) use of leaded petrol until 2000 (Lim et al., 2013).

Pb content in soil is highly variable, but it is present in almost all compartments of the biosphere: in a natural and undisturbed soil, Pb content ranges between 2 and 60 mg.kg^{-1}

(Pettinelli, n.d.). However, local geochemical anomaly and industrial processes can increase its presence to more than $1\,000 \text{ mg}_{\text{Pb}}.\text{kg}^{-1}$. In France, the RMQS estimated that Pb content in soil ranged between 3 and $624 \text{ mg}_{\text{Pb}}.\text{kg}^{-1}$ (Figure 1.4), but intense local contamination ($\sim 40\,000 \text{ mg.kg}^{-1}$) exists as shown for instance by Leveque in 2014 (Leveque, 2014). Indeed, the RMQS specification requires the sampling areas either to be a natural soil or arable lands (*i.e.* it excludes anthropic soils such as industrial areas which are usually the more contaminated).

Lead is one of the most measured contaminant in the environment because of numerous industrial activities. As for Cd, ingestion might be the principal exposure way for Pb accumulation in living organisms, followed by dust/particles inhalation. Lead intoxication is usually called saturnism with nerve, brain and kidney diseases (Navas-Acien et al., 2009).

Actually, it is classified 2B by the CIRC (potentially carcinogenic for human beings). Lead presents a lower mobility than Cd at the soil-water interface and generally binds more strongly to mineral surfaces (such as phosphates) than to soil organic matters (Quenea et al., 2009). Its speciation and mobility depend on iron and aluminum oxi-hydroxide contents (Dumat et al., 2001) and as for most of trace elements, pH is one of the major factors of influence in soil (Cecchi, 2008; Leveque, 2014). Hence, plant and microorganism exudates can affect Pb mobility and phytoavailability (Khan, 2005; Lin et al., 2004). Lead accumulation by plant can be high, with no general pattern: some plants such as *Allium cepa* L. accumulate in root (Wierzbicka, 1987) while other translocate Pb to their leaves, such as maize (Huang and Cunningham, 1996).

These characteristics, linked with Pb elevated toxicity, led to set up legal thresholds for Pb in food products and drinking water (Table 1.1). For soils, in France, only sewage sludge inputs present legal threshold for cultivated soils, but due to the bioavailability concept, no legal threshold values were fixed for the other contexts.

Table 1.1: Legal threshold of Pb in drinking water, soil, and aliments. Adapted from Cecchi (2008)

Law (Source)		Maximal threshold
Drinking water (µg.L ⁻¹)		
Decree 2003-461 & 2003-462 (2003)	France	10
EU Directive 98/83/CE (1998)	European Union	
Directive drinking water quality (1996)	World health Organization	
Soil (mg.kg ⁻¹)		
US EPA	Play areas	400
	Other	1200
AFNOR NFU 44-041	France, agricultural soil (when sewage sludges are willing to be spread)	100
Aliments (mg.kg ⁻¹ fresh weight)		
CE/466/2001	Fruit juice	0.05
	Oil & Fat, Vegetables (except leafy vegetables, Brassicaceae, aromatics, mushroom), Fruits (except small fruits & small berries)	0.1
	Wines, small fruits & small berries, cereals, leguminous crops and vegetables	0.2
	leafy vegetables, Brassicaceae, aromatics, mushroom	0.3

1.2.5. Antimony: emerging metalloid; emerging risks?

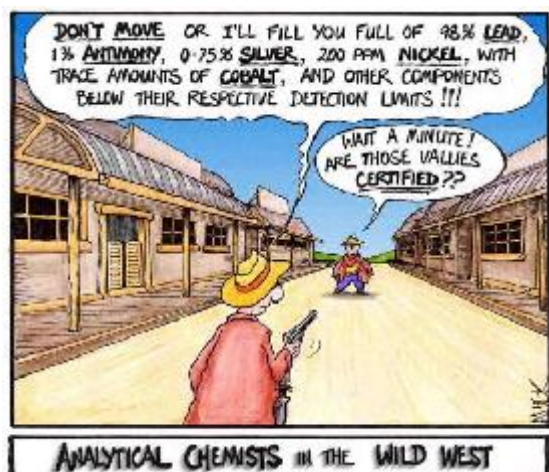


Figure 1.5: Antimony, an alloy in ammunitions,
<http://www.lab-initio.com/>

From the Latin Stibnium, Antimony (Sb) is a metalloid from the nitrogen group. It has been given such a name because it can only be found combined with other elements (Anti-monos = opposite to solitude). It is mostly present in two stable isotopic forms ^{121}Sb (57.36%) and ^{123}Sb (42.64%). Antimony's concentration in earth crust has been estimated to range between 0.2-0.3 mg.kg⁻¹. Its background concentrations in uncontaminated soil vary between <0.3 and 8.4 mg.kg⁻¹ (Hammel et al., 2000).

However, its increased use in industrial processes (battery manufacture, fire retardant, ammunition alloy..., Figure 1.5) led to a 50% increase in arctic snow records during the last 30 years (Krachler et al., 2005).

An excessive intake or long time exposure to Sb might cause vomiting, diarrhea, respiratory alterations and arrhythmias (Gebel et al., 1998; Kuroda et al., 1991; Winship, 1987), but it has also been used in medicine for years (Wang, 1919) and investigation is still ongoing (Hadjikakou et al., 2015). Both the United States Environmental Protection Agency (US EPA) and the European Union (Filella et al., 2007) classified antimony as a priority pollutant. However, legal thresholds are still scattered, with heterogeneous norms for water quality (from 5 to 20 µg.L⁻¹ depending on the country (WHO, 2008) and none for soil and vegetable.

Antimony can form compounds which oxidative states can vary between -III, 0, III, and V in the environment. However, in water and soil, Sb is mainly present as Sb^{III} under oxidative soil conditions and Sb^V when soils present reductive characteristics. Generally, Sb^{III} is a less mobile form due to its strong sorption to Fe^{III} compounds (Mitsunobu et al., 2010, 2006). As for Cd and Pb, pH might be the principal factor affecting Sb speciation and mobility.

Plant accumulation has been fully reviewed by Tshan et al in 2009 (Tschan et al., 2009a, 2009b) and more specifically in edible plants by Pierart et al in 2015 (Chapter 1 – Section 1.3), revealing an important lack of data concerning the role of arbuscular mycorrhizal fungi in the fate of Sb at the soil-microorganism-plant interface (Pierart et al., 2015) for which recent interest has been shown (Wei et al., 2016).

1.2.6. How are such metal(loid) contaminations managed?

As the contaminated surfaces have been increasing since the industrial revolution and due to the persistence of inorganic pollutants, cleaning soils for residential and agricultural purpose is a challenge (Wong et al., 2006). Remediation strategies are numerous (Khan et al., 2004) and the main ones are presented in Figure 1.6. These technics are mainly divided in two groups whether the targeted contamination is treated on site (*in situ* treatments) or outside the area, in a specialized place (*ex situ* treatment). Actually, *ex-situ* treatments with soil excavation process affect local ecosystems and are generally costly to set up, but they quickly remove the metals and are therefore interesting for places of high economic interest. However, these technics are generally too expensive to apply in large areas. *In situ* solutions are usually cheaper and certainly better from an environmental point of view, but less efficient for high contamination rates. The use of chemicals gives rapid results but also impacts ecosystems, while biological solutions are less stressful for the environment but with a much longer time of depollution.

Finally, for numerous sites, a complementary approach is generally developed based on a screening of the areas in function of the degree of pollution. In the case of urban and peri-urban agriculture areas, the gardeners' will to continue to cultivate their land is a key element to consider when choosing the remediation technic (Dumat et al., 2015). Hence, the investigation and development of (1) non-destructive (2) affordable and (3) 'permitting access during treatment' solutions is part of the objectives of the present study.

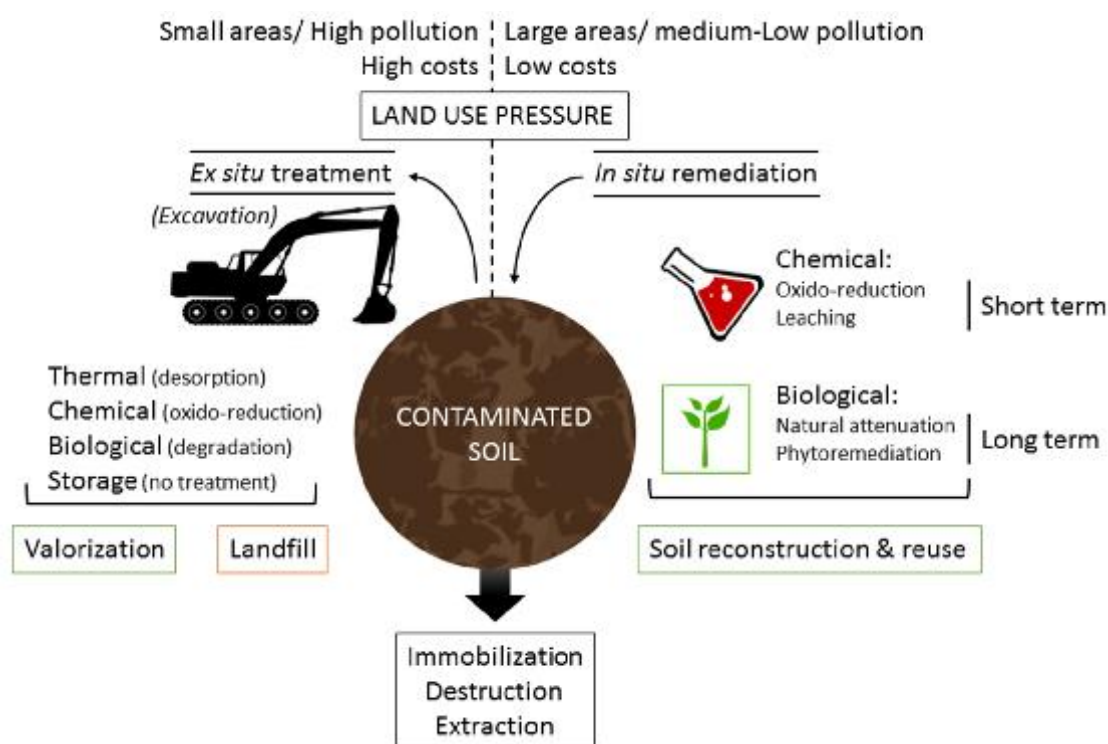


Figure 1.6: How to treat a polluted or contaminated soil? Adapted from Foucault, 2013

1.2.7. If you want more!

- Antimony:

Antimony: Its history, chemistry, mineralogy, geology, metallurgy, uses, preparations, analysis, production, and valuation; with complet bibliographies (Wang, 1919). An ancient book full of interesting data about Sb historical discovery and use.

The thorough reviews of Filella et al (Filella et al., 2007, 2002) and Tschan et al (Tschan et al., 2009b) describe precisely the fate of Sb at the soil-water and soil-plant interface.

- Cadmium:

Cadmium in Soils and Plants (McLaughlin and Singh, 1999). A very complete book about Cadmium in the environment. Due to its age, it lacks some details discovered more recently, but the basis are solid.

Root responses to cadmium in the rhizosphere: a review (Lux et al., 2011). An interesting article dealing with root growth and Cd uptake in Cd-rich medium, with a strong focus on physiological/histological response to Cd stress.

Root excretion and plant tolerance to cadmium toxicity-a review (Dong et al., 2007). Another review focused on Cd fate in the rhizosphere.

- Lead:

Review of Pb availability and toxicity to plants in relation with metal speciation; role of synthetic and natural organic ligands (Shahid et al., 2012). A complete review dealing with (1) Pb speciation and mobility as affected by humic substances and chemicals in soil and (2) Pb phytoaccumulation influenced by its speciation.

Devenir du plomb dans le système sol-plante. Cas d'un sol contaminé par une usine de recyclage du plomb et de deux plantes potagères (Fève et Tomate) (Cecchi, 2008). A thesis focused on Pb fate in the soil-plant system, with a very complete Pb-orientated state of art.

1.3. Sb bioavailability: Knowledge and research perspectives

This second part of the first chapter, which is presented as a published article entitled *Sb bioavailability: knowledge and research perspectives for agriculture*, is a review of the actual available literature concerning Sb fate at the soil-microorganism-plant interface, with special focus on:

- Antimony accumulation in edible plants, as it is very variable
- The lack of information regarding the role of arbuscular mycorrhizal fungi in its mobility and eventual accumulation by plants,
- Sb accumulation along food web and human bioaccessibility.

The article is followed by its supplementary data, also available at:

<http://dx.doi.org/10.1016/j.jhazmat.2015.02.011>

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Review

Antimony bioavailability: Knowledge and research perspectives for sustainable agricultures

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HIGHLIGHTS

- This paper reviews Sb in edible plants in relation to sanitary consequences.
- Sb contamination in urban areas has been increasing for 50 years.
- Sb values in edible plants are very scattered.
- A serious lack of data exists about Sb behavior with arbuscular mycorrhizal fungi.
- There is no legal threshold for Sb in edibles, but potential human risk can occur.

GRAPHICAL ABSTRACT



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ABSTRACT

The increasing interest in urban agriculture highlights the crucial question of crop quality. The main objectives for environmental sustainability are a decrease in chemical inputs, a reduction in the level of pollutants, and an improvement in the soil's biological activity. Among inorganic pollutants emitted by vehicle traffic and some industrial processes in urban areas, antimony (Sb) is observed on a global scale. While this metalloid is known to be potentially toxic, it can transfer from the soil or the atmosphere to plants, and accumulate in their edible parts. Urban agriculture is developing worldwide, and could therefore increasingly expose populations to Sb.

The objective of this review was in consequences to gather and interpret actual knowledge of Sb uptake and bioaccumulation by crops, to reveal investigative fields on which to focus. While there is still no legal maximal value for Sb in plants and soils, light has to be shed on its accumulation and the factors affecting it. A relative absence of data exists about the role of soil flora and fauna in the transfer, speciation and compartmentation of Sb in vegetables. Moreover, little information exists on Sb ecotoxicity for terrestrial ecosystems. A human risk assessment has finally been reviewed, with particular focus on Sb bioaccessibility.

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1. Introduction

Antimony (Sb) is a metalloid occurring naturally as a trace element in soils [1,2]. Its deposits are scattered worldwide, but differ greatly in terms of concentration. According to Krachler et al. [3], for the last 30 years Arctic snow and ice have recorded a 50% increase in Sb accumulation, mainly from anthropogenic sources [4]. Actually, it has been estimated that the total remaining world pool of Sb is equivalent to about 12 years of consumption at the current anthropogenic rhythm [5]. Antimony is used in numerous human activities, including smelting and mining, but its use as a fire retardant is the most important [6]. In addition, Sb contamination comes from shooting ranges (because of Pb–Sb alloys used in munitions) [7]. It has been reported that 5.56 mm bullets are comprised of almost 0.7 wt.% antimony [8], while 9 mm rounds contain up to 1.8 wt.% [9,10]. Antimony compounds are also used to treat Leishmaniasis, AIDS, and cancer [11,12]. Moreover, in the past, agricultural lands have faced large-scale Sb inputs, with the presence of this persistent element in the manufacture of pesticides and/or herbicides, and applications of sewage sludge [13,14].

Today, agricultural and urban lands with farms and gardens are facing new Sb contamination sources. In recent decades, the increased use of Sb in old batteries, and as a lubricant and alloy in brake linings, has been causing contamination through manufactures recycling battery and road traffic dust [15,16]. Airborne particles enriched with metal(loid)s can pollute soils [17,18], and metal(loid)s can accumulate in plants, both through root [19] and foliar uptake [20–23]. While agricultural lands present a contamination risk through aerial deposition and water, urban areas are facing a new important challenge: in reaction to the worldwide economic crisis, people are showing a clear desire to grow their own food in public, associative, or kitchen gardens [24]. Indeed, having a garden to produce healthy vegetables is one of the objectives highlighted by urban gardeners [25], but these areas are often either directly in contact with roads and/or industries, or set up on old industrial soils (*i.e.*, with a high risk of contamination) [26].

According to Winship [27], Sb is a toxic element, and excess intake by humans may cause vomiting, diarrhea, skin rashes, and respiratory symptoms, such as a cough. Other studies have also demonstrated the toxicity of Sb to human beings [28,29]. Cardiotoxicity has also been reported, with arrhythmias and cardiac arrest. According to the German Research Council, inhalation of Sb

compounds, as well as metallic Sb dust, may cause lung tumors [12,30].

Since 1979, antimony has been considered as a priority pollutant by the United States Environmental Protection Agency (US EPA), as well as the European Union [31]. Antimony has therefore been studied widely in the water compartment [32], and the results have led to different thresholds (from 5 to 20 $\mu\text{g L}^{-1}$) for drinking and agricultural water in some countries [33]. Subsequently, as food represents the main source of human exposure to environmental pollutants [34], with fruits and vegetables making up the world population's major dietary components, scientists have become more and more interested in Sb transfer in soil–plant–water systems [35,36]. The consumption of polluted plants could therefore have a strong impact on human health [37,38]. Recently, Feng et al. [39] reviewed Sb interactions with terrestrial plants. However, the data dealing with edible plants are widely scattered, and unlike lead (Pb), cadmium (Cd), or mercury (Hg), there is no regulation concerning maximal Sb concentrations in marketed consumer produce.

Agricultural lands and urban areas allocated for agriculture and gardening activities are also places where the soil biodiversity can be enhanced by sustainable cultural practices, based on organic matter inputs [40]. Consequently, these areas may have an important biological activity. A better knowledge of biotic factors influencing the transfer of Sb in the soil–plant system appears as an important goal. Earthworms are known to play a key role in metal(loid) bioavailability in soils [41], as do mycorrhizal fungi, which are used as biofertilizers in current agriculture practices [42]. Both interact with different soil compartments (*i.e.*, transfer from soil solution through fungus to plant for mycorrhization for the former, and solid-phase-to-earthworm transfer for the latter).

In this review, we investigate the sanitary risks induced by human exposure to Sb, owing to the ingestion of polluted vegetables or soils (in the case of accidental soil ingestion). The scientific questions raised are therefore: (1) What are the mechanisms involved in Sb transfer to vegetables and soil fauna? (2) What is the influence of plant–earthworm, or plant–fungi interactions on the transfer? The main factors affecting Sb bioavailability in relation to its compartmentalization and speciation are summarized first. Then, relevant data, and gaps concerning edible plant and Sb bioaccumulation, are studied, with a focus on biological factors influencing such transfers (plant mycorrhization and earthworm

Table 1

Minimal and maximal Sb values in selected soil and living organisms.

Media	Min (mg kg ⁻¹)	Max (mg kg ⁻¹)	References
Soil	0.3	2095	[43]
	Continental crust	Battery manufacture soil	[48]
Sediments	0.3	2122.8	[50]
	Uncontaminated sediment	Contaminated stream sediment	[46]
Mining site	1291	5045	[47]
	Mine tailing	Mining soil	
Urban soil	0.46	4.4	[16]
	Roadside soil	Road dust	
Shooting ranges	500	15000	[46]
Plants	0.004	5112	[39]
	Garlic	Pterisfauriei	
Edible plants	0.004	2236	[51]
	Garlic	Colza	
Terrestrial invertebrates	0.04	30.4	[52]
	Earthworm	Spider	
Aquatic invertebrates	0.43	3.35	[53]
	Grammarus	aquatic sowbug	
Small mammals	<0.02	8.6	[54]
	Mice	Vole	
Human body	0.045	0.8	[29]
	Hair sclape	Urine	

bioturbation in soils). Finally, data on the trophic chain and the risks threatening human health when producing edible plants in polluted areas are exposed.

2. Factors affecting Sb bioavailability

Antimony and its compounds occur naturally in relatively small quantities in rocks (0.15–2 mg kg⁻¹) [31], non-polluted water (<1 µg mL⁻¹) [31] and soils (0.3–8.6 mg kg⁻¹) [43]. While soil is the main plant-developing medium, it is also a major contaminant sink in various ways: fertilizers and amendments inputs, aerial deposition, and water percolation. According to the Toxics Release Inventory [44], in 1987, almost 3,061,036 pounds of Sb was released into the environment by different industrial activities in the United States. Of these Sb releases to the environment, 2% was to water, 4.4% to air, and 92.9% to land. Urban areas allocated for gardening practices and arable lands cultivated in ancient contaminated sites present then an important health risk.

Metal bioavailability can be expressed as the part of the total soil metal content that can interact with living organisms [45]. Generally, metal availability to plants and other living organisms in the soil is controlled by the pseudo-equilibrium between aqueous and solid soil phases [45]. Part of the soil metal introduced by anthropogenic activities is more mobile than naturally occurring metal in soil. The bioavailable part of a metal can be taken up by plants and other living organisms. Bioavailability of Sb in soil is controlled by several processes, such as adsorption/desorption, precipitation/dissolution, and Sb–ligand complex formation [19]. These processes depend mainly on soil characteristics, such as cation exchange capacity, soil pH, soil texture, biological and microbial conditions, amount of metals, organic and inorganic ligands, and competing cations [45]. These processes and parameters, either separately or in combination with each other, may affect Sb behavior in soil. This section reviews the actual knowledge about Sb behavior in soil.

2.1. Antimony consideration is variable

As shown in Table 1, because of numerous anthropogenic activities, Sb concentration is highly variable in the environment [46]. As a non-exhaustive example, its concentration in mining site, mine

tailing, industrial soil (battery recycling factory), shooting range, road side soil, and road dusts can reach, respectively, (in mg kg⁻¹): 5045 [47], 1291, 2095, 13 800, 0.46, and 4.4 [16,47–49].

Interestingly, great asymmetry can be observed between the geographic areas with high Sb levels in the soils, and the scientific studies performed all around the world, so we can effectively conclude that Sb contamination often seems not to be taken into account worldwide. Countries such as China (87% of the world's Sb production [5]) have been studying Sb behavior at the soil-edible plant interface to a large extent. In the same way, the number of publications about Sb content in edible plants has doubled since 2010, with an average number of accepted publications of 2.4 publications per year (1.25 during 2000–09; none before). At the opposite end of the spectrum, Bolivia, which possesses large Sb deposits, and is the second largest Sb producer (3.3% of the world's production), neither studies nor publishes data about Sb contamination.

There is a contradiction arising around Sb with respect to its importance in remediation and risk-assessment studies. Some studies highlight the fact that this toxic element can accumulate in plants [55], especially in the case of Chinese studies, and focus mainly on mining sites with major contamination [1,36]. But on the other hand, in the same country, this element is not even systematically measured. This is the case with the recent study of Huang et al. [56], which investigated the bioaccumulation of several heavy metal(loid)s in more than 300 vegetables of 11 common types, from a large geographic area, including Zhejiang province. They reported that Sb values in water and sediments could be high [1], and were potentially responsible for contaminating arable lands and urban gardens through irrigation.

2.2. Antimony solubilization in the soil

The behavior of Sb in soils has gained considerable attention over the last decade. Generally, the water, Na₂HPO₄, and NH₄NO₃ extractable fraction of Sb is considered to represent the soluble and bioavailable form of Sb in soil [57–60]. Wilson et al. [2] reviewed Sb behavior in soil systems and concluded that soil pH greatly affects its speciation and solubility. In addition, Sb background concentration, soil mineralogy, the presence of organic and inorganic ligands, soil organic matter contents (which can be

abnormally high in urban gardens: values such as 15–20% are commonly observed in comparison with 2–3% for agricultural soils), and amount and type of co-oxidants/co-reductants also influence Sb solubilization/mobilization in soil systems [2]. The reduced solubility and mobility of Sb in soil is already well documented: (1) due to partitioning to metal (hydro) oxides of Al, Fe, and Mn [61,62], (2) to secondary mineral precipitation [63,64], (3) humic acids [65] and clay mineral interactions [66,67]. The scientific community agrees that generally Sb is not highly soluble and available, but its solubility can vary widely depending on the soil's natural characteristics [68]. For example, Hou et al. [69] showed that mobilizable Sb varies in soils as follows: Primosol > Ferrosol > Isohumosol. Flynn et al. [58] reported less than 1% Sb solubility in soil near mining and smelting sites in the United Kingdom.

Antimony has been classified as easily phytoavailable, moderately phytoavailable, and not phytoavailable, as a function of its complexation with organic and inorganic ligands, as well as its extractability from these compounds [47]. The effect of As on the uptake and bioaccumulation of Sb by plants has also been reported [70]. Biochar is reported to enhance Sb mobility in soil [71,72], which might be a result of electrostatic repulsion between anionic Sb and negatively charged biochar surfaces [72]. The water-soluble fraction of Sb (Sb_{sf}) is calculated as the ratio between the water extractable Sb and the total Sb in soil. From available data in the literature, this ratio has been estimated between 0.5 and 2.9% (complete results available in Supplementary material Table A.1).

2.3. Antimony oxidative state and speciation in soil

It is well-known that metal speciation plays a key role in determining the biogeochemical behavior (bioavailability, bioaccumulation, toxicity, and detoxification) of metals in soil–plant systems [73–75]. Different forms of a metal are not always equally bioavailable and toxic. Therefore, total soil metal concentration is not considered a good proxy to estimate metal bioavailability and toxicity in living organisms [17,19]. Antimony is also reported to exist in different chemical forms (organic and inorganic) in soil. It occurs in various oxidation states in environmental systems (–III, 0, III, and V), but only two of them (III and V) are found mainly in soils [10,31]. The behavior of Sb depends largely on: (1) its oxidation states [77]; for example, Sb^{III} is reported to be 10 times more toxic than Sb^V ; and (2) the plant species [35], which may more readily take up either Sb^V or Sb^{III} . Some studies compared accumulation of Sb^{III} and Sb^V species by plants. They showed that its accumulation is both Sb species and plant species dependent (cf. 3.1., 3.5., and 4.2.). Most commonly, Sb occurs in soil as oxides, hydroxides or oxyanions [78,79]. Inorganic species of Sb are well-known and reported compared to organic species, but the latter are reported to exist. For example, it has been shown that organic Sb species such as soil trimethylantimony dichloride – $(CH_3)_3SbCl_2$ – probably exist in solution as $[TMSbOH]^+$ [80]. The existence of Sb in different chemical forms is considered to depend on the soil's physicochemical properties, adsorption/desorption processes, and the presence of inorganic and organic ligands [2,50,77,81]. As an example, strong humic acid–Sb interaction was currently observed in polluted soils.

Moreover, these humic acids participate to the oxidation level of antimony (between Sb^{III} and Sb^V species) with consequences on its toxicity [82] and mobility during lixiviation processes [83].

However, to date, there is very little data regarding the biogeochemical behavior of Sb in soil–plant systems, in relation to its chemical speciation. Precisely which chemical forms of Sb are more mobile, available and toxic are questions that always need to be explored, in order to better understand the biogeochemical behavior of Sb.

2.4. Antimony from soil to plant: an oxidative state dependent pathway

Although Sb does not have an essential role in plant metabolism, this metalloid can easily be taken up by plant roots from soils [1,39,84]. Different pathways have been suggested concerning Sb transporters in plants [39], but only a small amount of data exists. It has been proposed that Sb accumulation in plants seems to occur via a passive pathway, especially in its Sb^{III} form. But some cues indicate that an active pathway could also exist [85]. The first identification of a cellular transporter for Sb^{III} accumulation was done in *Escherichia coli* [86,87]. Kamiya and Fujiwara [88] identified an Sb transporter in *Arabidopsis thaliana* while studying the reactions of plants mutated in As^{III} transporters. They focused on the nodulin 26-like intrinsic proteins (NIPs), and showed that the NIP1;1 As^{III} transporter also transports Sb^{III} and influences *A. thaliana* sensitivity to antimonite. The problem is that, to date, an Sb^V pathway from soil to plant remains undiscovered, whereas the mechanism of uptake of As^V has been determined to occur via phosphate transporters [89]. The relationship between Sb speciation and localization/compartimentation inside the plant is also still to be established. Is there any link between organic or inorganic ligands and Sb speciation in soil and its bioaccumulation by plants and soil microorganisms? To what extent would gardeners' cultural practices affect Sb speciation? Finally, what are the consequences in terms of human exposure and Sb bioaccessibility if crops are cultivated in Sb-contaminated soils?

3. Antimony bioaccumulation in edible plants

3.1. Bioaccumulation factor

The bioaccumulation factor (BAF) has been defined by the US Environmental Protection Agency as: “the ratio of the contaminant in an organism to the concentration in the ambient environment at a steady state, where the organism can take in the contaminant through ingestion with its food as well as through direct content”. Data in Table 2 show a high variability of BAF for Sb in edible plants. It is expressed here using two different ratios: BAF, which is the ratio between Sb concentration in plant and the total Sb concentration in soil; and BAF_w , which is calculated as the ratio between plant Sb content and the soluble fraction of Sb in soil. The limitation is that the so-called soluble fraction is obtained through numerous different extraction protocols in the literature, such as shaking soil sample with ultrapure or bi-distilled water [36], or with KNO_3

Table 2
Antimony Bioaccumulation Factor (BAF) in edible plants.

	Plant	Organ	Sb specie	BAF	BAF_w
Sb present in soil	Peanut	Shoots		0.17	9.5
	Colza	leaves		0.02	1.08
Experiment with spiked Sb	Sunflower	Leaves	Sb_2O_3 -potting mix	0.07	10.19
	Spinach		KSbO-tartrate, $3H_2O$	0.8	9.07
	Maize	–	Sb_2O_3 -agricultural soil	0.03	6.66

BAF represents the ratio $[Sb]_{plant}/[Sb]_{total\ soil}$. BAF_w represents the ratio $[Sb]_{plant}/[Sb]_{soluble\ soil}$. When known, the bio-accumulative organ and Sb species are specified. Complete table available in Supplementary material Table A.2.

salt [85], leading sometimes to potential mistakes in results, and in comparison experiments.

In their review, Feng et al. [39] reported that Sb phytoavailability depends mainly on its solubility, and its facility of transfer from the solid phase to the soil solution. They concluded that Sb solubility in soils is generally very low, but highly dependent on soil characteristics (cf. §2.1). This concentration is usually less than the total Sb in the soil, but this fraction is the most easily phytoavailable, and gives a better idea of the real mechanism of bioaccumulation. It is still imperfect because it only considers the Sb soluble fraction, while the phytoavailable fraction is composed of both the water-soluble and cation-exchangeable fractions (i.e., the concentration extracted from soil with MgCl_2 or CaCl_2 for example). However, insufficient data was available in Sb-related publications. Such heterogeneity in protocols and lack of data are quite common. These results are therefore to be considered cautiously. Although Sb speciation is known to be an important factor affecting its solubility, very poor information is usually provided, except concerning its oxidative state [35] (cf. §2.2). Various Sb speciation analyses have been tested to date. They were reviewed with stresses on their advantages and difficulties in early 21st century by Krachler et al. [90]. Major difficulties come from both the lack of suitable Sb standards and the particular Sb chemistry complicating the simultaneous separation of its chemical species without changes (in comparison with the initial speciation in the environment). The development of certified materials (root, leaves, seeds, fruits etc.) for bioaccessibility assessment could therefore be of great interest for the scientific community.

Values range from 0.001 to 1.4 for BAF, and 0.04 to 10.19 for BAF_w , showing the potential risk of cultivating vegetables in Sb-contaminated soil (Table 2 and Supplementary material Table A.2). Such results also highlight the essential role of the Sb species in its uptake and bioaccumulation. Indeed, for the same vegetable, two different Sb species led to highly variable BAF and/or BAF_w while the exposure concentration was the same. Moreover, when exposed to SbCl_3 or SbCl_5 , rye (*Secale cereal* L.) and wheat (*Triticum aestivum* L.) presented two opposite absorption preferences: rye and wheat accumulated more Sb in the presence of SbCl_5 and SbCl_3 , respectively [91].

Consequently, since agricultural lands and urban gardens are submitted to frequent and sometimes major watering (i.e., water-logging), a fraction of the soil Sb pool could solubilize and convert in more phytoavailable species [35], and this has to be taken into account in both environmental and sanitary risk assessments.

3.2. Antimony in edible plants

Metal(loid) accumulation by plants occurs in different organs so it is crucial to distinguish the whole plant accumulation from the edible part one, which is the hazardous one. Table 3 summarizes the main information to date cited in the literature about Sb in edible plants. It shows that Sb can be found in every plant tissue, from roots [91] to fruits [92]. However, its range of concentration is very large ($0.004\text{--}1400\text{ mg kg}^{-1}$). Thus, in regard to the potential health risks induced by Sb exposure, the high concentrations sometimes observed in consumed plant parts highlight the interest in determining a threshold concentration for vegetables in the context of European regulation and beyond, to ensure food safety. However, although some studies found no obvious relationship between Sb concentrations in soil and plants [57,93], perhaps as a result of foliar pollution highlighted by Schreck et al. [22], others seemed to identify one. For example, a positive correlation between $[\text{Sb}]_{\text{soil}}$ and $[\text{Sb}]_{\text{roots}}$ ($R = 0.959$, p value = 0.01) has been found in an old mining site in Spain [26].

Antimony speciation in plants has not yet been studied in detail. The current state of knowledge has been reviewed by Feng et al. [39]

with often a mixture between precise chemical compounds and oxidative degree identification. For instance, four Sb species (Sb^{III} , Sb^{V} , $\text{Sb}(\text{CH}_3)_3$ and an unidentified Sb compound) have been found in *Pteris vittata* L. when grown in the presence of Sb^{V} [51]. Moreover, the origin of $\text{Sb}(\text{CH}_3)_3$ often observed in aboveground plant parts is still unclear. But some results showed the capability of fungi and bacteria to methylate Sb in soil (cf. 4.2). The question remains however, if the plants can methylate Sb as it has been shown for arsenate [94], or if they accumulate it through their endophytic fungi. In some edible plants, it has been shown that the main Sb species is Sb^{V} (~95%) [95] but its uptake pathway is still unclear (cf. 2.4).

The truth is that roadside soils do not generally have a very high Sb contamination. However, in some cases, when arable lands or urban gardens are set up in the direct vicinity of a road, or in old industrial/mining areas, long-term exposure could lead to an increased accumulation of Sb in their soils (and then to the harvests), leading to food-chain accumulation, human exposure and, in the worst cases, disease.

As a result of this synthesis, some plants seem to fit the role of Sb phyto-extractors because of their high capability to take up and stock Sb in their shoots (rapeseed, peanut, English mace and Bladder campion, in which Sb values have reached, 2236, 340, 1367 and 1164 mg kg^{-1} , respectively; see Table 3). However, it is still necessary to measure Sb values in peanuts, which is the edible part of this plant.

On the other hand, some plants reveal a potential capacity to grow on Sb-contaminated soil without any increment in their uptake of this metalloid. These plants could, therefore, be recommended to be grown when the soil cannot be remediated (i.e., in the case of a lithogenic Sb contamination). Such is the case for onions, cucumber, sunflowers and wild rosemary (Table 3).

Since farmers and gardeners can buy and grow many different varieties (cultivars) of each vegetable, investigations also need to be performed at the varietal scale, to know if different varieties of the same species behave similarly or not, with regard to Sb uptake and accumulation, as has been shown for other metal(loid)s such as Pb and Cd [102].

Differences between perennial and annual plants have also been shown [103]. For example, in seasonal plants, Sb concentrations ranged from $1\text{ to }447\text{ mg kg}^{-1}$, and in perennial plants its value was between $1\text{ and }20\text{ mg kg}^{-1}$, which is 20 fold less. Such differences highlight the potential sanitary risk, both in urban gardens and in agriculture, where seasonal plants are mainly cultivated.

3.3. Antimony in rice

Rice is one of the most consumed food crops for 3 billion people in the world. Nowadays, its cultivation in areas near towns (in Japan, Malesia, etc.) is seriously asked from a sustainable point of view [104]. But, the Sb accumulation by rice may cause human health threats, especially in Asia where such food could represent about 33% of direct Sb intake [95] and even more if, in addition, the possible entrance of Sb (as other potentially harmful element such as As) into food chain through cattle fed with rice straw is considered [105]. This section focuses therefore on the data concerning Sb availability and accumulation in rice plant. Actually, the relationship between rice and Sb has already been intensively studied, revealing that Sb concentration in edible parts could be potentially high. According to Ren et al. [106], rice plant can accumulate Sb up to 5.79 mg kg^{-1} in seeds. However, as shown in Table 4, Sb concentration in rice is generally relatively low. Iron plaques developed around rice root seem to play a major role in the alleviation of Sb contamination [107] through a strong bondage between Sb and Fe, leading to a decrease of Sb in plant. A competition in absorption of Sb^{III} and As^{III} has also been identified [108]. As for other edible

Table 3
Review of Sb content in edible plants in relation with the form of Sb and type of experiment.

Common name	Plant species	Sb exposed levels (mg kg ⁻¹ /mg L ⁻¹)	Chemical form	Maximum Sb concentration (mg kg ⁻¹ DW)	Accumulation organ	Experiment	Additional information	References
Apple	<i>Malus domestica</i>	–	–	0.011	Fruits	–	Retail network	[80]
Barley	<i>Hordeum vulgare</i>	67.3	–	0.02	Grains	F	Agricultural soil	[47]
Beet	<i>Beta vulgaris</i>	0.41	–	0.03	–	F	–	[16]
Cabbage	<i>Brassica oleracea</i>	–	–	0.021	Leaves	–	Retail network	[80]
		266.3	–	0.28	–	F	Garden soil	[47]
Carrot	<i>Daucus carota</i>	–	–	1.13	–	F	–	[82]
		–	–	0.008	Roots	–	Retail network	[80]
		159.4	–	0.03	Storage organ	F	Garden soil	[47]
		159.4	–	0.8	Leaves	F	–	
Celery	<i>Apium graveolens</i>	–	–	3.44	–	F	–	[82]
Chili pepper	<i>Capsicum annuum</i> Linn	–	–	2.87	–	F	–	
Chinese cabbage	<i>Brassica rapa</i> subsp. chinensis	–	–	3.33	–	F	–	
Colza	<i>Brassica campestris</i>	1 600	KSBO-tartrate	2236	–	P	–	[83]
		5 045	–	121	Leaves	F	–	[60]
		–	–	2.84	–	F	–	[82]
		–	–	6.65	–	F	–	
Coriander Herb	<i>Coriandrum sativum</i>	–	–	1	Leaves	F	Garden soil	[47]
Corn salad	<i>Valerianella locusta</i>	166.3	–	~600	–	P	gd	[83]
Cucumber	<i>Cucumis sativus</i>	1 600	KSBO-tartrate	–	–	–	Retail network	[80]
		–	–	0.01	Fruits	–	No detail for each plant	[84]
Dill	<i>Anethum graveolens</i>	40.6	–	0.14	–	F	–	[16]
Eggplant	<i>Solanum melongena</i>	0.41	–	0.03	Fruits	F	–	[47]
Endive	<i>Chicorium endiva</i>	266.3	–	2.2	Leaves	F	Garden soil	[80]
Garlic	<i>Allium sativum</i>	–	–	0.004	Clove	–	Retail network	[82]
		–	–	3.41	–	F	–	
Green bean	<i>Phaseolus vulgaris</i>	–	–	9.87	–	F	–	
Lucerne	<i>Medicago sativa</i>	14	–	1.75	–	F	–	[85]
Maize/Corn	<i>Zea mays</i>	28.75	Mining drainage	~100/78	Shoots/roots	P	–	[86]
		57.7	–	0.35	Shoots	F	Agricultural soil	[47]
		57.7	–	0.02	Grains	F	Agricultural soil	
	<i>Zea mays</i> cv. Magister	5 000	Sb ₂ O ₃ KSb(OH) ₆	~170 ~180	–	P	Agricultural soil. gd	[87]
		–	–	0.72	–	F	No specie details	[82]
Mung bean	<i>Phaseolus radiatus</i>	1 600	KSBO-tartrate	~1400	–	P	gd	[83]
Oat	<i>Avena sativa</i>	67.3	–	0.06	Grains	F	Agricultural soil	[47]
Onion	<i>Allium cepa</i>	–	–	0.011	Storage organ	–	Retail network	[80]

Table 3 (Continued)

Common name	Plant species	Sb exposed levels (mg kg ⁻¹ /mg L ⁻¹)	Chemical form	Maximum Sb concentration (mg kg ⁻¹ DW)	Accumulation organ	Experiment	Additional information	References
Oregano	<i>Oregano vulgare</i>	40.6	-	0.14	-	F	No detail for each plant	[84]
Parsley annual	<i>Petroselinum crispum</i>	94.2	-	0.03	Storage organ		Garden soil	[47]
Parsley biennial		0.41	-	0.46	-		-	[16]
Peanut		159.4	-	0.42	Leaves		Garden soil	[47]
Pepper	<i>Arachis hypogaea</i>	94.2	-	1.73				
Peppery bolete	<i>Piper nigrum</i>	1 837	-	314	Shoots		-	[36]
Potato	<i>Chalciporus piperatus</i>	-	-	0.016	Bay	-	Retail network	[80]
Prickly lettuce	<i>Solanum tuberosum</i>	82.5	-	1423	Fruits body	F	Mushroom	[88]
Radish	<i>Lactuca serriola</i>	-	-	0.02	Storage organ		Garden soil	[47]
Red beet	<i>Raphanus sativus</i>	-	-	5.12	-		-	[82]
Rice	<i>Beta vulgaris</i>	159.4	-	2.06	-		-	
Rye	<i>Rice</i>	75	-	0.09	Storage organ		Garden soil	[47]
	<i>Secale cereale</i>		SbCl ₃	52.5/26.6/12.3	Roots/seeds/leaves	H	No specie details	[79]
			SbCl ₅	73/44.9/44.4			-	
Shallot	<i>Allium fistulosum</i>	142.3	-	<0.02	Grains	F	Agricultural soil	[47]
Soybean	<i>Glycine max</i>	-	-	3.57	-		-	[82]
Spinach	<i>Spinacia oleracea</i>	500	KSbO-tartrate	1.01	-	P	-	[47]
Sugar beet	<i>Beta vulgaris</i>	266.3	-	399	Leaves		Garden soil	
		34.3	-	1.13	Storage organ	F	Agricultural soil	
Sunflower	<i>Helianthus annuus</i>	10 000	-	0.02	Leaves	P	potting mix. gd	[87]
			Sb ₂ O ₃	~700	-		Agricultural soil. gd	
			KSb(OH) ₆	~200	-		-	
Sweet potato	<i>Ipomoea batatas</i>	5 000	-	~40	-	F	-	[82]
Sword bean	<i>Canavalia gladiata</i>	-	-	~25	-		-	
Tangerine	<i>Citrus tangerina</i>	-	-	2.26	-		-	
Tomatoes	<i>Lycopersicon esculentum</i>	-	-	4.14	-	-	Retail network	[80]
			-	0.02	Fruits		Garden soil	
Water spinach	<i>Ipomoea aquatica</i> Forsk	266.3	-	0.014	-	F	-	[47]
Wheat	<i>Triticum aestivum</i>	-	-	<0.02	-	F	-	[82]
			-	7.27	Roots/stems/grains		Agricultural soil	[26]
			-	<0.02	Grains	P	gd	[47]
			KSbO-tartrate	~400	-	H	-	[83]
			SbCl ₃	80.3/17.8/4.06	Roots/seeds/leaves		-	[79]
			SbCl ₅	42.3/12.4/2.82	-		-	
White radish	<i>Raphanus sativus</i>	-	-	1.8	-	F	-	[82]
Wild Rosemary	<i>Rosmarinus officinalis</i>	539	-	~0.48/0.8	Stems/leaves		gd	[81]
Yacon	<i>Polymnia sonchifolia</i>	-	-	1.83	-		-	[82]
Yellow boletus	<i>Suillus luteus</i>	-	-	225	Fruits body		Mushroom	[88]

- = No information available; F = Field survey; P = Pot; H = Hydroponic; gd = graphical.

Table 4
Review of Sb content in rice in relation with the form of Sb and type of experiment.

Plant species	Sb exposition (mg kg ⁻¹ or mg L ⁻¹)	Chemical form	Maximum Sb concentration (mg kg ⁻¹ DW)	Sb specie	Accumulation organ	References
<i>Oryza sativa</i> L. cv. Jiahua	1562	Sb ^V (~80%)	511/11.5	Sb ^V	Root/shoot	[109]
–	–	–	0.93	–	–	[95]
<i>O. sativa</i> cv. Nanjing 45	0–5	KSb(OH) ₆	1.4/0.30/0.28	Sb ^V (>75%)	Root/stem/leave	[106]
		KSbO-tartrate	12.5/1.30 /0.30	Sb ^V (>78%)		
<i>O. sativa</i> cv. Yuhong No. 1	0–1	KSbO-tartrate	~1	–	Root/stem–leave/seed	[110]
		KSb(OH) ₆	~1	–		
–	–	–	0.013	–	seed	[111]

– = No information available.

plants (cf. 3.2.), Sb accumulation in rice is both Sb species and rice cultivar dependent [106,107], which has to be carefully taken into account in risk assessment studies [1]. Finally, Sb human bioaccessibility in rice seems not to have been studied at all to this point, the authors suggest therefore focusing further studies on that topic to assess the risk of Sb human exposure in eating rice grown on a Sb contaminated site.

3.4. Antimony in herbs

In urban areas many people grow herbs *via* various methods: either on their balconies or in their gardens. These plants may be either cooked or eaten fresh. They are not always well washed, which can increase the ingestion of Sb present in soil particles. While there is no data available about Sb in such urban-grown herbs, its transfer from wild rosemary harvested in contaminated soil has been studied [93], either when used as an essential oil or in herbal tea. In the first case, the very low Sb content found in wild rosemary oils indicates that it can be used even if it grew on a highly contaminated soil ([Sb]_{soil} = 309 mg kg⁻¹). Concerning the risk of contamination by boiling to prepare infusions, it depends on the elemental and leaf concentration of Sb. Under experimental conditions, the transfer ratio was low, and the final Sb value in the infusion was below the official threshold [33]. To reach this permissible value (5–6–20 µg L⁻¹) depending on different legislation [112–114], one would have to drink at least 387 L of herbal tea coming from the contaminated site. Although their results reveal no important health risk in their conditions, they ask for further research focused on Sb speciation, because it is already known that an inorganic pollutant's bioavailability is directly linked to its speciation (cf. §2.3 and [17,45,115,116]). The results of Affholder et al. [93] showed that for some herbs (i.e., wild rosemary), Sb seems not to be a threatening element. Results on coriander, dill and parsley seem to show a low accumulation of Sb (6.65, 0.14, and 1.73 mg kg⁻¹, respectively; Table 3), but not enough detail is available concerning the accumulating organ, except for parsley, in which As has been measured in leaves. Additional studies should be performed to confirm whether or not herbs present a risk regarding Sb bioaccumulation and bioavailability. If that is not the case, such plants could be proposed to gardeners when their soils present a proven risk of Sb contamination.

3.5. Lack of data about Sb localization in plant organs

One of the issues regarding metal(loid) accumulation in edible plants is their localization (organs, tissues, etc.). Since different edible types exist (leaves, roots, fruits, stems, etc.) and some vegetables are eaten peeled and others not, for these vegetables it is necessary to determine which tissues and/or organs are Sb sinks. Many studies represent plants only as a two-compartment organism (shoot/root) (Table 3), leading to a lack of data about the real Sb-accumulating organ (stem, leave, fruit, etc.). This shows that actual knowledge

about Sb accumulation and compartmentation in edible plants is still fuzzy. It seems essential to homogenize the methods, in order to make the results comparable.

What we actually know is that Sb localization in plant organs is variable and species-dependent. For example, in the case of some plant species, such as *Achillea ageratum* L., high Sb concentrations have been found, either in basal leaves (>1367 mg kg⁻¹) or in flowers (1105 mg kg⁻¹) [117]. On the other hand, some fern plants, such as *Pteris cretica* Retz, can accumulate up to 6405 mg kg⁻¹ of Sb in their root system [55]. Recently, it has been shown that in the hyperaccumulator *P. vittata*, Sb^{III} accumulates more than Sb^V, with all the Sb accumulating only in the roots [89]. This fern species was also reported to hyperaccumulate different species of arsenic, mostly in its fronds (93%) [118]. Recently, Affholder et al. [93] reported 309 mg kg⁻¹ of Sb in wild rosemary (*Rosmarinus officinalis* L.) roots, cultivated on multi-metal contaminated soil in southern France, under dry conditions. However, the translocation of Sb from roots to aerial parts was very limited. Similar results of reduced Sb translocation to aerial parts were also reported by Pérez-Sirvent et al. [119] for yellow fleabane (*Diurichia viscosa* L.), when grown in mining-affected semiarid soils in southeast Spain.

Another point is that one-quarter of the above Sb values (Table 3) comes from measurements performed on fruits and vegetables, taken directly in the retail network [92], which give no information about soil content, pesticide exposure, and proximity to roads and/or factories. In some cases, Sb has been detected inside fruits (for example, apple pulp – 0.011 mg kg⁻¹; pepper bays – 0.016 mg kg⁻¹; and tomatoes – 0.014 mg kg⁻¹). These concentrations cannot only be credited to soil-to-plant transfer. Aerial deposition onto leaves and fruits could explain such results [21,22]. Moreover, with the increasing quantities of ultrafine atmospheric particles in urban and peri-urban areas, foliar plant exposure could sometimes be the main route of plant pollution in aerial organs [23,120].

Antimony is often considered to behave similarly, but not always, to arsenic (As) [2,121]. Concerning As localization, one study showed that As accumulates more in open-leaf vegetables (e.g., lettuce, spinach, etc.) than in others [122]. Underground products, such as carrots and potatoes, seem to stock more As in their skin than in their flesh, which is not true for aboveground products, such as apples. Such specific studies have not been done for Sb, for which data are still scattered, as shown in Table 3. Nevertheless, in Table 3, Sb preferential accumulation seems to occur more in root than in shoot, suggesting a small translocation factor, except for maize, where accumulation is higher in leaves.

All these data highlight the urgent need to define maximal Sb values for food safety in urban areas, and to determine the key relationships between the different Sb compartments (soil, water, plants, atmosphere, etc.), in order to develop provisional models of Sb behavior. Although various models exist to simulate and foresee metal(loid) speciation and behavior in soils and factors affecting it [123], Sb is not yet available in such tools.

Table 5
Mycorrhizal response under ETM stress.

Mechanism	Action mode	Molecules	Metal(loid) studied
Extracellular inactivation	Hyphal exudation of complexing agent Association with bacteria Exudation of redox enzymes	Glomalin, phenolic, citric, malic or oxalic acids aggregates chelating agents Superoxide dismutase	As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Zn Cd, Mn, Pb, Tl, Zn Zn
Binding in fungal walls	Structure of cell wall with ETM binding sites	glucans, chitins and galactosamines polymers, small peptides and proteins, glomalin	Cd, Cu, Ni, Zn
Intracellular inactivation	Increase of ETM efflux Intracellular compartmentation: vacuoles, vesicles, spores	Protein carriers or permeases Chelators and then transporters: same molecules as above, fungal AND plant metallothionein	Cd, Cu, Zn As, Cd, Cu, Ni, Zn
Response to oxidative stress	Synthesis of molecules of resistance to oxidative stress (enzymatic and non-enzymatic pathways)	Glutathione, vitamin C, E and B6, catalase, superoxide dismutase, thiol reductase	As, Cd, Cu

4. Bioaccumulation in microorganisms and soil fauna

As mentioned earlier, as Sb is not known as an essential element for living organisms, and as its toxicity is lower compared to Pb or Cd, it has been poorly studied. Its increase during the last decades has recently led to heightened awareness in the scientific community. Data about its accumulation in different organisms are still weak and scattered, but are gradually being enriched. Here is gathered the current knowledge concerning Sb bioaccumulation by soil flora and fauna.

4.1. The fungal case

For millions of years, terrestrial plants have developed close relationships with different kinds of bacteria and fungi, seeking to increase their performance, particularly in terms of inorganic element absorption [124], resistance to stresses such as metal(loid)s, and development capabilities through their symbiosis [125]. Mycorrhizal symbionts are associated with plant roots, and are present in almost every terrestrial ecosystem. A detailed classification of symbiotic fungi exists, and can be summarized as follows: trees are associated mainly with ectomycorrhizal fungi, while about 94% of angiosperms are associated with endomycorrhizal fungi [126]. The main difference is that ectomycorrhizal fungi develop hyphae that surround root cells, but do not enter inside, while endomycorrhizal species penetrate into cortical root cells. Plants usually present these symbioses, and non-mycorrhizal ones are very rare. This symbiosis is a mutual exchange, where the plant transfers a portion of its photosynthesized carbon compounds, while the fungus enhances phosphorus and other nutrient absorption, soil exploration [127,128], and drought tolerance. It is also well-documented that these symbioses induce physicochemical changes in the mycorrhizosphere [129].

In agriculture, such symbiosis usually leads to increased harvest yields, but sometimes the mycorrhizal growth response is negative. This can occur for different reasons, such as arbuscular mycorrhizal fungi parasitism, where the benefits of increased nutrient uptake do not outweigh the fungus' carbon sink [127].

Mycorrhizal fungi have also been studied at the soil–plant interface for their capability to be either a barrier or an enhancer of metal transfer, through a large range of metabolic pathways. These results were reviewed few years ago [130] and Table 5 presents a synthesis of current actual knowledge. For example, the study of As transfer at the soil–AMF–plant interface led to a wide range of results, showing either accumulation enhancement permitting phytoextraction/remediation [131], or a phytoprotective role with decreased As in plants [132]. In lettuce, the combined addition of phosphorus and AMF could reduce As transfer from a contaminated soil (250 mg kg^{−1} As) to plant: −34% in roots, and −60% in leaves, in comparison with the control [133].

Mechanisms of interaction between AMF and plants, with regard to metal(loid)s, have been widely described [134], but Sb never appears in such publications. Thus, even if Sb behavior in soil has been compared to As in many ways [2], further research has to be done to clearly define under which conditions and parameters it is possible to transpose As behavior to Sb at the soil–plant interface.

4.2. Antimony and fungal relationships

Very poor information has been published concerning the Sb–fungal relationship. Research has been done on non-symbiotic fungi, such as *Penicillium notatum* and *Scopulariopsis brevicaulis* [79]; the latter being already known as a methylator of As inorganic compounds [135]. *S. brevicaulis* has the capacity not to accumulate Sb, but rather to synthesize trimethylantimony – (CH₃)₃Sb – in the presence of inorganic Sb (both Sb₂O₃ and Sb₂O₅), under aerobic conditions (detailed results in Supplementary material Table A.3) [135]. Interestingly, in the same year, different results were published for the same fungus, with no (CH₃)₃Sb detected, with the addition of another Sb^V species – Sb(OH)₆ – [136]. Even if the fungus studied was the same, it is rather difficult to compare these experiments because both the growing conditions and Sb species differed, as well as the fungal form used (spores vs. mycelia ball). The important Sb biosorption capacity (>90%) of three macro-fungi (*Agaricus campestris*, *Amanita muscaria*, and *Trametes gibbosa*) from Sb-contaminated water has also been reported, but further investigation on metal-binding mechanisms is still needed [137].

Concerning the relationship between mycorrhizal fungi and Sb in soils, one study has been reported on ectomycorrhizal fungi [101]. These organisms, as well as endomycorrhizal fungi, are already known as metal(loid) hyperaccumulators [138]. In their study, the authors sampled fungi on tailing piles and slag dumps (old As/Sb mining sites), and found no major differences for Sb concentration between ectomycorrhizal and saprobic fungi. Interestingly, among all their samples Sb content in the soil was higher than in fruit bodies, which could indicate that mycorrhizal fungi play a barrier role against this metalloid. However, the case of some ectomycorrhizal fungi (*Chalciporus piperatus* and various *Suillus*) able to accumulate Sb up to 10³ mg kg^{−1} is also mentioned. This suggests that these genera possess a specific biological metabolism to mobilize and concentrate Sb from the soil, while Sb is generally present in species with poor solubility (cf. §2.2, [57]). How then do some mycorrhizal fungi influence Sb speciation in the soil compartment? What about the reactivity of these newly formed Sb species under the influence of mycorrhizal fungi?

Other recent studies found (CH₃)₃Sb and another unidentified Sb species in some herbaceous plants [51], suggesting the role of the microbial community in the synthesis and transfer of these compounds. As stated earlier, it is well-documented that herbaceous plants generally develop more endomycorrhizal symbioses (such as AMF), suggesting the role of AMF in the biosynthesis and transfer

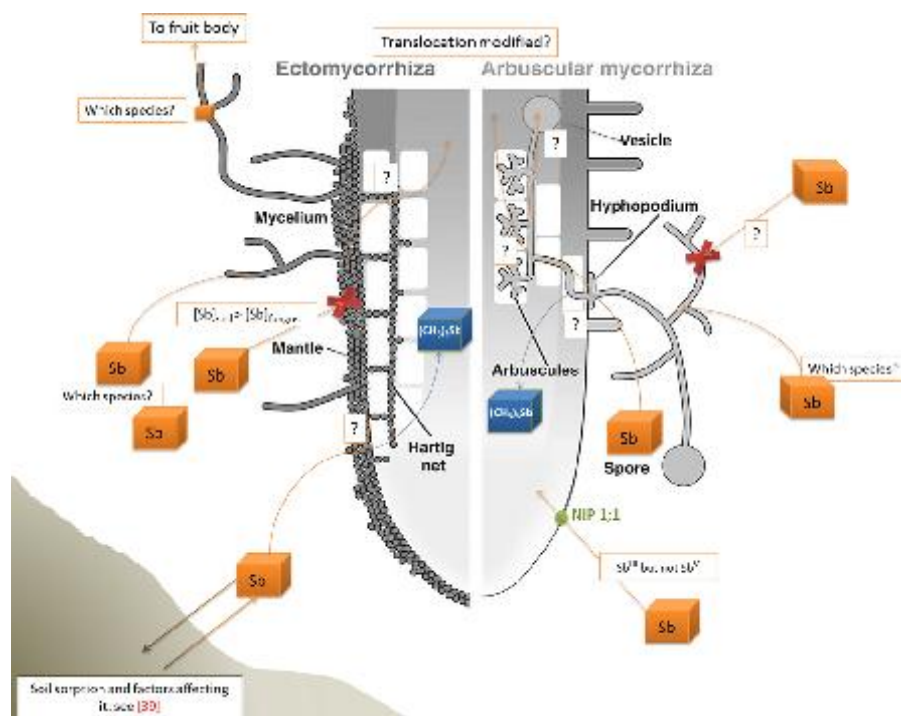


Fig. 1. Mycorrhizal role in Antimony (Sb) transfer from soil to plants.

[?] Represents the actual mechanisms to be elucidated. Sb regroups the different Sb oxidative states and species.

of $(\text{CH}_3)_3\text{Sb}$ to herbaceous plants. Fig. 1 presents the current state of knowledge of the possible ways for Sb to gain entry from soil to plants, either when associated with mycorrhizal fungi or without these organisms. 'Sb' refers without distinction to the various Sb species which have been found in soil and soil water, because not enough data exist about specific pathways for any Sb species. The different possible fluxes of Sb from soil to both plants and fungi are represented by arrows. As shown in Fig. 1, and summarized in this review, portions of these mechanisms are already known [39,88], but others have not yet been described (mostly the entirety of the fungal pathway). Nothing is known about how mycorrhizal fungi either absorb, adsorb and/or transform Sb at the soil–fungal–plant interface.

4.3. Biofertilizers in agriculture

With the development of biological agriculture, arbuscular mycorrhizal fungi are now produced and sold in almost every garden center, both for agriculture and casual gardeners, as biofertilizers [139]. As mycorrhizal symbiosis is known to affect different physiological parameters, such as stomatal conductance and fruit development [140–143], it might participate in increasing the entry of metal(loid)s through stomata (i.e., leaves) and fruits. Consequently, in some cases, these biofertilizers could also be factors in the increase of metal(loid) uptake by plants [144]. Therefore, research needs to be conducted, to determine if most fungi able to methylate As have the same capability with regard to Sb, especially in the case of AMF, which could be used as a barrier between edible plants and soil on Sb-contaminated soils. Such results would also allow conclusions to be drawn as to whether or not the knowledge we already have about the relationship between AMF and As is transferable to Sb compounds, and to what extent.

4.4. Bioaccumulation by the soil fauna

In addition to their key role in soil fertility, earthworms, as the major living organisms in soils, influence metal(loid) behavior in

soil through bioturbation [145]. In the case of Sb, Nannoni et al. [145] concluded that soil ingestion is the predominant means of exposure and absorption (Pearson correlation (PC) between [Sb] in earthworms and Sb extractable fraction = 0.88, $p < 0.001$). Nevertheless, they also indicated that skin penetration is not negligible (PC = 0.62, $p < 0.05$). In their experiment, total Sb concentration in earthworms varied from 0.04 to 1.1 mg kg^{-1} (on clean and contaminated soils, respectively). High Sb concentrations could also cause morphological abnormalities and low activity in *Perionyx excavates* [96]. Such inhibitory effects on earthworms might cause a loss of fertility. In the case of Sb, the BAF is very low, indicating that, for earthworms, the total Sb concentration in the soil is not a good predictor of their possible contamination, while the extractable fraction seems to fit this role better. It also indicates that these species do not accumulate Sb intensively from the environment, so that Sb will not spread and accumulate through food webs via these organisms. Moreover, as shown for other metal(loid)s, such as Pb, Cu, Cd, Zn, Cr, Co, and Ni [18,52], earthworms can modify metal bioavailability in soils. For example, earthworm activity on a contaminated soil led to a 46% increase in Cd and Pb in lettuce leaves, owing to improved soil–plant transfer [41]. As earthworms are mainly interested in soil organic matter, the same authors also discussed Sb–soil organic matter interactions. *Eisenia fetida* has also been shown to biotransform As without excreting it after exposure, until its death [146]. This led to a decline in the As concentration in the soil during this period, but no data was given about As speciation or transfer when these organisms die and decompose in the soil. Such effects have not yet been demonstrated for Sb. Fig. 2 represents the actual state of knowledge about Sb behavior in soil–earthworm systems with excreted castings. It shows that such organisms can absorb Sb and further change its bioavailability, but the Sb species involved have not yet been clearly identified.

Typically, the bioaccumulation of Sb by soil microfauna varies with their habitat and species type. Antimony concentrations in terrestrial invertebrates (30.4 mg kg^{-1} dry wt.) are generally higher than those in aquatic invertebrates (5.2 mg kg^{-1} dry wt.) and

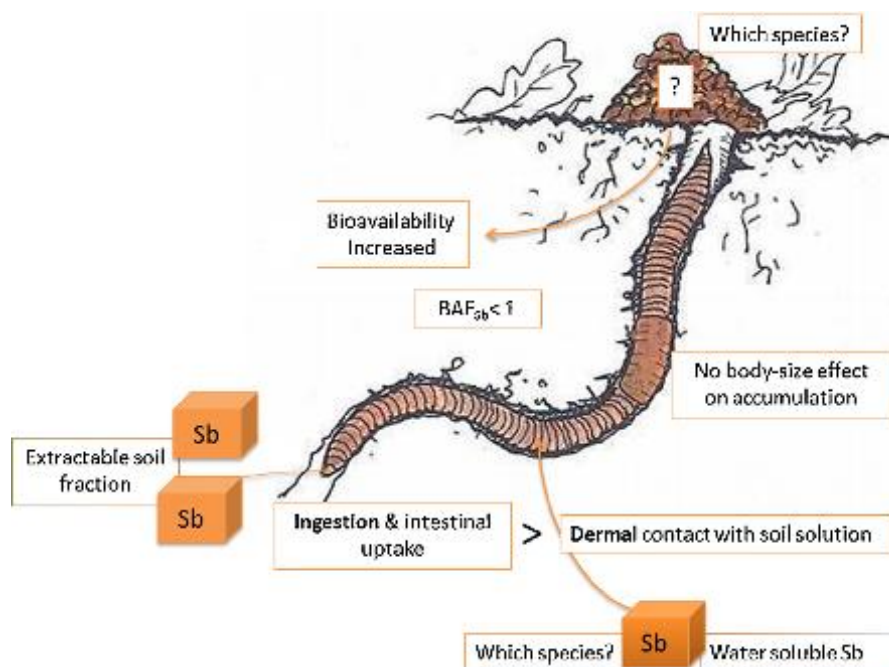


Fig. 2. Antimony bioaccumulation in earthworms.

Original earthworm drawing (www.onf.fr). BAF_{Sb} = bioaccumulation factor of Sb.

amphibians (2.3 mg kg^{-1} dry wt.) [147]. Some terrestrial invertebrate such as earthworms have already been shown to accumulate Sb [54]. Such disparity could be explained by differences in Sb compartmentation (in particular, soil organic matter influence) or speciation and in diet of these living organisms (soil consumption, water filtration, etc.). The same authors [147] also reported high Sb concentrations in *Acrida chinensis* and *Pheretima aspergillum*: 17.3 and 43.6 mg kg^{-1} , respectively, within 1 km of an Sb mining area. Pauget et al. [148] noted the high availability of Sb to snails, at three industrially impacted sites in northern France. They studied Sb accumulation kinetics from the soil into these organisms, and showed that CaCl_2 extract concentrations were the best predictors of Sb bioaccumulation. As noted earlier, organic matter (OM) participates in Sb availability, and the relatively high level of OM in their study area (up to 10%) could partially explain such results.

Up to the present, there is no data available concerning Sb accumulation in other living macro/meso-organisms in the soil compartment. It is, therefore, difficult to precisely identify the possible pathway of Sb through the food chain.

5. Human health risks assessment

People working with Sb compounds are subject to Sb inhalation, mostly antimony trioxide. For the rest of the population, food represents the predominant source of Sb exposure. Its absorption through the digestive tract has been estimated between 5 and 20% of the total Sb content ingested [149]. In 1992, urban dwellers were exposed to about $60\text{--}460 \text{ ng day}^{-1}$ through inhalation [150]. Nowadays, this value has certainly increased with the increase of Sb uses (since 1992) around the world.

5.1. Food-chain biomagnification

Antimony biomagnification has not been investigated much as yet, but some studies have intended to evaluate this parameter [151,152]. However, these studies did not discover any evidence of Sb accumulation across the food chain, but their intention was not necessarily to assess trophic linkage. Therefore, this conclusion is not guaranteed. In any case, biomagnification only considers the

xenobiotic accumulation in an organism through its daily alimentation [153]. For example, as shown earlier (cf. §4.2), some fungi have been identified as Sb hyperaccumulators [101], with concentration exceeding 1400 mg kg^{-1} in the fruiting body of *C. piperatus*. Consequently, they can become Sb sources in the food web, through slugs, then ducks or chickens, and then humans (or directly to human beings in the case of mushroom consumption). Nevertheless, these organisms are more sensitive to soil contamination than aerial deposition because of the short fruiting period (10–14 day) in which they could accumulate metalloids from dusts. However, it would be necessary to consider other sources of Sb exposure (inhalation, skin contact, etc.), and to focus not only on the biomagnification factor but also on the bioaccumulation factor, which takes into account every kind of exposure. Investigations need to be performed on the transfer of Sb from cereals, such as wheat and maize (which are known to accumulate Sb up to 700 mg kg^{-1}), to poultry and livestock, in order to give clues about the risk of transfer through the plant–meat–human food chain. However, little risk of Sb bioaccumulation seems to exist for herbivores, even when their grassland diet suffered major contamination near an Sb smelter [154]. Indeed, rabbits and voles presented relatively high levels of Sb in different organs (0.30 and 0.68 mg kg^{-1} DW in voles and rabbit Liver respectively) when they fed in contaminated sites ($<250 \text{ m}$). However, these concentrations were considered not be harmful as laboratory animals (mice and voles) presented no visible diseases when fed with even higher concentrations than in contaminated grasslands ($<0.02\text{--}8.6 \text{ mg kg}^{-1}$ DW when fed with 6700 mg of Sb per kg DW); except in mice liver which could accumulate Sb up to 46.2 mg kg^{-1} DW [84]. Same authors nonetheless suggest investigating longevity, resistance to stresses and breeding success in field experiment to complete their study. It will also be necessary to focus on soil–livestock Sb transfer, because their daily soil intake can be potentially high (up to 30% for sheep) [155]. Although no data have been published on Sb transfer from soil to cattle, the previous study on As also showed that 34 to 90% of animal intake comes from ingestion of polluted soil particles.

Concerning aquatic ecosystems, much information can be found in the review published by Filella et al. [32] about the different types of Sb speciation, transfer and pathways, with a focus on microbiota

interactions. At the macrobiotic scale [156], antimony concentration in freshwater fish (Crucian, carp, wild carp, grass carp, herring, and bighead carp) can reach $809 \pm 360 \mu\text{g kg}^{-1}_{\text{DW}}$. This might be due to highly contaminated algae ($>11 \text{ mg kg}^{-1}$) consumption. This demonstrates that Sb could transfer and accumulate through these fish up to human beings.

Antimony can also reach human directly through food packages (plastic, ceramic, drinking cup, etc.) as it is used in alloy during the production processes. An accurate method has therefore been developed to measure Sb in the leaching of these packages and showed concentrations about 1.6 mg L^{-1} [157].

Consequently, many fields of investigation need to be clarified, to permit a good understanding of Sb behavior in the environment (soil, water, and living organisms), and through the food chain.

5.2. Human bioaccessibility of Sb

The human bioaccessible fraction of a metal(loid) is defined as the fraction extracted by the entire digestive system when it is ingested with polluted soil [158], or polluted vegetables [20,21]. A standardized bioaccessibility test has been developed by the BioAccessability Research Group of Europe (BARGE): the Unified BARGE Method (UBM) [159]. The UBM simulates the digestion process with synthetic digestive solutions (mouth, gastric and gastrointestinal). It mimics all the chemical reactions occurring throughout the digestive tract, with appropriate physiological transit times and temperatures [160]. It has been validated with *in-vivo* tests for As, Cd and Pb in contaminated soils, with the measurement of these elements at four endpoints (kidney, liver, bone, and urine), in swine grown and fed with such polluted soils in their diet [161]. However, the UBM for Sb did not achieve validation *in-vivo*, except in urine, because of its low concentration in soil samples. Nevertheless, using the measured bioaccessibility it is then possible to evaluate and approach the human bioavailability of Sb.

Different studies focused on the daily intake of Sb by measuring the total Sb concentration in aliments eaten by Chinese people (between 0.252 and $9.3 \mu\text{g}(\text{kg bw})^{-1}$; details in Supplementary material Table A.4) [95,162]. However, as with almost every value cited in the literature (Table 3), they were concerned with total Sb content, but give very little information as to speciation and human bioaccessibility and bioavailability in the case of consuming polluted vegetables.

Recently, some studies focused on Sb bioaccessibility and ecotoxicity, in relation to soil remediation [159] and Sb bioaccessibility in vegetables, with regard to the context of the pollution (polluted soil or atmosphere) and plant species [20,21]. Gastric Sb bioaccessibility was 14% and 43% for spinach and cabbage, respectively. Such variations could come from differences in leaf morphology, and/or changes in Sb speciation throughout the plant [23].

Currently it remains unclear how Sb will transit through the different nodes of the food web. The question arising then is: are these Sb compounds bioaccessible to humans when digested? If they are, in what proportions, and what are the species absorbed? Finally, what about their toxicity, and what are the risks of eating vegetables grown in contaminated soils, such as urban and peri-urban soils, where the metal(loid) levels are increasingly alarming?

6. Conclusion and perspectives

The present review gathers the current state of knowledge on Sb behavior in edible plants, and the factors affecting it in the context of polluted arable lands and urban areas. While a portion of urban and peri-urban soils are used for agriculture, Sb production has been increasing continuously for 50 years ($\sim 55,000 \text{ t/year}$ in 1960; $163,000 \text{ t/year}$ in 2013) leading to an increasing risk for

human health. Many contamination sources exist, and gardeners' cultural practices could participate in transferring Sb from soil to plants. Such practices can lead to sanitary consequences, as shown by Sb values found in some edible plants grown on polluted soils (Tables 3 and 4).

The major issue is the lack of data concerning Sb values, speciation and compartmentation in vegetables, as well as its behavior with arbuscular mycorrhizal fungi, such organisms being known to play a major role in metal(loid) transfer at the soil–plant interface. Actually, there is still no legal threshold for Sb in edible plants. Thus fields of investigation are proposed to complete our understanding of the health risks when growing food in Sb-contaminated soils.

Research needs to focus on the immobilization and transfer mechanisms of metal(loid)s such as Sb, in order to develop future strategies and guidelines for sustainable agriculture and urban agriculture. For example, a better understanding of the different pathways and interactions could lead to solutions for gardeners, such as crop association or rotation adapted to their soil contamination. To choose plant species according to their potential to absorb or exclude a few inorganic elements and to choose fertilizers and amendments in function of their composition, could enable plant quality and soil ecosystemic services to be optimized. Measuring Sb human bioaccessibility in edible plants, and modeling such transfers at soil–plant–human interfaces are finally fields of investigation to integrate into future experiments and risk assessments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhazmat.2015.02.011>.

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Antimony bioavailability: Knowledge and research perspectives for sustainable agricultures.

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Supplementary material

Table A.1: Soluble antimony fraction, expressed as the ratio ($Sb_{\text{water-extractable}}/Sb_{\text{total}})_{\text{soil}}$

	References	Sb species	[Sb_{total}] in soil, mg.kg^{-1}	Sb_{sf} %
Contaminated soil	51	-	5 045	2,2
	36	-	1 837	1,8
Experiment with spiked Sb	54	$KSb(OH)_6$ -potting mix	10 000	2,9
		Sb_2O_3 -potting mix	10 000	0,7
		$KSb(OH)_6$ - agricultural soil	5 000	0,6
		$KSb(OH)_6$ -agricultural soil	5 000	0,6
		Sb_2O_3 - agricultural soil	5 000	0,5
		Sb_2O_3 -agricultural soil	5 000	0,5

Table A.2: Antimony Bioaccumulation Factor (BAF) in edible plants. BAF represents the ratio $[Sb]_{\text{plant}}/[Sb]_{\text{total}}_{\text{soil}}$. BAFw represents the ratio $[Sb]_{\text{plant}}/[Sb]_{\text{soluble}}_{\text{soil}}$. When known, the bio-accumulative organ and Sb species are specified.

	Plant	Organ	Sb specie	max [Sb] _{total} _{soil} mg.kg ⁻¹	max [Sb] _{soluble} _{soil} mg.L ⁻¹	[Sb] in edible	BAF	BAFw
Sb present in soil	Peanut	Shoots	-	1837	33.07	314	0.17	9.50
	Maize		-	-	28.75	100	-	3.48
		roots	-	-		78	-	2.71
	Colza	leaves	-	5045	112.30	121	0.02	1.08
	Oregano	-	-	0.41	-	0.46	1.12	-
	Beet	-	-	0.41	-	0.03	0.07	-
	Eggplant	-	-	0.41	-	0.03	0.07	-
	Lucerne	-	-	14	-	1.75	0.13	-
	Cucumber	-	-	40.6	-	0.14	<0.01	-
	onion	-	-	40.6	-	0.14	<0.01	-
	Wild rosemary	leaves	-	539	-	0.8	<0.01	-
		Stems	-		-	0.48	<0.01	-
	Sunflower	Leaves	Sb ₂ O ₃ -potting mix	10000	68.73	700	0.07	10.19
Experiment with spiked Sb	Spinach		KSbO-tartrate, 3H ₂ O	500	44	399	0.80	9.07
	Maize	-	Sb ₂ O ₃ - agricultural soil	5000	25.51	170	0.03	6.66
		-	KSb(OH) ₆ - agricultural soil		29.27	180	0.04	6.15
	Sunflower	-	Sb ₂ O ₃ -agricultural soil	5000	25.51	40	0.01	1.57
	Wheat	Roots	SbCl ₃	-	75	80.3	-	1.07
	Rye		SbCl ₅	-	75	73	-	0.97
	Sunflower	-	KSb(OH) ₆ -agricultural soil	5000	29.27	25	0.01	0.85
	Rye	Roots	SbCl ₃	-	75	52.5	-	0.70
	Sunflower	Leaves	KSb(OH) ₆ -potting mix	10000	286.53	200	0.02	0.70
	Rye	Seeds	SbCl ₅	-	75	44.9	-	0.60
		Leaves		-		44.4	-	0.59
	Wheat	Roots	SbCl ₅	-	75	42.3	-	0.56
	Rye	Seeds	SbCl ₃	-	75	26.6	-	0.36
	Wheat			-	75	17.8	-	0.24
			SbCl ₅	-		12.4	-	0.17
	Rye	Leaves	SbCl ₃	-	75	12.3	-	0.16
	Wheat			-	75	4.06	-	0.05
			SbCl ₅	-		2.82	-	0.04
	Colza	-	KSbO-tartrate, 3H ₂ O	1600	-	2236	1.40	-
	Mung bean	-			-	1400	0.88	-
	cucumber	-			-	600	0.38	-
	Wheat	-			-	400	0.25	-

Table A.3: Presence or absence of trimethylantimony compounds when *Scopulariopsis brevicaulis* is exposed to different Sb species under different experimental conditions. (III, V) represent the oxidative state of the Sb.

Major differences in experimental conditions	Sb form added (oxidative state)	(CH ₃) ₃ Sb	Reference
Aerobic, 1-3.10 ³ spores.ml ⁻¹ , 200ml Oxoid malt extract broth (Unipat Ltd Basingstoke U.K.)	Sb ₂ O ₃ (III)	Yes	92
	KSbO-tartrate (III)	Yes	
	Sb ₂ O ₅ (V)	Yes	
Aerobic, mycelia ball submerged in 400ml Cox & Alexander solution	KSbO ₃ (III)	Yes	110
	Sb(OH) ₆ (V)	No	

Table A.4: Daily intake of antimony in China with regard to Tolerable Daily Intake (TDI). The TDI is expressed in µg per kg of body weight (kg_{bw}) per day.

References	138	137
Sb Daily intake, µg.(kg bw) ⁻¹	9.3	0.252 – 0.567
TDI ³³ , µg.(kg bw) ⁻¹	6	
Localization	Near the world's largest Sb mine: Xikuangshan	Hong Kong city

1.4. Urban agriculture, to a safe auto-consumption model

1.4.1. What are we talking about?

As a very large definition, one could say that urban and peri-urban agriculture (UPA) is the act of producing food inside or in the direct vicinity of the cities. However, UPA covers a larger objective: its development reveals also its crucial role in ecological, social and individual development (McClintock, 2010). To summarize briefly, UPA began in the 19th century with the industrial revolution and the rural exodus it generated (Boulianne, 2001). Indeed, cities provided arable spaces for workers to grow their own food with the objective to maintain and develop the work power and productivity. Then, during and between World War I and II, UPA areas had an obvious alimentary role. The economic boom which occurred after World War II and in the early 70's transformed the consumption habits because of the development of industrialized agriculture and supermarkets. Hence, UPA interest decreased and shifted more to a recreational activity. This period continued until the last decade, which marked a break in UPA perception. Currently, UPA seems to gain interest because of the economic crisis and the rejection of economic and urban development models. UPA is now a complex and diversified theme, gathering multiple types of gardeners and areas with various objectives (production, social linkage, recreational ...). Currently, urban agriculture projects and literature associated are sprouting as fast as the crops themselves.

1.4.2. Urban agriculture's investigation program



As said earlier, research programs dealing with urban agriculture have been developed all around the world for the last few years¹. In France for example, the National Research Agency funded a three year investigation program called JASSUR (ANR-12-VBDU-0011), as an acronym for **J**ardins **A**SSociatifs **U**Rbains (Associative urban gardens). The main objective was to study the services provided by urban gardens in cities' sustainable development. This interdisciplinary project aimed to study urban agriculture in its entirety through five major axes:

- I Project coordination and communication.
- II Associative gardens: stakeholders for local governance & urban environment.
- III Study of the production and consumption functions.
- IV Study of ecosystemic services of regulation and support of associative gardens.
- VIII Management of soil TM pollution in urban gardens.

¹ <http://www.inuag.org/>, International Network for urban Agriculture

1.4.3. How to safely cultivate downtown?

While urban and peri-urban areas are known to be places more and more contaminated (Werkenthin et al., 2014), citizens are usually poorly aware about the risks associated with growing their own food (Dumat et al., 2015). All around the world, air, water and soil quality has been decreasing with a fast increase of both TM and other contaminants concentrations (dust, petroleum, and polycyclic compounds) (Borrego et al., 2006). Chemicals (natural and synthetic) have been accompanying the development of our societies for centuries, with their advantages (tools manufacturing, medical drugs...) and drawbacks (intoxication, drugs side-effects...). During the past decades, the regulation concerning the production, use and emission of chemicals has been reinforced with the European REACH² or the French ICPE³ regulations for example. The « Global Harmonized System » has been set up worldwide to ensure equal « environment-health » information for chemicals. We can consider therefore that progresses were made in terms of awareness concerning the potential risks to use chemical substances.

As urban soils are subject to a high economical pressure for cities development, areas available for UPA projects are often old brownfields, highly anthropic soils, or places near industries and traffic where no real estate project could set up. Hence, the question of soil, water, and air quality is crucial in these areas. Furthermore, it ought to be treated collectively and with transparency in order to permit to UPA projects to play their role of well-being vector and reducer of ecological inequalities.

Moreover, gardeners can also improve or worsen their lands through their agricultural practices. Indeed, the quality and quantity of the fertilizers, amendments, and treatments they use generate questions such as over-fertilization or the toxicity of pesticides (for both gardeners and ecosystems). For the latter, even biological agriculture can face this problematic since copper has been authorized in some chemicals while it presents a known ecotoxicity.

² REACH - Registration, Evaluation, Authorization and restriction of CHemicals

³ ICPE - Installations Classées pour la Protection de l'Environnement

To recycle organic wastes and to develop ecological cultivation practices, the use of biofertilizers and bioamendments, 'home-made' or not, such as microorganisms or compost is quite common in UPA. However, limited literature have been published concerning their influence on TM mobility at the soil-plant interface, which is the main objective of this thesis ; especially for antimony (emerging pollutant), lead and cadmium (which are often detected in urban areas).

1.4.4. If you want more!

L'agriculture urbaine au sein des jardins collectifs québécois: Empowerment des femmes ou « domestication de l'espace public » ? (Pourrias, 2014). A thesis comparing canadian and french UPA models, with a very interesting state of art on the history and concepts of UPA.

INUAG⁴: The International Network for urban Agriculture, to follow the development of UPA projects worldwide.

Réseau AGRIVILLE: A french association providing an openaccess courses platform on urban agriculture and agronomic basis. <http://reseau-agriville.com/>

JASSUR program: all the details, publications, and valorisations developed all along the project (2012-2015). <http://www6.inra.fr/jassur>

1.5. Using of biofertilizers and organic amendments to deal with metalloid contamination

Plenty of different biofertilizers and amendments (organic or mineral) are available and are used in agriculture, such as plant growth promoting bacteria, symbiotic fungi and bacteria, compost, and nitrogen fixing crops... Choosing between this pool is generally a compromise between economical cost and benefits, while environmental cost might also be considered as represented in Figure 1.9. All these products and technics will not be discussed in this manuscript as we decided to focus only on three elements: the use of symbiotic fungi, one type of compost, and one biochar amendment.

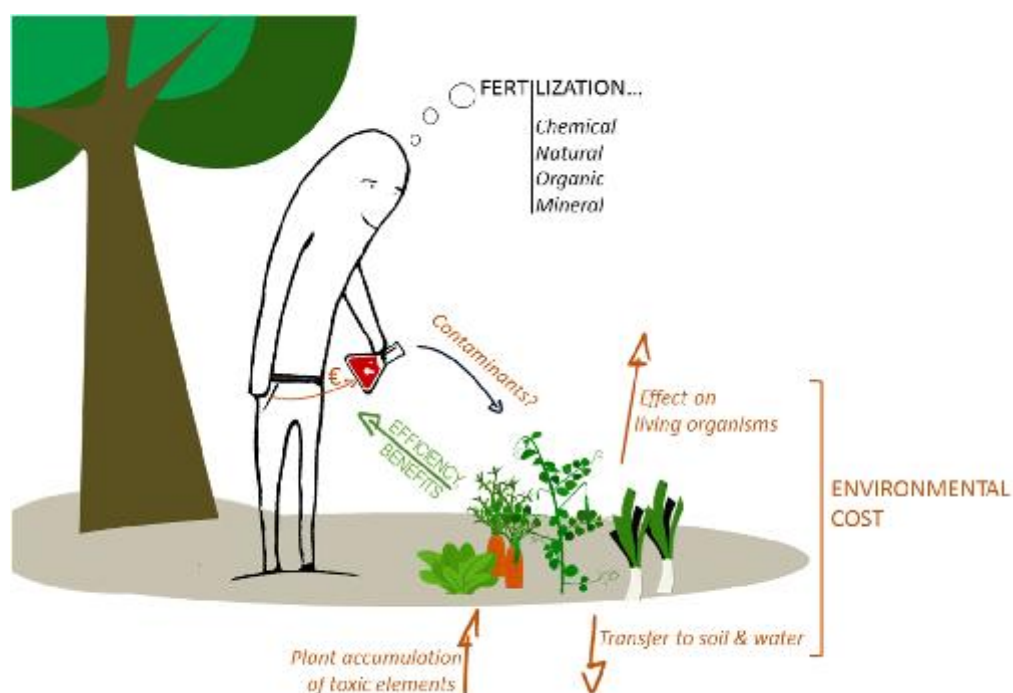
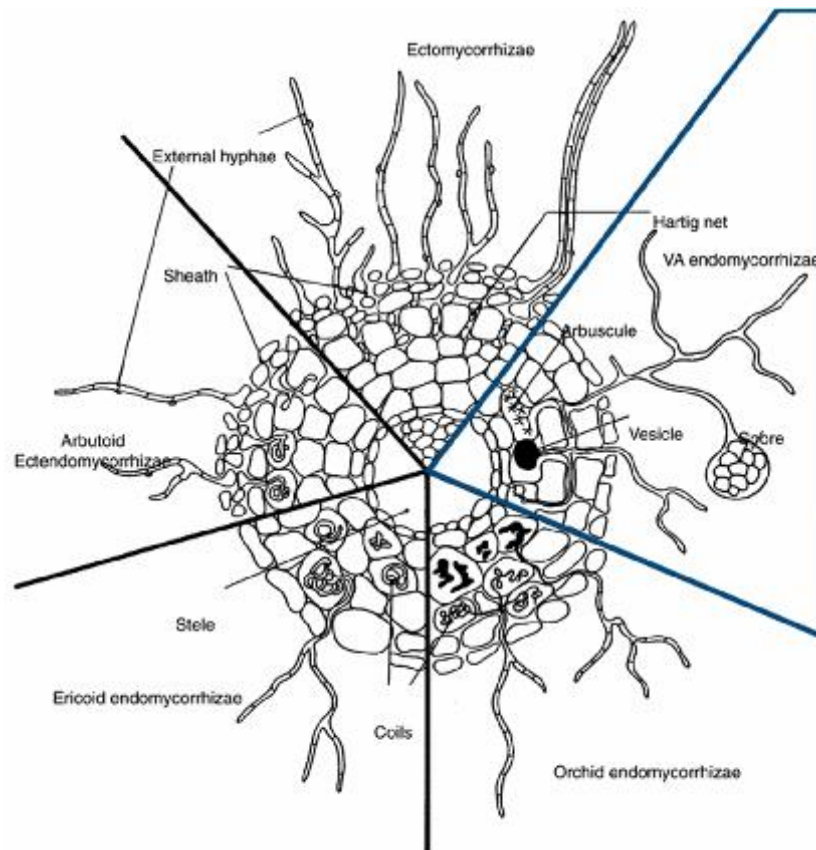


Figure 1.9: Fertilizing crops, costs and benefits.

1.5.1. Arbuscular mycorrhizal fungi

Symbiotic fungi are numerous, with a wide range of structure and physiology (Figure 1.10). To summarize, ectomycorrhizal fungi mainly associate with trees while about 94% of angiosperms are associated with endomycorrhizal fungi (Brundrett, 2009). The main difference is that ectomycorrhizal fungi develop hyphae that surround root cells but do not enter inside, while endomycorrhizal species penetrate into cortical root cells. Plants usually present these symbioses, and non-mycorrhizal ones are very rare. Indeed, this is generally considered as an evolutionary loss of this capability. The following manuscript will focus especially on one predominant group: the arbuscular mycorrhizal fungi (AMF). Their apparition is evaluated around 450 millions of years (Remy et al., 1994), playing in that time a key role in land colonization by plants.

As summarized by Selosse & le Tacon (Selosse and Le Tacon, 1998), $1 + 1 > 2$ in the case of these symbiotic associations. Indeed, such mutualistic exchanges enclose different but complementary genetic materials, permitting the emergence of beneficial traits. For example, AMF are able to solubilize inorganic phosphorus and transfer it to the host plant with water and other nutrients in exchange of photosynthetic carbon compounds (Li et al., 2013; Smith and Smith, 2011).



Selosse & Le Tacon, 1998.

'VA endomycorrhizae' corresponds to AMF

AMF symbiosis has been used for agricultural purpose for years, with the objective to decrease human's dependency on mineral fertilizers. Indeed, their use in agriculture usually leads to an increased harvest yield. However, the mycorrhizal growth response can be negative (lower yield) for various reasons, such as AMF parasitism, where the benefits of increased nutrient uptake do not outweigh the fungus carbon sink (Smith and Smith, 2011). Qualified as biofertilizers, numerous firms intend to develop fungal-based products, either in agriculture or gardening. The main interest of such fungi comes from their ability to explore a

huge proportion of soil with hyphae, permitting plants to have access to a bigger nutrient pool (Figure 1.11), especially phosphorus while the world phosphorus stock is decreasing drastically (Vaccari, 2009).

AMF have also been studied at the soil-plant interface for their capability to be either a barrier or an enhancer of metal transfer, through a large range of metabolic pathways (binding in fungal wall, excretion of organic acids and glomalin...) (Amir et al., 2014) (a detailed Table of fungal mechanisms is presented in Section 1.3). These pathways reveal either accumulation increase permitting phytoextraction/remediation (Danh et al., 2014), or a phytoprotective role with a decrease of TM in plants (Rangel et al., 2014). Thus, the mechanisms of the AMF-plant interaction concerning TM mobility have been widely described (Table 1.7). But, (1) mechanisms are highly complex and no general pattern have been found yet and (2) Sb was poorly described in these publications (Pierart et al., 2015). We focused therefore on these living organisms in the case of Pb, Cd, and Sb.

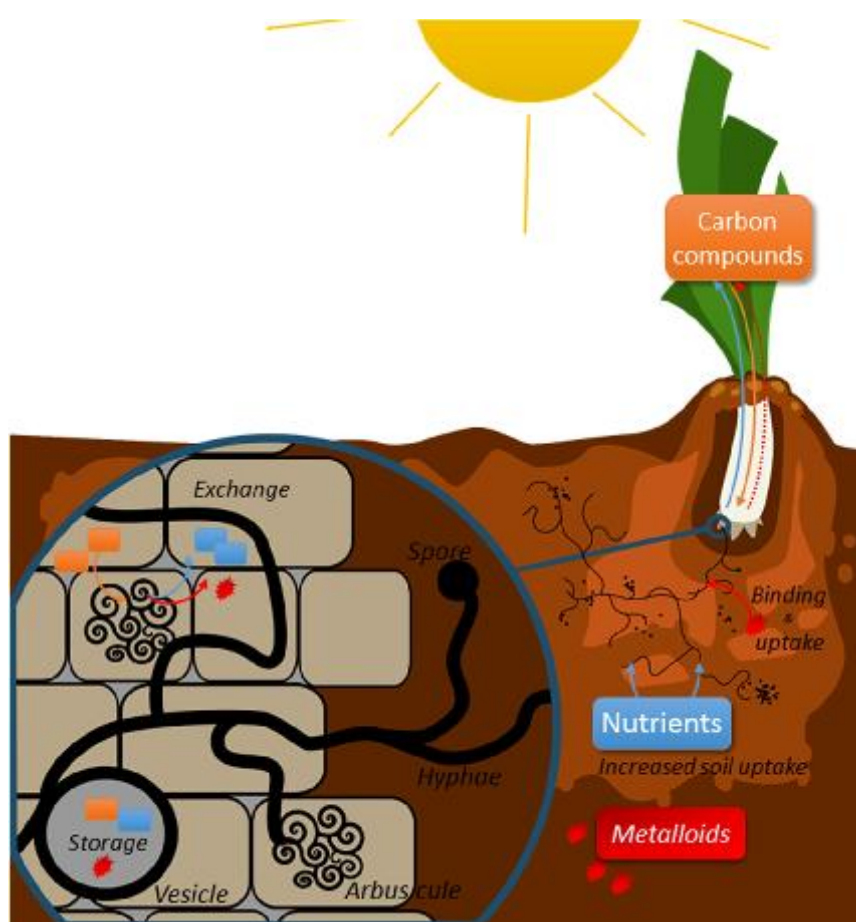


Figure 1.11: Arbuscular mycorrhizal fungi symbiosis in a nutshell-

Table 1.7: Synthesis of AMF impact on TM mobility in soil and phytoaccumulation

Reference	Exp.	[TM], mg.kg ⁻¹	AMF species	Host plant	Main results of AMF addition
Jiang et al., 2016	Pot exp., Sterilized soil (autoclave), +AMF, + TM	CdCl ₂ .H ₂ O: 5-10-20-40	<i>F. mosseae</i> BGC XJ02	<i>Solanum nigrum</i>	Increased Cd in shoot and root
Wei et al., 2016	Pot exp., Natural soil, +AMF, + Sb	Sb(V): 500-1000	<i>F. mosseae</i>	<i>Cynodon dactylon</i>	Increase of Sb in shoot and root, Increase of BCF and TF Increases Sb exchangeable Decreased carbonate-bound Sb in rhizosphere, Decreased of Sb(III) fraction compared to total Sb, AMF might inhibited Sb(V) to Sb(III) reduction processes
Xu et al., 2016	Pot exp., Sterilized soil (autoclave), +AMF, + Pb	Pb(NO ₃) ₂ : 50-500-1000	<i>F. mosseae</i> BGC XJ01A	<i>Sophora viciifolia</i>	Decreased Pb in shoot and root
Degola et al., 2015	Pot exp., Hydropony, +AMF, + Pb	CdCl ₂ Na ₂ HASO ₄ .7H ₂ O 1-30 µM	<i>F. mosseae</i> (local) <i>F. mosseae</i> (commercial inoculum, INOQ GmbH)	<i>Nicotiana tabacum</i> L. cv. Petit Havana	Decreased Cd in leaves, Decreased As in roots
Firmin et al., 2015	Field exp., TM-rich Agricultural soil, Local AMF,	Cd ~ 13 Pb ~ 750 Zn ~ 950	Commercial inoculum (Solrize, Agrauxine), <i>F. mosseae</i> (local)	<i>Miscanthus × giganteus</i>	No effect on TM in root or leaf, Decrease oxidative stress in leaf
Garg et al., 2015	Pot exp., Sterilized soil (autoclave), +AMF	Na ₂ HASO ₄ .7H ₂ O, CdCl ₂ 30-60	<i>F. mosseae</i> UTMU 128 WM1/11	<i>Cajanus cajan</i> L. Millsp. 'Sel 85N', <i>Pisum sativum</i> L. 'PB 89 2008'	Decrease of As and Cd in both plants
Liu et al., 2015	Pot exp., Sterilized soil (autoclave), +AMF, + TM	CdCl ₂ .H ₂ O: 25-50-100	<i>G. versiforme</i> BGC GD01C	<i>Solanum nigrum</i>	Increased Cd at Cd25 and Cd50 Decreased Cd at Cd100, Increased DTPA extract (phytoavailable) at Cd25/50

Aghababaei et al., 2014	Pot exp., Sterilized soil, +AMF, + Cd	CdCl ₂ : 10-20	<i>R. irregularis</i> , <i>F. mosseae</i>	No effect on the soluble/exchangeable/organic-bound fraction in soil, Decreased inorganic-bound fraction, Increased residual fraction
Liu et al., 2014	Pot exp., Sterilized soil (autoclave), +AMF, + Cd	CdCl ₂ : 0.02-0.2 mM	<i>R. irregularis</i> (BGC USA05)(1), <i>Zea mays</i> L. <i>G. constrictum</i> (BGC USA02)(2), <i>F. mosseae</i> (BGC NM04A)(3)	Low Cd: (all) increased Cd in plant, High Cd: (all) decreased Cd in plant, (2) increased Cd in root, (3) decreased Cd in root
Hassan et al., 2013	Pot exp., Sterilized soil, +AMF, + Cd	CdSO ₄ : 0.75-10-40 Zn ~290	<i>R. irregularis</i> DAOM-234328 (1), <i>F. mosseae</i> BEG12 (2)	Cd in shoot: (2) < control ≤ (1), Zn in shoot: (2) < control = (1)
Chen et al., 2012	Pot exp., Hydropony, +AMF, + As	NaAsO ₂ : 2-8 μM	<i>R. irregularis</i> (AH01)	Decrease of As in root at 2 μM As, no effect at 8 μM, No effect of As in shoot, No organic As species in shoot/root
Garg and Singla, 2012	Pot exp., Sterilized soil (fumigated), +AMF, + As	Na ₂ HAsO ₄ ·7H ₂ O: 30-60-90	<i>F. mosseae</i>	Decrease of As in shoot and root
Shahabivand et al., 2012	Pot exp., Sterilized soil (autoclave), +AMF, + Cd	CdCl ₂ : 34-67-100	<i>F. mosseae</i>	Decreased Cd in root
Yu et al., 2011	Pot exp., γ-radiation Sterilized soil, +AMF, + Se	Na ₂ SeO ₃ : 20	<i>F. mosseae</i> BGC XJ01	No clear effect on soybean, Decrease of Se in shoot and root, Increase of the % of inorganic Se in shoot and root, Decrease of the % of organic Se in plant, However: plant effect
Arias et al., 2010	Pot exp., Hydropony, +AMF, + Pb	Pb: 10-50-100 Cr: 20-40-75-125 (mg.L ⁻¹)	<i>Zea mays</i> L. cv. ND108 <i>Glycine max</i> L. cv. Zhonghuang No. 17 <i>Medicago sativa</i> L. cv. Chuangxin <i>Prosopis</i> sp.	Increase of Cr and Pb in root, Increase of TF for Cr and Pb

Cozzolino et al., 2010	Pot exp., contaminated soil, +AMF,	As ~ 250	<i>R. irregularis</i> (AEGIS, Italpollina)	<i>Lactuca sativa</i> L.	Increased Cd in root and leaf
Punamiya et al., 2010	Pot exp., Sterilized soil (autoclave), +AMF, + Pb	Pb(NO ₃) ₂ : 400-800-1200	<i>F. mosseae</i>	<i>Chrysopogon zizanioides</i> (L.)	Increased Pb in root, Increased Translocation to shoot
Christophersen et al., 2009	Compartmented Pot exp., uncontaminated soil, +AMF, + As	As: 2.08-4.16	<i>R. irregularis</i> (DAOM181602)	<i>Hordeum vulgare</i> L. cv. Golden Promise	Decrease of As in shoot and root
Hua et al., 2009	Pot exp., Sterilized soil (autoclave), +AMF,	As ~ 40	<i>G. spp. and Acaulospora spp. (HN), Acaulospora mellea</i> (n° 40), <i>G. versiforme</i> (n° 90001), <i>G. caledonium</i> (n° 90036)	<i>Nicotiana tabacum</i> L. cv. Yunyan 85	All strains: Decreased As in shoot and root, Decreased pH & H ₂ O-extractable As, Increased As bound to Fe/Al.H ₂ O-oxides fractions
Wu et al., 2009	Pot exp., Hydropony, +AMF, contaminated vs clean sand, + As	Na ₂ HAsO ₄ .7H ₂ O: 50-200-400-1000 (μM)	Isolates from clean () or contaminated (TM) soil: <i>F. mosseae</i> (1)(1TM) <i>G. geosporum</i> (2)(2TM) <i>G. etunicatum</i> (3TM)	<i>Pteris vittata</i> L.	<400μM: (1)&(1TM) increased As in plant, >400μM: only (1) increased As in plant, No clear effect of other species
Yu et al., 2009	Pot exp., γ-irradiation Sterilized soil, +AMF, + As	As: 25-50-100	<i>F. mosseae</i>	<i>Zea mays</i> L.	Decrease of As in shoot and root, Decrease of As(V) kinetic uptake, No effect on As(III) kinetic uptake
Sudová et al., 2008	Pot exp., Sterilized soil (autoclave), +AMF, + TM	Pb(NO ₃) ₂ : 50-100-500 ZnSO ₄ .7H ₂ O: 0.2-450-700-1000 CuSO ₄ .7H ₂ O: 25-50-100 Cd(NO ₃) ₂ : 25-50-100 (mmol.l ⁻¹)	<i>R. irregularis</i> PH5 (contaminated soil) <i>R. irregularis</i> BEG75 <i>R. irregularis</i> LT (clean soil)	<i>Agrostis capillaris</i> (6 clones from contaminated soils or not)	Clones from clean soil: Higher accumulation in shoot and root, no effect of AMF, Clones from polluted soil: Results varied from negative to positive, (depending on the fungal isolate, plant clone and TM)

Chen et al., 2007, part 1	Pot exp., natural soil from Cu mine tailings, +AMF	Cu: 232 Pb: 81 As: 54 Cd: 2.5 Zn: 189	<i>F. mosseae</i>	<i>Coreopsis drummondii</i>	Decrease of Cu in shoot, no effect on Cu in root, No effect on Zn in shoot/root, Decrease of As in shoot, no effect in root, Decrease of Cd in shoot, no effect in root
				<i>Pteris vittata</i>	Decrease of Cu in shoot, no effect on Cu in root, Decrease of Zn in shoot, no effect in root, Decrease of As in shoot, no effect in root, Decrease of Cd in shoot
Chen et al., 2007, part 2	Pot exp., Soil from Cu mine tailings, +AMF	Cu: 232 Pb: 81 As: 54 Cd: 2.5 Zn: 189	<i>F. mosseae</i>	<i>Lolium perenne</i>	No effect on Cu in shoot/root, Increase of Zn in shoot, no effect in root, No effect on As in shoot/root, No effect on Cd in shoot/root.
				<i>Trifolium repens</i>	Decrease of Cu in shoot, no effect on Cu in root, No effect on Zn in shoot/root, Decrease of As in shoot/root, Decrease of Cd in shoot, no effect in root
Munier-Lamy et al., 2007	Pot exp., Sterilized soil (pasteurized), +AMF, + Se	Na ₂ SeO ₄ : 12	<i>F. mosseae</i> P2 BEG69	<i>Lolium perenne</i> cv. Barclay	Decreased Se in plant, Small increased of H ₂ O-soluble fraction of Se, Tend to increase residual fraction,
Chen et al., 2006	Pot exp., Hydropony, +AMF, contaminated sand	As: 106	<i>F. mosseae</i> BEG167, <i>G. caledonium</i> , <i>R. irregularis</i> BEG87	<i>Pteris vittata</i> L.	No effect on As content
Janouskova et al., 2006	Pot exp., Sterilized soil, +AMF, + Cd	Cd(NO ₃) ₂ : 1.2-2.4-4.8	<i>R. irregularis</i> PH5	<i>Nicotiana tabacum</i> L., var. Wisconsin 38	Decreased Cd in root and leaf, Suggest Cd immobilization in rhizosphere
Ma et al., 2006	Pot exp., Sterilized soil, +AMF,	Pb Zn	Commercial mix (Endorize-Mix2, Biorize Sarl): <i>F. mosseae</i> , <i>R. irregularis</i>	<i>Leucaena leucocephala</i>	25% decrease of Pb & Zn mobility (DTPA & NH ₄ OAc extracts), No effect on Pb/Zn in leaf, Decreased Pb/Zn in root

Marques et al., 2006	Pot exp., contaminated soil, +AMF,	Zn ~ 430	G. sp. BEG140 (1), <i>G. claroideum</i> (2), <i>F. mosseae</i> (3), <i>R. irregularis</i> (4), A Mix of all these species (5)	<i>Solanum nigrum</i>	(2&4): Increased Zn in leaf, root and stem, (all): root > stem > leaf, decreased TF
Trotta et al., 2006	Pot exp., Hydropony, +AMF, + As	Na ₂ HAsO ₄ ·7H ₂ O: 25	Commercial inoculums (Biorize, Dijon): <i>F. mosseae</i> BEG12(1), <i>Gigaspora margarita</i> BEG34(2)	<i>Pteris vittata</i> L.	Decreased As in root (1>2), Increased TF (1>2),
Chen et al., 2005	Pot exp., Hydropony, +AMF, + Pb	Pb(NO ₃) ₂ : 300-600	Mix, no detail	<i>Kummerowia striata</i> (Thunb.) Schindl, <i>Ixeris denticulate</i> L., <i>Lolium perenne</i> L., <i>Trifolium repens</i> L., <i>Echinochloa crusgalli</i> var. mitis	Increase of SPb in shoot and root, Increase of the root/shoot ratio in highly mycorrhized plants, No effect on the ratio in poorly mycorrhized plants
Citterio et al., 2005	Pot exp., Sterilized soil (autoclave), +AMF, + TM	Cd: 100 Cr: 300 Zn: 100	<i>F. mosseae</i>	<i>Cannabis sativa</i> L. cv Carmagnola	No effect of TM bioavailability, Increased Ni in stem/leaf Trend of increased TF
Vogel-Mikuš et al., 2005	Field exp., Mining soil, Local AMF,	Cd ~ 68000 Pb ~ 5500 Zn ~ 400	No detail	<i>Thlaspi praecox</i> Wulf.	Hyperaccumulation of all TM, No control (AMF-free) to compare
Yu et al., 2005	Pot exp., Sterilized soil (autoclave), +AMF, + Cd	CdCl ₂ : 5-10-20	Commercial mix (Endorize-Mix2, Biorize Sarl): <i>F. mosseae</i> , <i>R. irregularis</i>	<i>Lolium multiflorum</i> L.	Increased Cd in root and shoot
Andrade et al., 2004	Pot exp., Sterilized soil (fumigated), +AMF, + Pb	Pb(NO ₃) ₂ : 150-300-600	<i>G. macrocarpum</i>	<i>Glycine max</i> L. Merrill var. IAC-14	30% decrease of Pb in shoot
Malcová et al., 2003	Pot exp., Hydropony, +AMF, + Pb	Pb(NO ₃) ₂ : 0.01-0.1 mM	<i>R. irregularis</i> PH5 (contaminated soil) <i>R. irregularis</i> BEG75	<i>Zea mays</i> <i>Agrostis capillaris</i>	0.1mM Pb: no effect, 0.01mM Pb: decrease of Pb in maize root and shoot (both strains), No effect in <i>Agrostis</i> shoot, Higher Pb in <i>Agrostis</i> root mycorrhized with PH5 compared to BEG75 and no-AMF

Vivas et al., 2003	Pot exp., Sterilized soil (autoclave), +AMF, + TM	CdSO ₄ : 30	<i>F. mosseae</i> (contaminated soil), <i>Trifolium repens</i> L. <i>F. mosseae</i> (BEG119)	Small decrease of Cd in plant, High decrease of Cd in plant when co-inoculated with <i>Brevibacillus</i>
Joner and Leyval, 2001	Pot exp., Zn smelter soil, +AMF	Cd, Zn, Pb, Cu	indigenous AM fungi (Ind), sterilized + <i>F. mosseae</i> P2 (P2) <i>Zea mays</i> L. cv. Fanion, <i>Trifolium subterraneum</i> , cv. Mount Barker	All treatments: High root/shoot ratio, Ind: lowest Cd content in shoot and root, Ind: increased Cd and Zn Root/shoot ratios, Ind versus P2 : Lower TM contents and higher root/shoot ratio, Pot size effect: larger pot = higher TM in plants
Tonin et al., 2001	Pot exp., natural soil, + Cd and Zn, +AMF	CdSO ₄ .7H ₂ O: 2 ZnSO ₄ .7H ₂ O: 200	<i>R. sp.</i> (4) from <i>Viola calaminaria</i> (1) <i>F. mosseae</i> P2 (BEG 69) (2)	(1) Increased Cd and Zn in root, (2) Increased Cd in plant, no effect on Zn.
Hildebrandt et al., 1999	Pot exp., contaminated soil, +AMF	Ni: 0.02 Cu: 0.13 As: 0.46 Zn: 1.41	<i>R. isolate Br1 from Viola calaminaria</i> <i>R. irregularis</i> Sy167, <i>F. mosseae</i>	Plants died when non mycorrhized. Maize, alfalfa, barley, and the zinc violet, grew on sterilized soil spiked with either Br1 or Sy167. No data about TM content in plants. No data about <i>F. mosseae</i>
Kaldorf et al., 1999	Pot exp., contaminated soil, +AMF	Ni: 0.02 Cu: 0.13 As: 0.46 Zn: 1.41	<i>R. isolate Br1 from Viola calaminaria</i> <i>R. irregularis</i> Sy167	With Br1 AMF strain (compared to no AMF or Sy167): Lower accumulation of TM, sequestration in root, Higher accumulation in cortex than in stele (except for Al, evenly distributed), Sy167 showed similar accumulation than the control
Joner and Leyval, 1997	Compartmented Pot exp., +AMF, + ¹⁰⁹ Cd radioisotope	CdCl ₂ .H ₂ O: 1-10-100	<i>F. mosseae</i> P2 (BEG 69)	No effect on Cd mobility in soil, No effect on Cd in shoot, increased Cd in root,
Diaz et al., 1996	Pot exp., Sterilized soil (autoclave), +AMF, + As	ZnSO ₄ : 10-100-1000 Pb(NO ₃) ₂ : 100-1000	<i>G. macrocarpum</i> <i>F. mosseae</i>	Low TM dose: (all AMF) Increased TM in plant Higher doses: <i>F. mosseae</i> decreased TM in plant, <i>G. macrocarpum</i> increased TM in <i>A. cytisoides</i> and had no effect in <i>L. spartum</i>

Guo et al., 1996	Compartmented Pot exp., contaminated soil, + Cd and Ni, +AMF	Cd, Ni, Cu, Zn	<i>F. mosseae</i> P2 (BEG 69)	<i>Phaseolus vulgaris</i> L. cv. Saxa, <i>Zea mays</i> L. cv. Zenit 200	No effect on Ni, Increase of Cd, Cu and Zn content in plant
Hetrick et al., 1993	Pot exp., Zn mining soil, +AMF	Zn: 750	<i>G. etunicatum</i> versus a mix of: <i>G. ambisporum</i> , <i>Septoglomus constrictum</i> , <i>Scutellospora pellucida</i> , <i>F. mosseae</i> , <i>G. etunicatum</i>		Similar results for the mix or <i>G. etunicatum</i> isolate, Increase of Zn in shoot of both plants

F. = Funneliformis; G. = Glomus; F. = Rhizophagus. BCF = bioaccumulation factor; TF = translocation factor; +AMF: enriched with arbuscular mycorrhizal fungi; exp.: experiment

1.5.2. Organic matter amendments: The case of lombricompost

Soils from urban gardens are particularly enriched with OM. In addition to improve soil fertility, this practice permits both to use the domestic organic wastes (and reduce in consequence the quantities to treat) and also to reduce the urban soil erosion with reduction in consequence of water pollution.

Organic matter amendments are numerous and widespread in UPA, with a wide range of physicochemical characteristics, but the present thesis focused only on one example of product, lombricompost as it has been gaining interest in UPA over the past few years (Moreno Flores, 2007). However, food safety remains a central topic for two major reasons: first, depending on the wastes' origin, contaminant (biologic or not), can be brought on crops when these products are applied (Armar-Klemesu, 2000; Jones and Martin, 2003). Indeed, even if the composting processes generally inactivate most of the pathogens through an important increase of temperature, some can survive and non-biological persistent harmful elements such as metalloid will stay.

Then, as described in [section 1.1](#), TM interaction with negative surfaces of organic matters plays a tricky role in their mobility as they can either be bound or released depending on soil pH for example (Gerritse and van Driel, 1984; Sauvé et al., 2000).

Consequently, because AMF are known to influence pH in the mycorrhizosphere (Hu et al., 2013), solubilization of TM could occur, leading to a possible increase of plant uptake. Hence, because both OM amendments and AMF fertilization are currently used in UPA with sustainable arguments, we decided to study such interaction.

1.5.3. An emerging amendment: the biochar

Biochar stands for *biological charcoal*, due to its fabrication process which is the pyrolysis of organic material (high temperature, no oxygen) to produce charcoal ([Figure 1.12](#)). Its use in agriculture is both very ancient and completely new (in terms of use for pollution management). Indeed, its first use goes back to the Amazonian Indians, who prepared it to create the *Terra preta* soils (Amazonian black soils), which are still more fertile than surrounding arable lands, more than 1 000 years after their creation (Lehmann and Joseph, 2015).

For the last few years, the scientific community intensified its research on the benefits of using biochar on agricultural soils (~ 2 700 publications before 2010; > 15 500 in August 2016 with 'Biochar+agriculture' keywords). Indeed, from a climatic and ecological point of view, its physicochemical properties reveal great potential to use it as a stable CO₂ storage material. In agriculture, its use can improve soil structure and fertility, leading to increased yields (45-250%) through an increase of soil water retention, CEC, and conductivity (Jha et al., 2010; Lehmann and Joseph, 2015).

In the case of contaminated soils with TM, its use also revealed good potential (Paz-Ferreiro et al., 2014), but with variable efficiency depending mainly on the preparation process (temperature and composition) (Uchimiya et al., 2011).

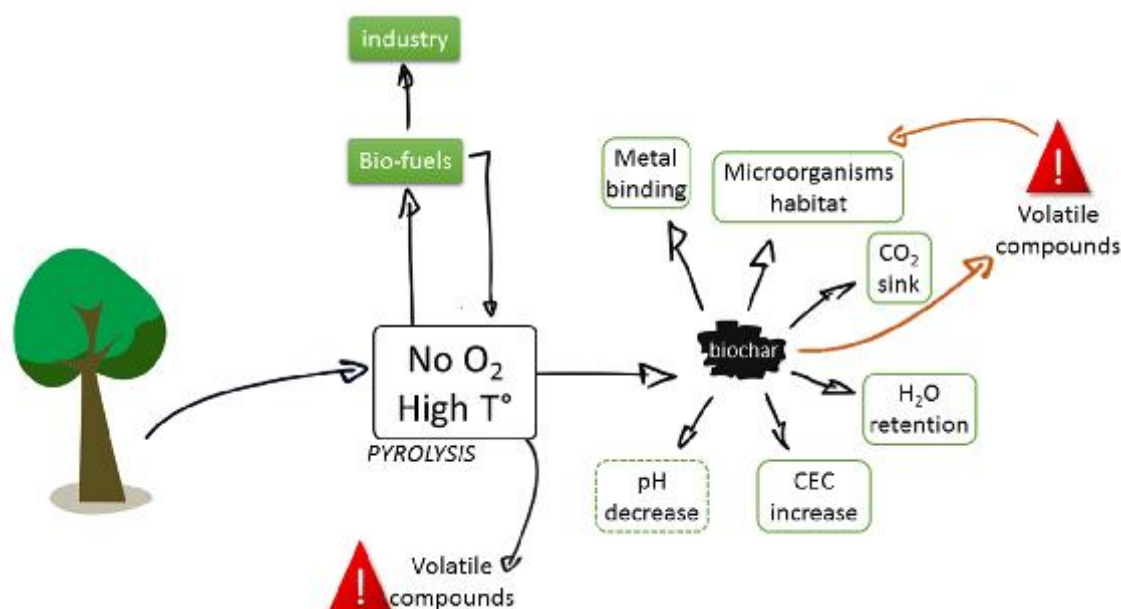


Figure 1.12: Simplified biochar production, from organic matter to biochar interests and risks*

1.5.4. If you want more!

A history of research on arbuscular mycorrhiza (Koide and Mosse, 2004). Everything is in the title. This article retrospects the main discovery steps of arbuscular mycorrhizal fungi.

Biochar for Environmental Management: Science, Technology and Implementation (Lehmann and Joseph, 2015). A very complete book on biochar, from its history to its production, uses and effects on soil and crops.

1.6. Health risk assessment & human bioaccessibility

Trace metals and metalloids present a high health risk because of their possible accumulation along food webs, even if part of them are degraded or excreted (Zhuang et al., 2009). Once in the organism, these toxic compounds are stored and metabolic pathways of stress alleviation are usually activated (Yadav, 2010). Moreover, biotransformation in plants could modify TM speciation and bioaccessibility as suggested by Schreck et al (Schreck et al., 2014). To study their accumulation, mathematical factors are usually employed. We focused here on two of them (1) to study the accumulation from soil to plants and (2) to follow the transfer through plants to edible parts. Finally, *as all the contaminants are not absorbed during digestion, the bioaccessibility of these compounds was also investigated.*

1.6.1. How to follow metalloid from soil to edible organs?

The bioaccumulation factor (BF) is defined as the ratio between an element concentration in a living organism and its concentration in the environment around him (Eq. 1). It represents in which proportion this element accumulates in the organism, and is an indicator of which quantity could potentially transfer along the food chain.

$$(Eq. 1) \quad BF = \frac{[TM]_{organism}}{[TM]_{environment}}$$

The Translocation Factor (Eq. 2) is a concentration ratio between two organs of an organism. It highlights the metabolic mechanisms of contaminant movement inside it. For example, in the study of atmospheric dust fall on aerial parts, it is interesting to study if contaminants stay there or if they are transferred to roots (Xiong et al., 2014a). In our case, as TM source came from the soil, we studied their translocation from roots to leaves. Hence, it is possible to assess whether or not it is hazardous to grow leafy vegetables (do they accumulate in leaves?) or root vegetables such as carrot (do they transfer TM to shoots, letting clean storage organs to eat?).

$$(Eq. 2) \quad TF = \frac{[TM]_{roots}}{[TM]_{leaves}}$$

1.6.2. Which proportion of pollutants is absorbed by the human body when digested?

As for any compound, the human digestive system does not extract the entire quantity of a TM when ingested: this extracted part represents the human bioaccessible fraction (**B**) of this TM (Pascaud et al., 2014; Xiong et al., 2014a, 2014b). In human health risk assessment, it is crucial to distinguish **B** from other TM fractions whether in soil or edible plants, as represented in Figure 1.13. **B** is expressed as the ratio between the bioaccessible concentration and the total concentration ingested (Eq. 3)

(Eq. 3)
$$B = \frac{[TM]_{bioaccessible}}{[TM]_{ingested}}$$

To assess **B**, a standardized chemical test has been set up and named the Unified BARGE Method (UBM) by the BioAccessibility Research Group of Europe (BARGE) (Foucault et al., 2013). They developed chemical solutions and a protocol (with adapted physiological time and temperature) permitting to simulate the reactions occurring all along the digestive tract (Denys et al., 2009), which is described in Chapter 2.

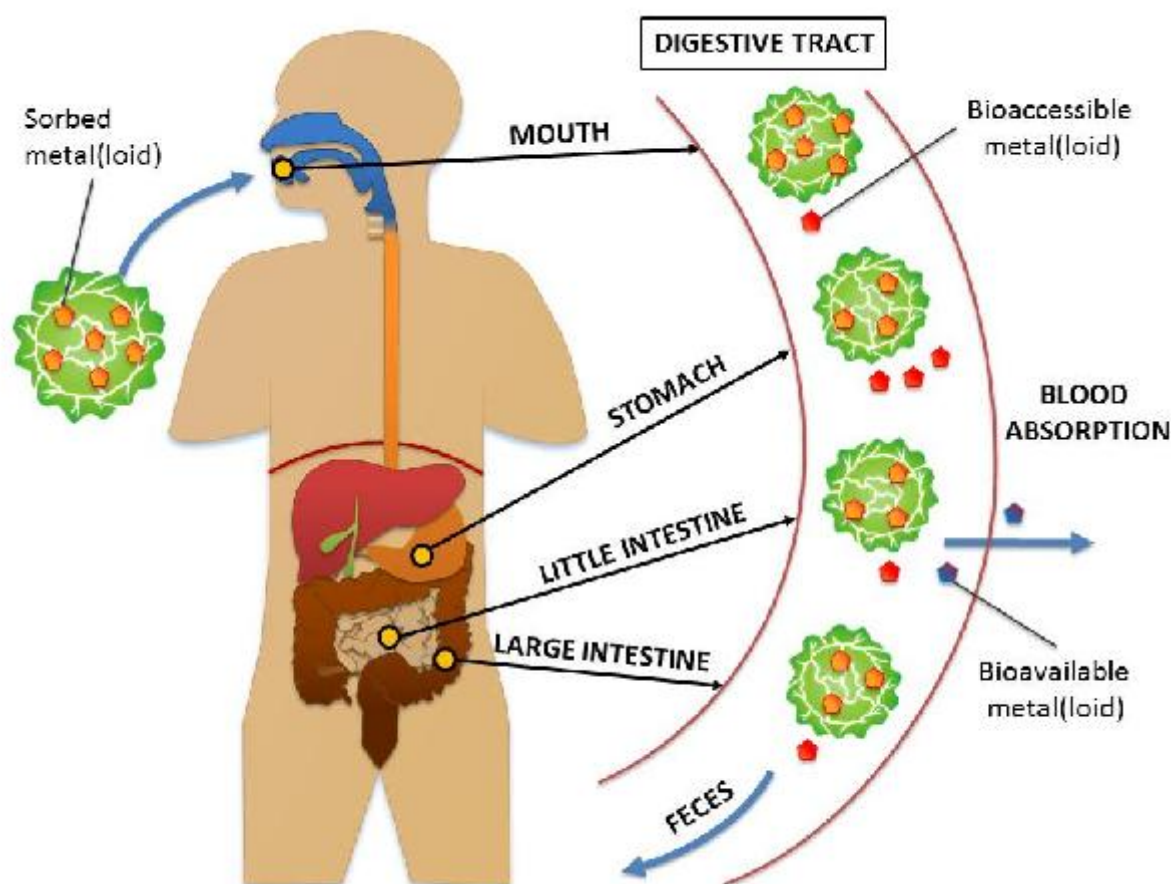


Figure 1.13: Schematic representation of the bioaccessibility of an element when ingested and digested

However, as shown in Figure 1.13, even if the bioaccessible fraction can be considered as the hazardous proportion of contaminant for the digestive tract, it doesn't fully transfer from the lumen to blood vessels, meaning that the real dangerous concentration for the whole body is lower than the bioaccessible concentration. This bioavailable concentration is usually expressed as (Eq.4); with **Ba** the bioavailable fraction, **B** the bioaccessible fraction (from Eq. 3), **Fa** the digestive tract absorption rate and **Fh** the transfer rate to blood vessels).

(Eq. 4)
$$Ba = B \times Fa \times Fh$$

Some toxicity tests were carried out on epithelial cells and showed that PbO, PbSO and Sb₂O₃ (which are mainly as Pb²⁺ and Sb³⁺ in solution) generated no toxicity while CdCl and CdO₂ were toxic, with a dose-dependent response (Leveque, 2014).

1.6.3. What does society do with all these pollution problems and complex data from research?

In the case of such close to the field studies, the ideal objective is to draw useful recommendations to gardeners willing to practice UPA and to give them clues to better understand the risks of their activity. Indeed, their will to cultivate a piece of land overpasses the risks for their own health. Consequently, the solutions and recommendations have to be brought anyway because closing these areas has to be considered as the very last option (Dumat et al., 2015). Moreover, scientific trials as these ones generally focus on thin details and analysis on representative conditions to try to extrapolate the results, but this step is highly delicate because of the numerous physicochemical and biological parameters involved, the interactions, and the uncertainty due to living organisms.

Meaning that most of the time, as stated by Stuart Firestein, '*knowledge generates ignorance*'. Hence, the discussion and perspectives will be the occasion to try a step back to generate recommendations (Chapter 7).

1.6.4. If you want more!

<https://www.bgs.ac.uk/barge/ubm.html>. The Bioaccessibility Research Group of Europe - BARGE) developed and certified a Unified method (UBM) to study the fraction of contaminants extracted during digestion. The website contains links to all the publications dealing with the development and validation of their method, which is used in this thesis.

1.7. What do we study and how do we proceed?

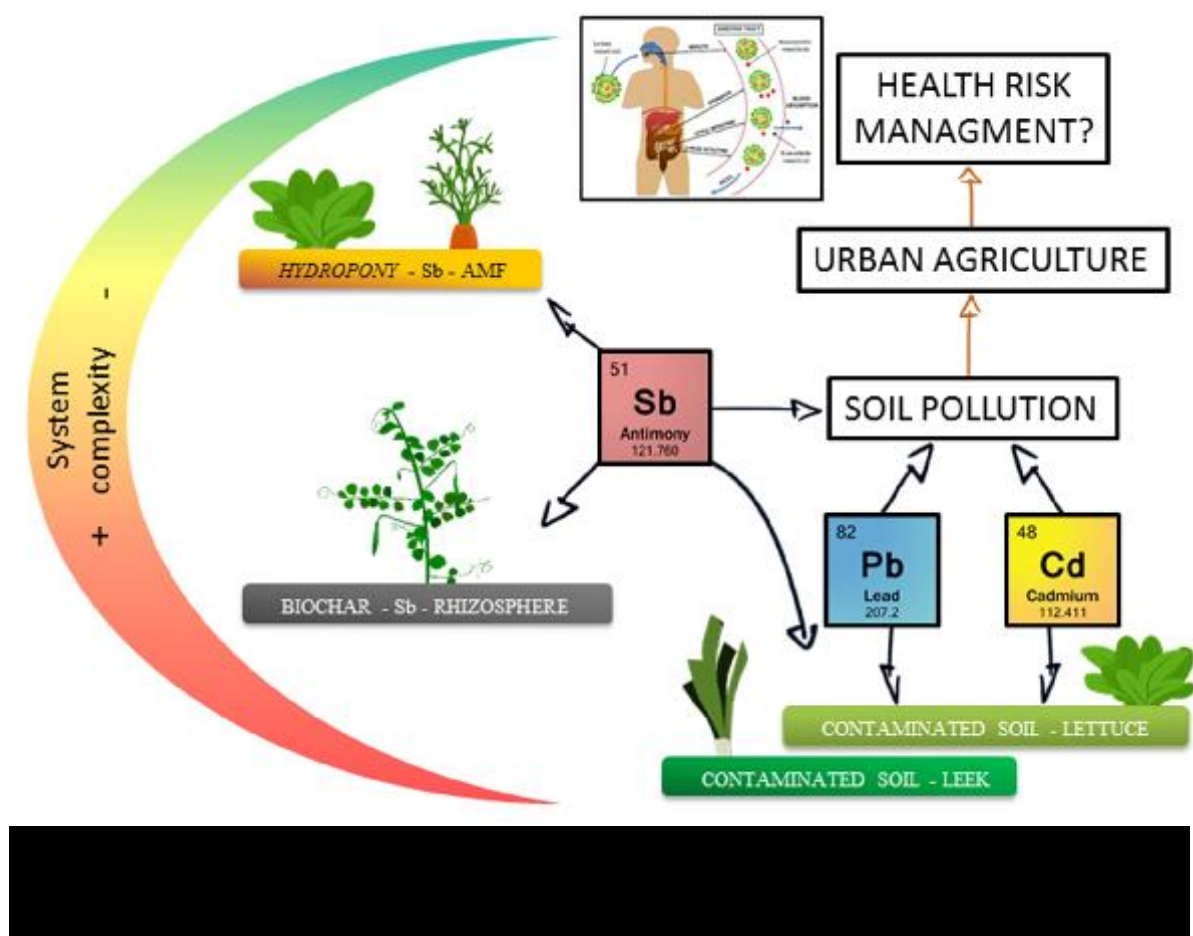
The general objectives of this thesis were to:

- Assess the phytoavailability and bioaccessibility of TM (Cd, Pb, and Sb) from different contamination origin (geogenic vs anthropic) in edible plants.
- Study the impact of arbuscular mycorrhizal fungi and organic matter amendment on TM transfers from soil to plant and within plant (translocation and compartmentation).
- Evaluate the influence of microbial community on TM fate at the soil-microorganism-plant interface.
- Study the influence of these physicochemical and biological parameters on human bioaccessibility of TM in soil and edible organs for health risk evaluation.

The complexity of the mechanisms involved in the fate of TM at the soil-microorganism-plant interface highlights the need to better understand the roles of each one through a multidisciplinary and multiscale complementary approach using tools and concepts from biogeochemistry, agronomy, metagenomic and (eco)toxicology.

[This work connects with](#) the approach of (1) Levèque (Leveque, 2014) on the ecotoxicology and biomonitoring of soil contaminated with TM and (2) Xiong (Xiong, 2015) on the fate of atmospheric TM pollution at the air-plant interface.

To study TM transfer from soil to plant and the impact of microorganisms and organic matter amendments, we chose different approaches with a gradient of complexity. As illustrated on the [Figure 1.14](#), in addition to complex experiments with the three TM, simpler controlled experiments were performed for Sb. This multi-scale approach allows to investigate TM fate at the soil-microorganism-plant (s-μ-p) interface. Hence, the simplest systems generate more fundamental results, without many parameter interactions, but their field reality always needs to be checked. On the other hand, the 'real soil' trials intend to approach field reality in a better way, with all the interactions which can occur at the s-μ-p interface. These results are generally more complex to interpret but the degree of confidence is considered higher.



The present manuscript is divided into 8 chapters as follow:

CHAPTER 1 is a contextual introduction, gathering essential information around the scientific topic treated in this thesis. A lack of literature on Sb bioavailability in edible plants and the role of AMF in its transfer has been evidenced. Hence, [a second part of the first chapter](#) reviews this topic through a published article entitled “Antimony bioavailability: knowledge and research perspectives for sustainable agricultures”. Then, every detailed method used to fulfill our objectives are presented in **CHAPTER 2**. Note that because the manuscript is written as a coherent sequence of publications, some methods will also appear in the next chapters. The main results about the fate of cadmium at the s-μ-p interface under different treatments (bioaugmentation with arbuscular mycorrhizal fungi, lombricompost amendment) on two contrasted peri-urban soils are presented in **CHAPTER 3**. It is presented as a submitted article entitled “How do soil biofertilizers and organic amendments influence Cd phytoavailability and human bioaccessibility?”. Then, the same experiment was also used to study Sb and Pb fate at the s-μ-p interface, with a more detailed approach (microscopy and fungal community analysis). It is developed in **CHAPTER 4**, an article in final writing phase before submission entitled “Pb and Sb accumulation influenced by arbuscular mycorrhizal fungi community and organic matter in a crop rotation (*Lactuca sativa* L. → *Allium porrum* L.)”. Even if these trials were not field experiments, the complexity of such systems made difficult the discussion and conclusion step. Thus, we developed a simpler approach dealing with AMF role in Sb transfer to edible plants in hydroponic conditions. The preliminary results are presented in **CHAPTER 5**. Until now, Sb impact on soil enzymatic activity (plant exudates and microbial activity) in relation with addition of biochar has not been described, which we began to investigate in **CHAPTER 6** with the study of the effect of spent coffee ground biochar amendment on Sb transfer from soil to plant. All these results, discoveries, and doubts are then discussed and perspectives are developed in **CHAPTER 7** before a conclusion and investigation openings in **CHAPTER 8**.

Chapter 2. MATERIAL AND METHODS

The second chapter gathers all the methods used to answer the scientific questions raised in Chapter 1, and summarized in Figure 2.1.

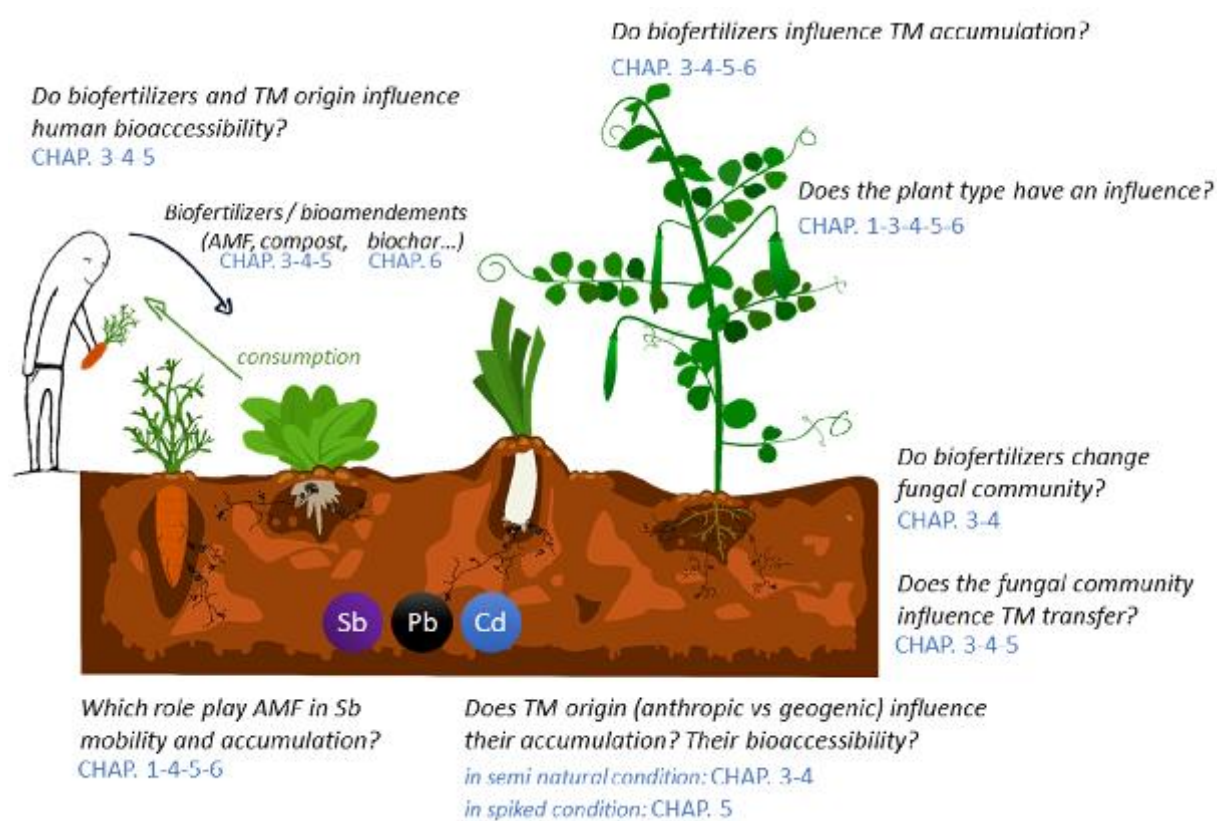


Figure 2.1: Scientific questions developed in the present thesis.

2.1. Soil description

To assess the role of AMF in the transfer of TM between periurban soils and edible plants, pot experiments were carried out with full plants growing on contaminated soils. Three different soils were studied, each one with specificities allowing the estimation of the role of soil characteristics in these mechanisms. Their localization is reported in [Figure 2.2](#).

- Arable soil with anthropic contamination (BZC)

This soil, described in [Table 2.1](#), came from a site impacted by a regulated factory previously documented on various ecotoxicological and physicochemical aspects (Cecchi, 2008; Leveque et al., 2013). The sampling area corresponds to the point T4-140m (Leveque, 2014). This area was exposed to important airborne particles fallouts coming from a battery recycling factory. These particles are highly contaminated in Pb, but also in Cd and Sb (among others), leading to a gradient of soil contamination along the dominant wind direction. These newborn particles have a low mobility in soil so their accumulation profile generally decreases drastically in the first 50 cm beneath the surface (Leveque, 2014).

- Urban garden soil with a geogenic anomaly (NTE)

This soil was collected from the urban garden “*Jardin des Eglantiers*” in Nantes. Its characteristics are gathered in [Table 2.1](#). The area revealed an important natural positive Pb anomaly, coming from the alteration of the mother rock beneath the surface. Because Pb comes from underground alteration, total depollution of the area is impossible. Various proposals were therefore tested to deal with the contamination without closing the gardens (detailed information are available on JASSUR¹ website). One of them was to use arbuscular mycorrhizal fungi as a biological barrier.

- Peri-urban soil from an agricultural land (TLD)

Organic olive orchards are grown on the collection area, near Toledo, Spain. The soil presents no Sb contamination and poor organic matter content (~1%).

¹ <http://www6.inra.fr/jassur>

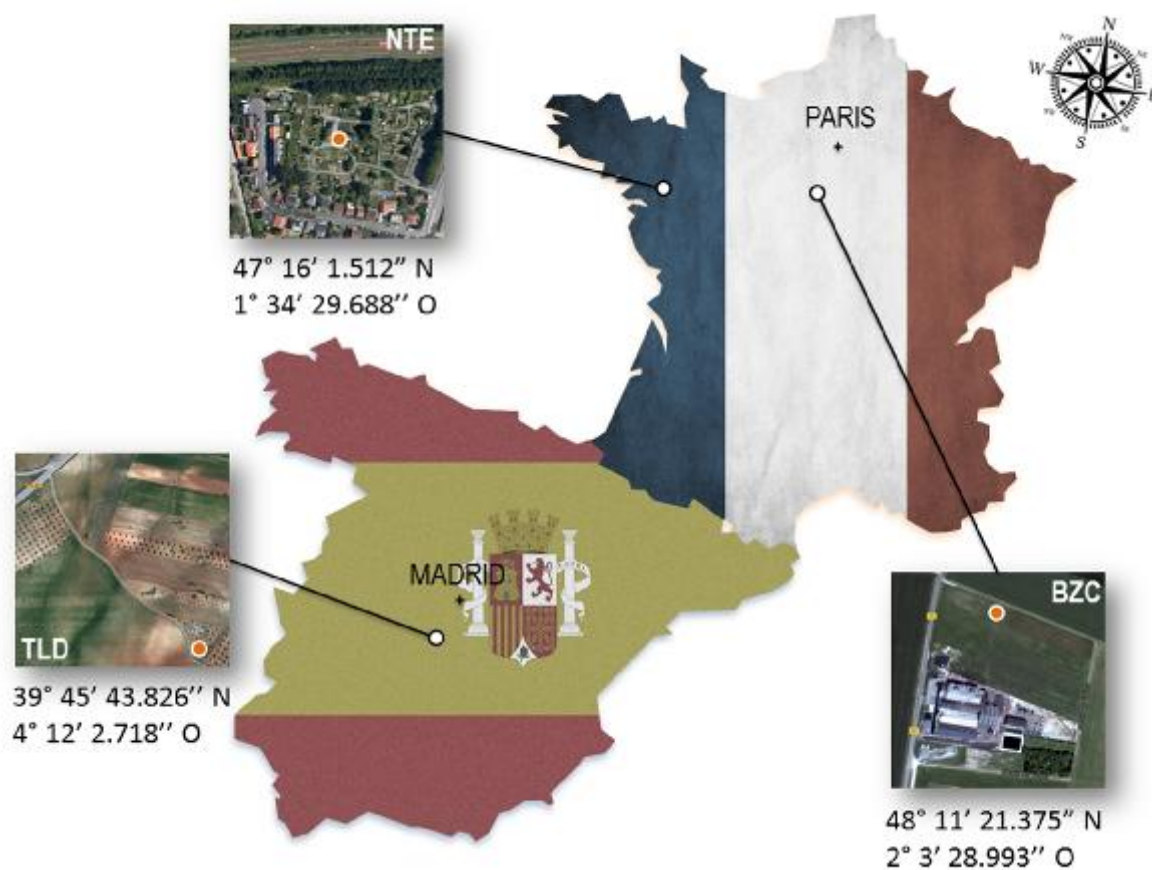


Figure 2.2: Studied soil localization. BZC, Bazoches; NTE, Nantes; TLD, Toledo.

Table 2.1: Bazoches (BZC) and Nantes (NTE) soil characteristics. P_2O_5 has been measured following the Joret-Herbert method

	pH	Initial H_2O	[Pb], mg.kg ⁻¹	[Sb], mg.kg ⁻¹	[Cd], mg.kg ⁻¹	Clay, %	Silt, %	Sand, %	CaCO ₃ , %	P ₂ O ₅ , mg.kg ⁻¹	CEC cmol+.kg ⁻¹
BZC	8.1	3.2	412	15,4	3.0	24.0	55.3	16.7	2.0	76.7	15.85
NTE	7.0	6.0	456	2,7	0.2	14.0	28.4	57.4	0.2	380	8.71

2.2. Full plant cultivated in pot under greenhouse condition

2.2.1. Plant cultivation

A two-step experiment was carried out to mimic a crop succession (lettuce followed by leek) (Figure 2.3). Although these two vegetables are usually highly cultivated by gardeners, they are subject to important contamination risks through both root transfer and aerial deposition on the edible organs. In this experiment, two soils were tested - BZC and NTE (Figure 2.2). Both soils were softly sieved with a 2mm steel sieve before the experiment.

POT PREPARATION. Two layers of wintering veil (Gerbaud®) were placed at the bottom of each 2L plastic pot (diam.17cm, Soparco®) to prevent roots from getting out. Each pot was filled with 1.9 kg soil. A regulated dripper line brought water to each saucer to fill it every three days. Watering the pots per capillarity limits spores and TM lixiviation processes.

TRANSPLANTATION. Before transplantation on contaminated soils, organic lettuces seeds (*Lactuca sativa* L., Laitue Pommée appia, Ferme de Sainte Marthe®) were sown in sterilized oildri® (20min, 121°C) and grew under natural light at ~17°C. After a 3 weeks growing period, seedlings were placed in a greenhouse (artificial lights turned on if brightness < 2800 lx, 18/20°C day/night - 50% relative humidity) for 1 week acclimation. Lettuces were then transplanted each in a pot.

BIOAUGMENTATION. For the bio-augmentation condition, 9 ml of a spore suspension were spread directly on the whole root system before filling the planting hole. Because of its high mycorrhization rate and its rusticity, organic leek (*Allium porrum* L., Poireau de Saint Victor, Ferme de Sainte Marthe®) was used to multiply the natural AMF inoculum from contaminated soils. Leeks were grown under the same greenhouse conditions in pots filled with a mix of NTE and BZC soils (~25%) and sterilized oildri® (~75%). This mix was done to obtain a biofertilizer composed of local fungi which might be tolerant to metalloid contamination. Spore extraction was performed with the same protocol used for lettuces (Cf. 2.6) to prepare a spore suspension (280 sp.ml⁻¹).

A fully randomized design was adopted to apportion on each modality the eventual greenhouse climatic heterogeneity.

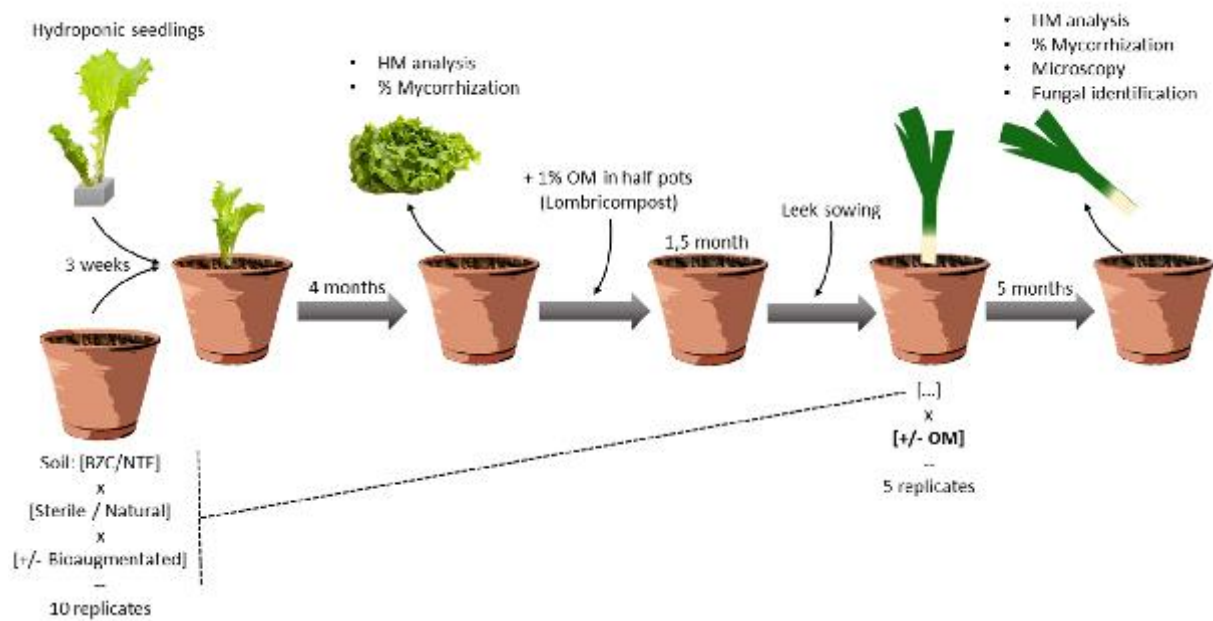


Figure 2.3: Experimental design of a crop succession on contaminated soils

2.2.2. Sampling and second crop cultivation

PLANTS. After a 4 months of growth period, lettuces were harvested with a ceramic knife by cutting the aerial part away from the root system. Leaves and roots were rinsed with distilled water and oven-dried (4 d, 50°C) in plastic bags. Some fragments of the root system were kept fresh for staining and mycorrhizal rate evaluation (Cf. 2.6).

SOIL. About 60 g of soil were sampled from each pot to assess sporulation and soil physicochemical properties (pH, %OM) after drying at room temperature for 10 days and 200 µm sieving.

ORGANIC MATTER ADDITION AND LEEK CULTIVATION. After lettuces harvest, each pot kept its soil and rested for 2 months. Then, sterilized lombricompost (organic matter NFU 44-051, Botanic®) was mixed with soil in half of the replicates of each modality, in order to increase soil OM content of 1%. The pots rested for stabilization during another month before leeks (*Allium porrum* L., Poireau de Saint Victor, Ferme de Sainte Marthe®) sowing, cultivation (25 weeks), harvest and analysis.

2.3. Sb transfer in simplified hydroponic systems

The experiment presented in [section 2.2](#) generated high amount of data concerning metalloid fate at the soil-plant interface, but also complex hypothesis related to the real role of AMF in there. This complexity is mainly due to all the interactions occurring between plants, microorganisms and soil physicochemical properties.

Hence, we developed a simplified hydroponic system to overcome the soil factors. In this study, we grew lettuce (same as before) and carrots (*Daucus carota* 'Rothild' L., Ferme de Sainte Marthe®) with the objective to compare the results with those from lettuce cultivation on natural soil ([Cf. 2.2](#)).

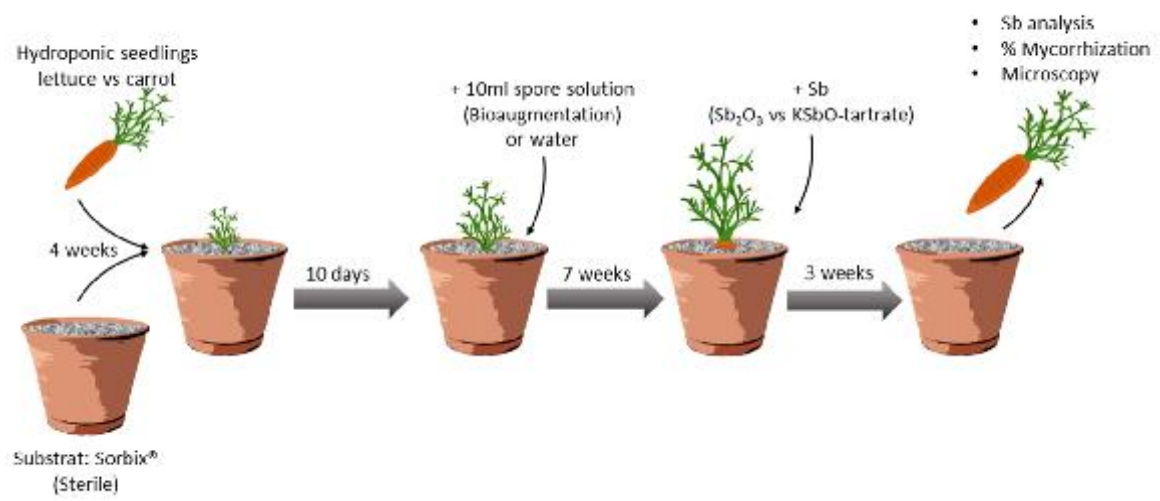
POT PREPARATION. Two layers of wintering veil (Gerbaud®) were placed at the bottom of each 1.5 L plastic pot (Ø 14 cm, Soparco®). Each pot has been filled with 1.3 kg of sterile (autoclaved for 20 min, 121°C) inert substrate (Sorbix®). A regulated dripper line brought water to each saucer to fill it every four days.

Before transplantation on the substrate in pot, organic lettuces and carrot seeds were sown in a germination plate filled with sterilized Sorbix and grew in the greenhouse (same conditions as in [section 2.2](#)) for 4 weeks acclimation. Two plants were transplanted in each pot and after 1 week acclimation, the smallest one was removed. A fully randomized design was also used. Every 2 weeks, plants received 150 ml of Long Ashton solution (low P).

MYCORRHIZATION. The mycorrhized condition was obtained by spreading 10 ml of a spore suspension (from the same leek trap cultivation as in [section 2.2](#)) directly underneath the substrate surface, near the root system. The spore solution was prepared following the same procedure as in [section 2.6](#), to obtain a spore suspension of 350 sp.ml⁻¹. 10 ml of sterilized water was spread on non-mycorrhized modalities. Plants grew during 7 weeks before harvest.

2.3.1. Antimony spiking

To compare the accumulation rate regarding Sb chemical form, we tested here two Sb^{III} species - trioxide antimony (Sb₂O₃) and potassium antimony tartrate trihydrate (KSb) – at the same dissolvable Sb³⁺ concentration (10 mg.l⁻¹). 100 ml of these solutions were brought to half the replicates of each modality. Plants were harvested 3 weeks after the spiking ([Figure 2.4](#)). The modalities were finally: [± MYC] × [Lettuce/Carrot] × [Sb₂O₃/KSb] × [4 replicates].

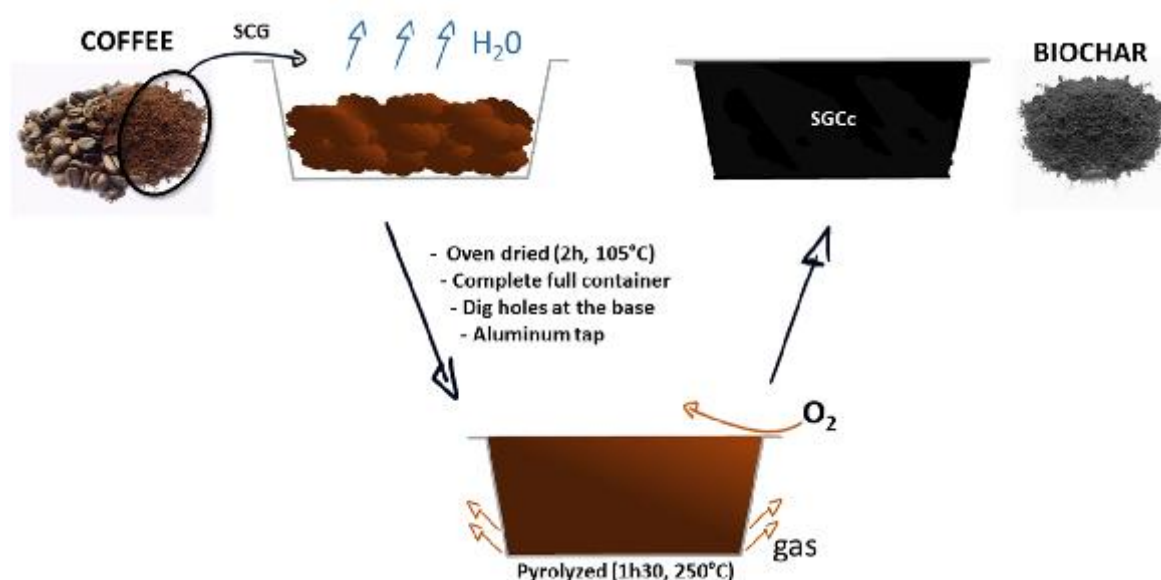


2.4. Influence of biochar and Sb concentration on soil microbial activity

These experiments and all the enzymes assays were carried out in the Ecotoxicology Laboratory (Environmental Science and Biochemistry Faculty, University of Castilla-La Mancha, Toledo, Spain) in collaboration and under the excellent supervision of Pr. Juan Carlos Sanchez Hernandez.

2.4.1. Biochar preparation, pyrolysis protocol

Spent coffee ground (SCG) were firstly oven-dried (2 h, 105 °C, Universal Stove Binder®) in aluminum container. Then, dry SCG was transferred in a new aluminum container previously drilled at the base to let the gases escape during the combustion. The coffee-full containers - taped with aluminum film to prevent air contact - were afterward oven-pyrolyzed (1h30, 250 °C, Muffle furnace Nabertherm® L-090H1SN) to obtain coffee-biochar (SGCc) through anoxic combustion ([Figure 2.5](#)).



2.4.2. Experimental design

The soil used in this experiment was the TLD soil, presented in [section 2.1](#), its characteristics are gathered in [Table 2.2](#).

ORGANIC MATTER ADDITION. Amendment was mimicked by adding 5% (%w/w) of Spent Coffee Ground (SCG) or 5% (%w/w) of Biochar made with these grounds (SGCc) to TLD soil. Such quantity is usually recommended as amendment to increase soil physicochemical properties.

Sb CONTAMINATION. As shown in [Figure 2.6](#), we focused in this experiment on one chemical specie, Antimony tartrate trihydrate ($C_8H_4K_2O_{12}Sb_2 \cdot 3H_2O$; noted KSb), with a range of concentration (0-5-10-20 $mg \cdot kg_{soil}^{-1}$ of dissolvable Sb^{3+}). The triplicates (pots) were first prepared all together in a bucket containing 3.6 kg of TLD soil (or 3.420 kg soil + 180 g of SCG or SCGc for the respective treatments). Each bucket received either 120 ml of the Sb solutions or the same volume of water and was then homogenized before transfer in each pot (1.2 kg per pot). After Sb spiking and OM addition, the pots rested for two months in a greenhouse to dry completely, mimicking thus a typical hot dry season from center/south Spain.

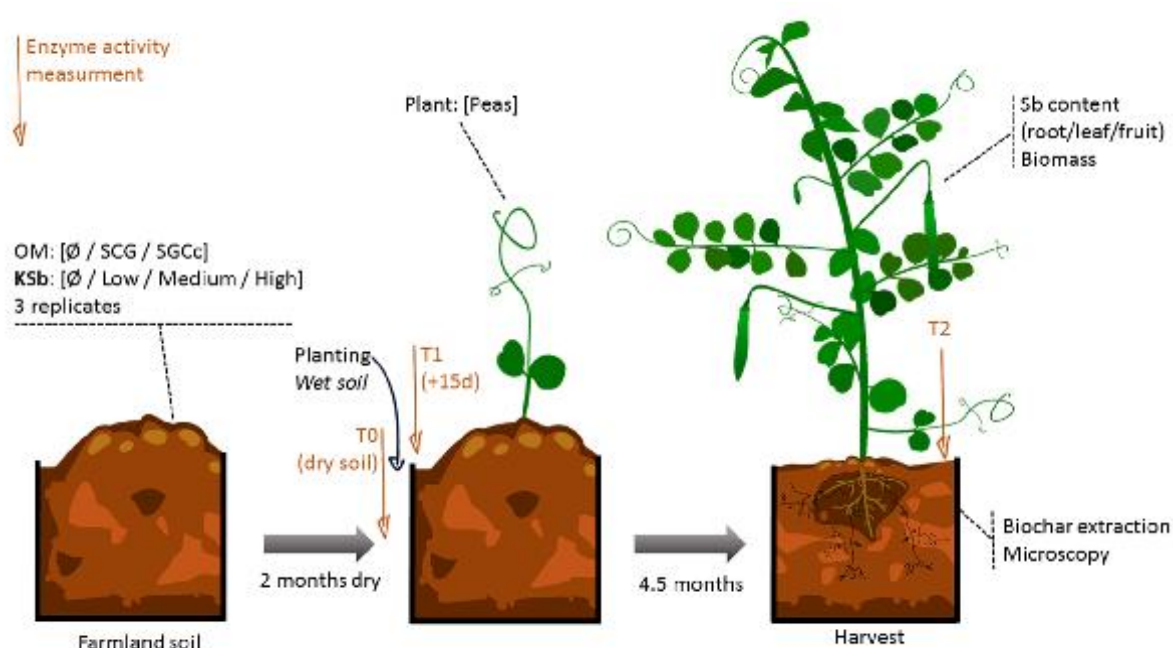
PEAS BREEDING AND TRANSPLANTATION. Pea seeds were sown (2 per pot) and after development of the seedlings only one was kept. Irrigation was made by capillarity, filling the saucers once a week, to limit soil (and Sb) lixiviation.

SOIL AND PLANT SAMPLING. After 4 months and a half, the plant organs (roots/aerial part/fruits) were harvested and oven dried (3 days, 50 °C) before being weighted and crushed for metal analysis. For this experiment, soil analysis were measured by Dr. Juan Carlos Sanchez Hernandez.

[Table 2.2](#) gathers soils characteristics at the end of the experiment. Total organic carbon (TOC, %) was determined in the air-dried soils according to the dichromate redox colorimetric method (Skjemstad and Baldock, 2007), and adapted to microplate format with sucrose as the standard to prepare a carbon calibration curve ranging between 0 and 16 $mg \cdot ml^{-1}$.

Table 2.2: Toledo soil characteristics at the end of the experiment. SCG: Spent Coffee ground; SCGc: Spent Coffee Ground charred; TOC: Total organic Carbon; EC: Electrical Conductivity.

OM	Sb	TOC, %	pH	EC ($\mu\text{S.cm}^{-1}$)
OM-	NO Sb	1.04 \pm 0.03	8.17 \pm 0.05	189.67 \pm 31.77
	LOW Sb	0.98 \pm 0.07	8.27 \pm 0.03	158.73 \pm 18.1
	MEDIUM Sb	0.97 \pm 0.09	8.31 \pm 0.03	147.23 \pm 13
	HIGH Sb	0.93 \pm 0.07	8.27 \pm 0.04	166.17 \pm 20.83
SCG, 5%	NO Sb	2.24 \pm 0.21	8.24 \pm 0.02	152.53 \pm 6.33
	LOW Sb	2.56 \pm 0.08	8.12 \pm 0.03	149.6 \pm 1.15
	MEDIUM Sb	2.82 \pm 0.11	8.11 \pm 0.02	141.43 \pm 3.87
	HIGH Sb	2.63 \pm 0.19	8.05 \pm 0.02	158.37 \pm 13.9
SCGc, 5%	NO Sb	3.69 \pm 0.14	8.06 \pm 0.08	168.77 \pm 9.91
	LOW Sb	4.08 \pm 0.25	8.02 \pm 0.06	164.63 \pm 9.02
	MEDIUM Sb	4.51 \pm 0.42	7.98 \pm 0.02	167.67 \pm 10.37
	HIGH Sb	4.39 \pm 0.24	8.01 \pm 0.02	164.97 \pm 10.29



2.5. Measurement of metal(loid) concentration

Most of the experiments involved TM content measurements in soil and/or plant organs. These analyses were performed following validated protocols from the literature to ensure data validity and comparison.

2.5.1. Total metal extract

As described in Foucault (Foucault, 2013), 0.125 ± 0.01 g were incubated with 5 ml HNO_3 2% (Pro Analysis Merck®) for 12 h in Digiprep tubes. After incubation and outgas, tubes were placed in a Digiprep® (80 °C, 1h30) before addition of 5 ml H_2O_2 30%. Two successive incubations in Digiprep® (55 °C, 25 min; 80 °C, 3h) were performed before outgassing and adjustment to 25 ml with ultrapure water and filtration on a 0.45 μm membrane.

2.5.2. CaCl_2 and EDTA extract

The CaCl_2 and EDTA fractions are currently used as pertinent predictors of respectively exchangeable and phytoavailable metal(loid) fractions in soil (Meers et al., 2007). The analyses were performed following the NFX-31-120 (AFNOR, 1992) and NEN-5704 (NEN 5704, 1996) norms respectively.

CaCl_2 : 10 ml of a CaCl_2 solution (0.01 M, Merck ENSURE®, pH 5.24) were added to 1 ± 0.01 g soil sample in 50 ml polypropylene tubes. All samples were shaken (50 osc.min^{-1} , 2 h) and then centrifuged (2000 g, 3 min).

EDTA: 10 ml of an EDTA- $\text{C}_2\text{H}_3\text{O}_2\text{-NH}_4$ (0.01M/1M) solution were added to 1 ± 0.01 g sample in 50 ml polypropylene tubes. Each soil sample was shaken (100 osc.min^{-1} , 2 h) and then centrifuged (2000 g, 10 min). The supernatant of CaCl_2 and EDTA extracts were then filtered on a 0.45 μm acetate membrane and each sample was acidified to 2% HNO_3 (Pro Analysis Merck®). All samples were stocked at 4°C in dark until analysis.

2.5.3. Bioaccessibility extract

The bioaccessible fraction was estimated following the unified Barge Method (Denys et al., 2012): 0.6 ± 0.01 g were incubated with 9 ml of synthetic saliva and shaken 10 sec in 50 ml polypropylene tubes. Then, each sample received 13.5 ml of synthetic gastric solution and was shaken 10 sec before adjustment of pH at 1.2 ± 0.05 with HCl 37%. To mimic digestion, tubes were shaken 1 h at 37 °C. After this step, if pH was >1.5 , samples were restarted from the beginning. Otherwise they were centrifuged (20 000 g, 15 min) before filtration of the supernatant on a 0.45 μm acetate membrane. Finally, each sample received 0.5 ml HNO_3 65% (Pro Analysis Merck®).

2.5.4. Metal analysis

Extracts were diluted with HNO₃ 2% and analyzed depending on their concentration either by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES – Thermo® Electron IRIS Intrepid II) or Mass Spectrometry (ICP-MS – PerkinElmer® Agilent 7500ce). Apple leaves (SRM-1515) and a lake sediment from Ontario (WQB-1) were used as certified material for plant and soil analysis respectively. Metalloid recovery rate from ICP MS analysis are reported in Table 2.3. Total metalloid in leaves were generally underestimated but similarity in replicates gave us great confidence in sample analysis repeatability. Indeed, Cd, Pb, and Sb recovery were 82%, 78%, and 67% respectively, in accordance with the results of Agnan (Agnan, 2013) performed in the same laboratory which presented a Cd, Pb and Sb recovery of 84%, 81%, and 66% respectively. Recovery rates in soils were more variable (127%, 75%, and 134% for Cd, Pb, and Sb respectively) but presented a good reproducibility too. Hence, we decided to apply a data correction to center our results on certified material theoretical concentrations.

Table 2.3: Metalloid recovery rate. Average \pm Standard error, %

Element	Recovery rate, %	
	Apple leave (SRM 1515)	Recovery rate (IAEA-336) (Agnan, 2013)
Cd	82 \pm 2.5	84 \pm 8
Pb	78 \pm 0.6	81 \pm 9
Sb	67 \pm 2.4	66 \pm 20
	Ontario lake sediment (WQB-1)	
Cd	127 \pm 0.9	-
Pb	75 \pm 0.6	-
Sb	134 \pm 3.6	-

2.6. Fungal analysis

2.6.1. Root staining and counting

To ascertain the presence of AMF in roots and estimate the mycorrhization rate, a staining protocol adapted from Vierheilig et al (Vierheilig et al., 1998) was carried out as follows:

Roots were bathed with a 10% KOH (w/v) solution and maintained at 95°C for 15min. Then, roots were rinsed with distilled water and bathed with a mix of 5% Jet Black Ink (Shaeffer®) in 5% acetic acid (95 °C, 3 min). After this step, roots were rinsed with successive 10 min bath of 5% acetic acid until a total disappearing of the color in the solution (Figure 2.7). Finally, roots were stored at 4 °C

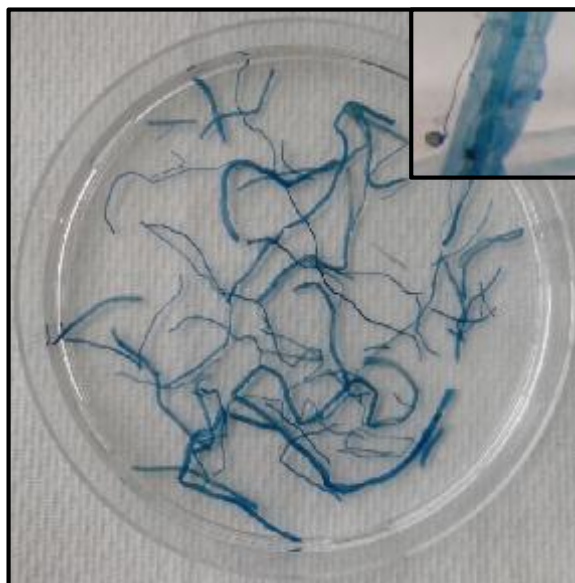


Figure 2.7: Picture of stained leek roots

in 5% acetic acid. The mycorrhizal rate (%M) was calculated within 15 days to prevent staining alteration. Counting was performed following the root intersect method (Giovannetti and Mosse, 1980) with a X65 stereomicroscope (Olympus SZX10). It is expressed as the ratio between the number of intersection showing a root colonized by AMF and the total number of intersection observed.

2.6.2. Spore isolation and counting

Because AMF spores size is usually in the range [40-180µm], extraction was performed by wet sieving using a triple sieve device (from 350>200>36 µm) to gather the spores in the smallest one. Their density allowed to separate them from soil particles with a sucrose gradient (50% v/v) or glycerol (50% v/v) after a short centrifugation (5 min, 2500 rpm). Spores were finally rinsed with ultrapure water and stored at 4°C before identification and biomolecular analysis. Spore concentration was estimated with a ×65 stereomicroscope (Olympus SZX10) by counting all the spores in 10 drops (100 µl) of the previously made spore solution. The concentration is usually expressed as a number of spores per 100 g of dry soil.

2.6.3. Fungal community identification through molecular biology

As the response of a host plant could depend on both the environment and the fungal community in symbiosis (Tonin et al., 2001), it is essential to estimate this community and study its variation between treatments. From this perspective, we intended new generation sequencing approach with Illumina MiSeq analysis.

DNA EXTRACTION AND SEQUENCING. Leek root fragments were collected for fungal DNA extraction using the PLANT KAPA 3G® Kit (Kapa Biosystems). To identify the fungal community brought with propagule solution in bioaugmented modalities, microbial DNA was also extracted from spore solution using the DNeasy® Plant Mini kit (Quiagen) with a pre-step as: (1) spores extraction; (2) Water take-out through deposition of the spore solution on a sterile membrane and vacuum depression; (3) Spore crushing in Fastprep 2 ml tubes containing silica fragments and spheres by vortexing the tubes 5 times (15 sec each) after liquid nitrogen freezing. DNA extracts were finally diluted 10× in DNA-free water before amplification procedure.

PRIMER CHOICE. Fungi amplicons library were obtained with three primer pairs as shown in Table 2.4, to amplify the ITS1 and ITS2 regions (Internal Transcribed Spacer). As the spore solution was not sterile, it is highly probable that we brought soil rhizospheric bacteria from the trap culture while extracting the spores. Thus, amplicon libraries were also produced by using bacteria-specific primers of the ribosomal V3-V4 region. All primer pairs (both forward and reverse) were barcoded with a unique (sample-specific) error-correcting 10-12 bases barcode for Illumina MiSeq.

Table 2.4: Primer pairs used for Illumina MiSeq identification of AMF communities.

Sequences came from Tedersoo et al., 2015

Region	Primer	Sens	Sequence (Bold, Illumina adaptor ; <i>italic, primer sequence</i>)
ITS1	ITS1F	Forward	CTTTCCCTACACGACGCTCTTCCGATCT <i>CTTGGTCATTTAGAGGAAGTAA</i>
	ITS2	Revers	GGAGTTCAGACGTGTGCTCTTCCGATCT <i>GCTGCGTTCTTCATCGATGC</i>
ITS2	ITS86F		CTTTCCCTACACGACGCTCTTCCGATCT <i>GTGAATCATCGAATCTTTGAA</i>
	ITS4		GGAGTTCAGACGTGTGCTCTTCCGATCT <i>TCCTCCGCTTATTGATATGC</i>
ITS2ngs	ITS3ngs4		CTTTCCCTACACGACGCTCTTCCGATCT <i>CATCGATGAAGAACGTAG</i>
	ITS4ngs		GGAGTTCAGACGTGTGCTCTTCCGATCT <i>TCCTSCGCTTATTGATATGC</i>
V3-V4			CTTTCCCTACACGACGCTCTTCCGATCT <i>ACGGRAGGCAGCAG</i>
			GGAGTTCAGACGTGTGCTCTTCCGATCT <i>TACCAGGGTATCTAATCCT</i>

AMPLIFICATION CYCLE. All amplifications were performed twice as follow: 25 µl reaction, 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.µl⁻¹), 12.5 µl of KAPA 3G Buffer mix with DNTPs, 0.4 µl of Taq DNA polymerase (2.5 U) and 7.6 µl of DNA-free H₂O.

The amplification cycle steps were: Initial denaturation at 95 °C (5 min), followed by 40 cycles of 30 sec denaturation at 95 °C, 30 sec primer annealing at 55 °C, 1 min extension at 72 °C, and a final extension at 72 °C for 10 min.

SEQUENCING MIX PREPARATION. The pooled samples were sent to the French GET-PlaGe platform (INRA Campus, 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.

SEQUENCE ANALYSIS. MiSeq sequences were analyzed with MothurGUI (V 1.0.0) (Schloss et al., 2009) for pairing, trimming and sequence cleaning and Usearch (V 8.1.1861) (Edgar, 2010) for OTU clustering with chimera removal, OTU abundance and taxonomy (using Unite+INSD V7 – 31/01/2016 and Maarjam ITS databases). The following section presents a detailed pipeline from raw data to OTU table and taxonomy assignation.

THE USE OF MOTHUR_GUI

Figure 2.8 shows the pipeline of analysis we developed on MothurGUI V 1.0.0 to (1) pair the forward and reverse sequences of each sample, (2) trim edges from MiSeq sequencing and (3) clean sequences from short artifacts.

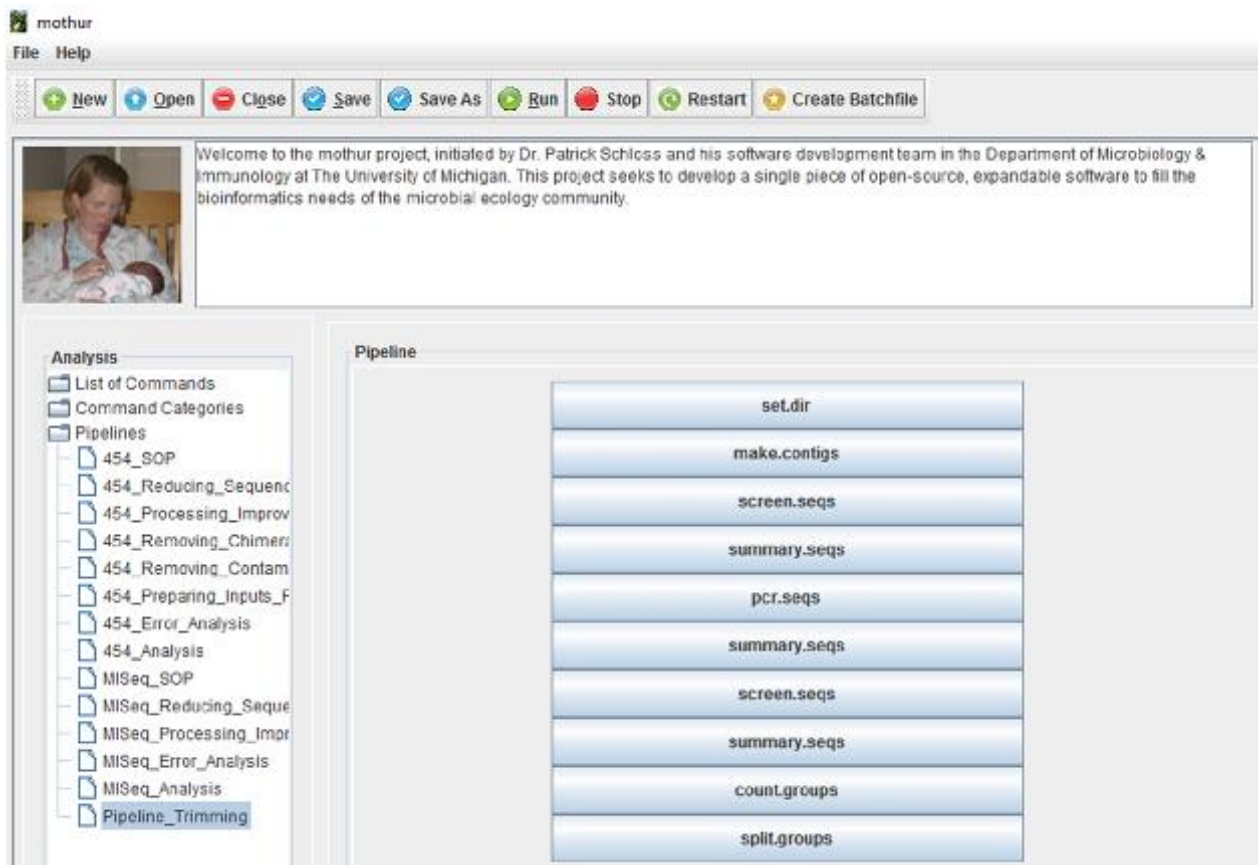


Figure 2.8: The MothurGUI interface and the pipeline of analysis developed and used for sequence cleaning

`set.dir` is used to define input and output folders for analysis. As a large number of files are created along with Mothur analysis, folders ought to be properly organized. The `modifynames` parameter is selected, to allow Mothur to change file names each time new ones are created. `make.contigs` need a `.file` document containing the list of samples to be analyzed. This file is a column document (tabulation spacer) with (per range of column) (1) sample name chosen by the user (2) forward (R1) sequence name and (3) reverse (R2) sequence name, both in `.fastq` format. The `format` is Illumina 1.8+ with a Needleman alignment protocol (`align`). As the datasets are very large, it is possible to use more than one processor core. However, when set to more than 4, some bugs can lead to a loss of sequences. Hence, we used only 2 or 3 processors at each step. Other parameters are set to default.

`screen.seqs` is set to default, except for the `minoverlap` parameter (10). This function checks and keeps the sequences for which forward and reverse are overlapping.

`summary.seqs` is a function which creates a summary file after each critical step (when the function is inserted in the pipeline).

`pcr.seqs` deletes sequence edges which correspond to the nucleotides present before the forward primer and after the reverse primer. These small sequences correspond to the MiSeq sample specific identification barcode. The `oligos` are needed, which is a file containing the primer sequences such as:

```
primer[TAB]GTGAATCATCGAATCTTTGAA[TAB]TCCTCCGCTTATTGATATGC
```

The `pdiffs` parameter is set to 4, meaning that 4 changes in the primer bases are accepted to recognize the sequence as the primer. This parameter could be a problem in the case of ITS sequence analysis as these regions are highly variable among fungi. However, the beginning and the end of these regions are generally more conserved than the central part. `Keepprimer` parameter is selected to conserve the primer sequence while `nomatch` is set to reject, to delete the outliers.

`screen.seqs` is then reused with everything to default, except for the `minlength` parameter (100). This function deletes all small sequences (length < 100 bases).

`count.groups` is added to count sequences from each set of groups (because Mothur is analyzing at the same time various samples (=various groups) thanks to the first `.file` document (see `make.contigs` above).

`split.groups` is finally added to separate the whole dataset into as many documents as the sample number.

As Mothur was coded for small datasets inducing extensive time of analysis, the last steps of the procedure (*i.e.* taxonomy assignment to the samples sequenced) was carried out using Usearch.

USEARCH (V 8.1.1861), AS A TOOL TO MAKE OTU AND THEIR TAXONOMY

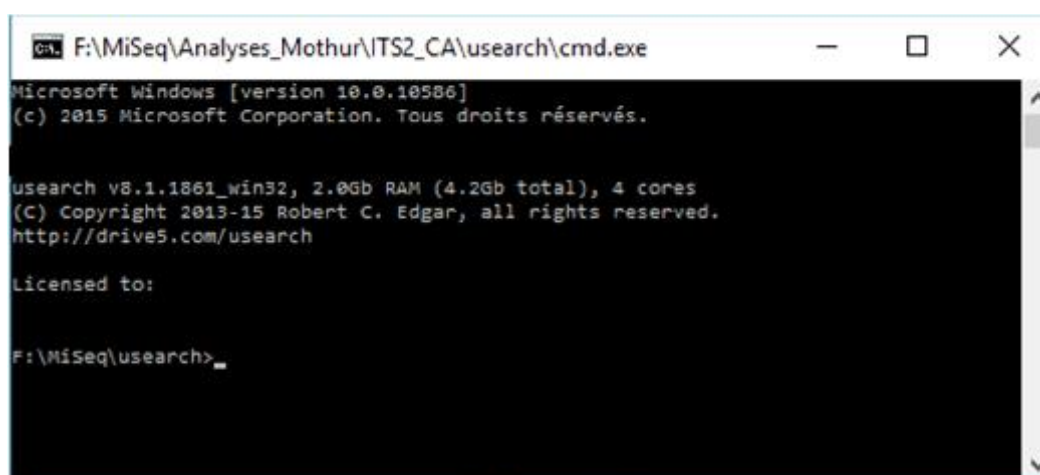


Figure 2.9: The Usearch user friendly interface

The files ultimately created by the Mothur pipeline were used in Usearch (whose interface is presented in Figure 2.9) for the following steps:

Dereplication: each sample file containing all the sequences needs to be summarized. Indeed, the Miseq technology amplifies thousands of times the sequences (but some more than others), so this step allows to count the 100% homological sequences and create a new file containing only unique sequences, with their abundance (called size in the program). The following command line was performed for each sample file:

```
-derep_fulllength Sample_file_from_mothur.fasta -fastaout new_file_name_derep.fasta
-sizeout -relabel new_name_for_sequences_into_derep_file
```

OTU clustering²: Clustering is a technic that gathers similar sequences into operational taxonomic units (OTU) depending on the chosen homology between them (generally fixed to 0.3, which means 97% of similarity). Different OTU clustering methods exist (Chen et al., 2013) but there are still controversies to determine which method is the best in new sequencing generation technics with large datasets. In this study, we used the Uparse clustering method detailed in footnote³ which has been validated for new generation sequencing on ITS region by Edgar (2013). Herein, all the sequences in an OTU were less than 3% different from the centroid sequence. This command also detects chimeras and deletes these sequences.

```
-otu_clustering new_file_name_derep.fasta -otus new_file_name_OTU.fasta -minsize 2 -
out_radius_pct 0.3 -sizeout -sizein -relabel new_name_for_sequences_into_otu_file
```

² Read this page for more information about OTU: http://drive5.com/usearch/manual6/otu_definition.html

³ For details about uparse clustering: http://www.drive5.com/usearch/manual/uparseotu_algo.html

OTU abundance table: To estimate the relative abundance of each OTU among treatments, an OTU table has to be built. This table represents all the newborn OTU in line and all the samples (= in our case CTR, OM+, B:OM+ treatments) in columns. To do so, various steps are needed:

- I. Put all the OTU sequences from samples in the same file, referenced here as *OTU_all.fasta*,
- II. Put all the sequences (*new_file_name_derep.fasta*) from samples in a same file, referenced here as *derep_all.fasta*,
- III. Blast all the derep sequences against the OTU_all database with 97% homology to create the OTU table:

```
-usearch_global derep_all.fasta -db OTU_all.fasta -strand plus -id 0.97 -otutabout
OTU_table.txt
```

Taxonomy assignation: This step allows to compare the set of OTU sequences with existing and identified databases, to name the OTU. In this study, we used various fungal databases of ITS sequences (Maarjam, utax, Unite+INSD) and finally selected Unite+INSD V7 – 31/01/2016, which gave the best taxonomic assignation. We used an 85% homology with the database as the intraspecific variation of fungal ITS sequences has been described as high. However, when assigned, the majority of the OTU were identified at more than 98% similarity.

```
-usearch_global OTU_all.fasta -db Unite+INSD_database.fasta -strand plus -id 0.85 -
uct taxonomy_assignment.txt
```

SEAVIEW (V 4.6) TO ALIGN OTU AND BUILT PHYLOGENIC TREES

Because the databases are not exhaustive, some OTU are not perfectly (or not at all) assigned to one or another taxa. Hence, to give better information of their similarity to other OTU, sequences need to be aligned and compared to build a similarity-based tree. These steps were done on Seaview v4.6. [Figure 2.10](#) to [2.12](#) are an example of the results of the different steps: [Fig.2.10 - Fig.2.11](#), alignment (with *muscle* algorithm, a Seaview internal tool) [Fig.2.12](#): tree representation and badly assigned OTU identification.

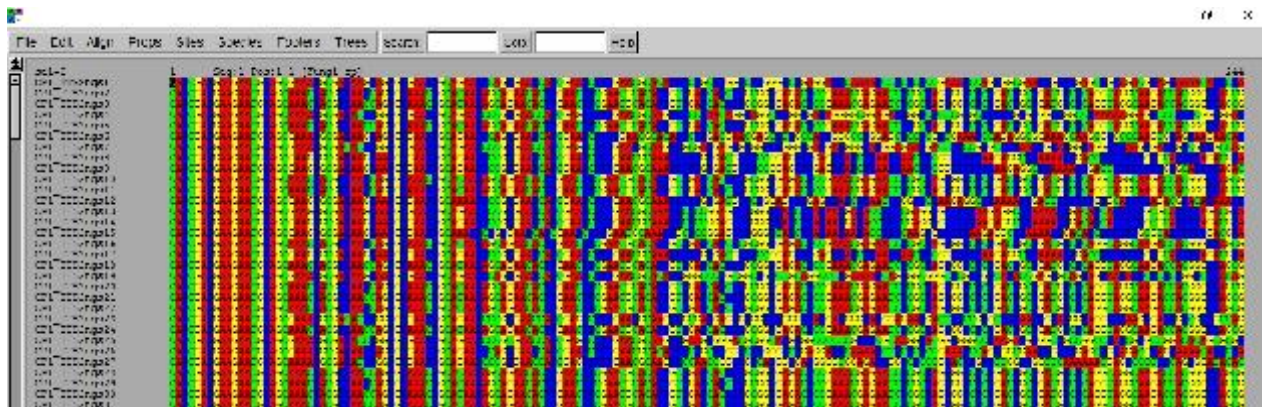
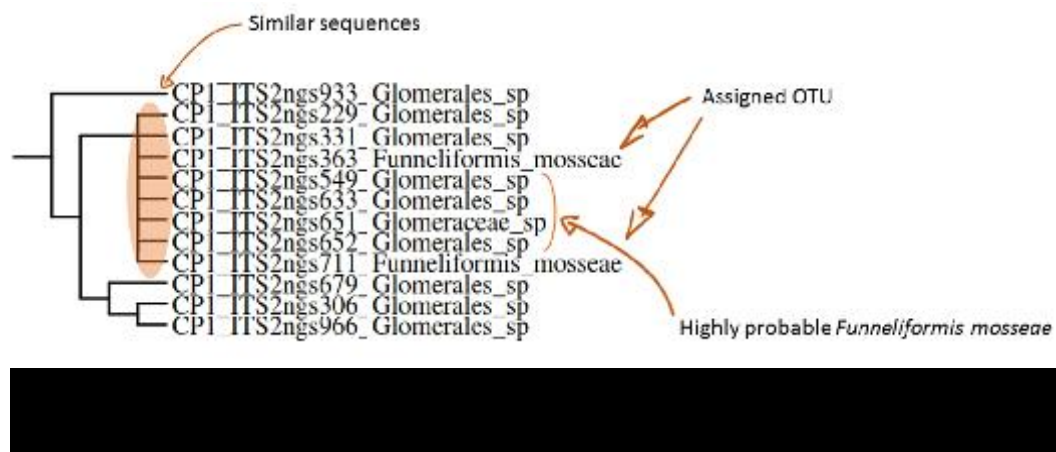


Figure 2.10: Seaview interface and non-aligned sequences (extract). Each color represent a nucleotide.



Figure 2.11: Aligned sequences, with muscle algorithm. This is only an example of extract of some sequences permitting the image to fit the page. It is completely normal that the alignment won't be perfect.



2.7. Enzyme assay and mathematical indexes

The enzyme activity measurement was carried out only in the Sb-biochar experiment (section 2.4, results in Chapter 6). To follow the temporal variation on enzyme activity, soil samples were collected and analyzed at T0 (dry soil, just before rewetting soil and planting), T1=T0+15 d, and T2 (harvest).

2.7.1. The study of soil enzyme activity

In order to study enzyme activities, a soil-water suspension (1:50; w:v; noted S-H₂O) was prepared according to Sanchez Hernandez et al (Sanchez-Hernandez et al., under review). One gram of wet soil was homogenized with 50 ml of deionized water for 30 min using an orbital shaker (Elmi® Intelli-mixer RM-2L, C2 program, 50 rpm) at room temperature ($\approx 20^\circ\text{C}$) in Falcon® tubes. As the reaction for acid phosphatase, β -glucosidase, urease and protease assays is a lengthy process (4 h), 1 ml of sodium azide (1 mM, final conc.) was added to each tubes to prevent microbial growth (Turner, 2010). However, NaN₃ was only added after Carboxylesterase (CbE) measurements as it interfered in the Fast Red-naphthol complex formation. These soil-water suspensions were stored at 4-5 °C for 3 days of analysis without change in enzyme activities. Studied enzymes are reported in Table 2.5, with main information (wavelength, substrate, and buffer).

Table 2.5: Potential enzyme activities measured in Peas experiment.

Adapted from Sanchez-Hernandez et al., under review. MUB: Modified Universal Buffer.

Enzyme (EC)	Substrate (final concentration)	Buffer	λ , nm
Carboxylesterase (3.1.1.1)	4-nitrophenyl butyrate (2.5 mM)	Tris-HCl 0.1 M (pH=6.5)	405
Acid phosphatase (3.1.3.2)	4-nitrophenyl phosphate (5 mM)	MUB* 20 mM (pH=6.5)	405
Alkaline phosph. (3.1.3.1)	4-nitrophenyl phosphate (5 mM)	MUB* 20 mM (pH=11)	405
β -glucosidase (3.2.1.21)	4-nitrophenyl β -D-glucanopyranoside (5 mM)	MUB 20 mM (pH=7.2)	405
Protease (3.4.21.92)	Casein (1% w/v)	Tris-HCl 50 mM (pH=8.1)	700
Urease (3.5.1.5)	Urea (40 mM)	Unbuffered method	690
Dehydrogenase (1.1)	Iodotetrazolium chloride (0.14% w/v)	Tris-HCl 1M (pH=7.0)	464
Catalase (1.11.1.6)	H ₂ O ₂ (8.8 mM)	Unbuffered method	505

HYDROLASES ACTIVITY MEASUREMENTS.

Carboxylesterase activity (3.1.1.1): The reaction mix was composed of 100 μl of S-H₂O, 140 μl of Tris-HCl 0.1 M (pH=7.6) and 10 μl of substrate (2 mM, final conc.). After incubation of the microplates with a thermostatically controlled orbital shaker Elmi® Skyline DTS-2 (800 rpm, 30 min, 25 °C), they were centrifuged (2500 rpm, 10 min, 10 °C). Then, 150 μl supernatants were transferred to a new microplate. Revelation was performed with addition of 75 μl of a solution containing 2% (w/v) SDS and 2% (w/v) Tris base and the absorbance was immediately read at 405 nm. A calibration curve was made with 4-nitrophenolate using S-H₂O (to correct the adsorption of the chromogenic substances onto soil colloids) to assess enzyme activity.

Controls (substrate-free) and blanks (soil-free) were respectively used to correct background absorbance and non-enzymatic hydrolysis of the substrates.

Acid phosphatase (EC 3.1.3.2), *Alkaline phosphatase* (3.1.3.1) and *β -glucosidase* (EC 3.2.1.21) assays: The reaction medium was prepared with 100 μ l of S-H₂O, 100 μ l of distilled water and 50 μ l of respective substrate (4-nitrophenyl phosphate.Na₂.6H₂O for both phosphatases or 4-nitrophenyl β -D-glucanopyranoside; 5 mM final conc.) dissolved previously in 20 mM modified universal buffer (pH = 6.5, =11 and =7.2 respectively). After 4 h of incubation (continuous shaking at 20 °C), microplates were centrifuged (2500 g, 10 min, 10 °C). Supernatant aliquots (150 μ l) were transferred to new microplates. Reaction was blocked with addition of 75 μ l of 0.5 M NaOH to wells (after this blocking step, blanks received 50 μ l of respective substrate for color correction). Then, the formation of 4-nitrophenolate was immediately (< 1 min) read at 405 nm. Standard calibration curves were made with 5–100 μ M of 4-nitrophenolate in the presence of S-H₂O, to correct potential matrix effect.

Protease (EC 3.4.21.92) and *Urease* (EC 3.5.1.5) activity were measured according to Schinner et al. (Schinner et al., 1996). Volumes were adapted using 1.5 ml microfuge tubes to save reagent consumption. For protease, reaction mixture consisted of 0.5 ml of S-H₂O and 0.5 ml of casein (2% w/v in 50 mM Tris-HCl, pH = 8.1). After 2 h of incubation (continuous agitation in a water bath at 50°C), excess of proteins was precipitated by addition of 0.5 ml of 0.92 M cold trichloroacetic acid followed by centrifugation (10 000 g, 10 min, 4°C). The aromatic amino acids formed were detected by mixing in microplate wells the aliquots of supernatants (80 μ l) and 120 μ l of an alkali solution (50:1:1, v/v, 0.47 M Na₂CO₃ containing 6 mM NaOH: 20 mM CuSO₄: 26 mM sodium potassium tartrate) followed by 80 μ l of 10% (v/v) Folin-Ciocalteu's phenol reagent (Sigma-Aldrich®). Microplates were kept in dark until complete color development (90 min) and spectrophotometry reading at 700 nm. Tyrosine (Sigma-Aldrich, Madrid) was used as the standard.

Urease (EC 3.5.1.5) activity was carried out studying the hydrolytic reactions by mixing 100 μ l ml 80 mM urea and 100 μ l of S-H₂O and 4 h incubation (orbital shaker) at room temperature (22 °C). Reactions were blocked adding 500 μ l of cold [1M KCl; 10 mM HCl]. Additional agitation (30 min) was performed for ammonium extraction before centrifugation (10 000 g, 5 min, 10 °C). Supernatants (150 μ l) were added to wells of microplates, and ammonium was measured after addition of (1) 75 μ l of 1:1 (v:v) 0.3 M NaOH: 1.06 M sodium salycilate containing 4.6 mM sodium nitroprusside and (2) 30 μ l of 39.1 mM sodium dichloroisocyanide. Microplates were left for 30 min in dark for color development before absorbance reading (690 nm). NH₄Cl was used for calibration curve.

DEHYDROGENASE AND CATALASE ACTIVITIES.

These oxidoreductases were studied as they present great potential as indicators of soil microbial activity (Moore and Russell, 1972).

Dehydrogenase activity (EC 1.1) was measured following the Von Mersi and Schinner method (Mersi and Schinner, 1991) with iodonitrotetrazolium chloride. The formation of reduced iodonitrotetrazolium formazan (INTF) was read at 464 nm after a 60 min reaction at 40°C.

Catalase (1.11.1.6) activity was determined according to Trasar-Cepeda et al. (Trasar-Cepeda et al., 1999) with H₂O₂ (0.3% w/v) as substrate mixed with 0.5 g wet soil and 8 ml H₂O. Incubation lasted 6 min in a water bath at 25°C before addition of 1 ml of 3 M H₂SO₄ to stop it. 1ml of solution was transferred in 1.5 ml microfuge tubes for centrifugation (10 000 g, 5 min, 10 °C). 10 µl of the residual substrate was transferred in microplate and decomposed by addition of 50 µl of 110 mM Na-phosphate buffer (pH=7.4) mixed with a 240 µl of an indicator solution containing 50 µl of 110 mM Na-phosphate buffer (pH=7.4), 30 mM 4-aminoantipyrine, 5.3% phenol and 1.2 U.ml⁻¹ of horseradish peroxidase type-I (5 KU, Sigma-Aldrich). Coloration was read at 505 nm. The calibration curve was performed with H₂O₂.

2.7.2. Mathematical indexes

Mathematical indexes developed in ecotoxicology studies were used to access the combined activities of enzymes and to highlight global changes in soil activity. Accordingly, we calculated the *GMean index* (Hinojosa et al., 2004), the *WMean index* (Lessard et al., 2013), the *T-SQI* (Mijangos et al., 2010), and the *IBRv2 index* which is a modified version (Sanchez et al., 2013) of the original IBR index (Beliaeff and Burgeot, 2002).

The GMean and WMean indexes were calculated as follow:

$$GMean = \left(\prod_{i=1}^n y_i \right)^{1/n}$$

$$WMean = \sum_{i=1}^n w_i \times y_i$$

$$w_i = \frac{u_i}{\sum_{i=1}^n u_i}$$

Where y_i is the enzyme activity, n is the total number of soil enzymes, and w_i is the weight of each soil enzyme with u_i being the eigenvector for each soil enzyme activity associated to the

first principal component obtained from a principal component analysis. High values of these two indexes indicate high microbial functional diversity.

The T-QSI was calculated using the following equation:

$$T_SQI = 10^{\log m + \frac{\sum_{i=1}^n (\log n_i - \log m) - \sum_{i=1}^n |\log n_i - \log \bar{n}|}{n}}$$

Where m is the reference soil (mean value of enzyme activity, set to 100%), and n is the mean value for each enzyme activity measured in treated soils as a percentage of the reference soil.

As some variations of interpretation and application of the formulas appear in the literature (Sanchez-Hernandez et al., under review), we used the detailed method of the latter authors to calculate the IBR v2. Individual enzyme activities from each treatment (X_i) were therefore compared to mean values of enzyme activity from the control plots (*i.e.* no OM, no Sb) (X_0) and its standardized value Z_0 .

$$IBR = \sum \left| \frac{(\log \frac{X_i}{X_0} - \mu)}{\sigma} - Z_0 \right|$$

Increase in IBR index values in contaminated sites when compared to reference ones is generally expected due to global changes in enzyme activity.

2.8. Microscopy

Scanning Electron Microscopy in low vacuum coupled with X-Ray analysis (ESEM Quanta 250 FEG; with Platine and cryo PP3000T module - Quorum; X-ray analysis Edax - APEX 2i; CMEAB, Toulouse, France) was used to evaluate metal(loid) localization in plant cells. The contact between an electron emitted with a microscope's cathode and one from each atom from the sample surface produces an element specific energy (-1- a retrodiffused electron after elastic interaction with a sample atom; -2- another electron tiered out from the sample surface and -3- X-ray energy, when excited atoms from the sample comes back to fundamental state of energy) permitting to localize and estimate these atoms relative abundance (Figure 2.13). The incident electrons penetrate at variable depth depending on (i) the initial energy of emission and (ii) the atomic number of the element composing the sample. This technic informs on localization of the elements and potential interactions. Unfortunately, it does not provide any information about metal(loid) chemical speciation.

Hence, fresh leeks root fragments were used to evaluate Cd, Pb, and Sb localization in plant cells and fungal structures. 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). Last steps consisted of (1) sublimation (10 min, with a temperature increase to -90 °C) to vaporize the water and (2) platinum covering (5-10 nm).

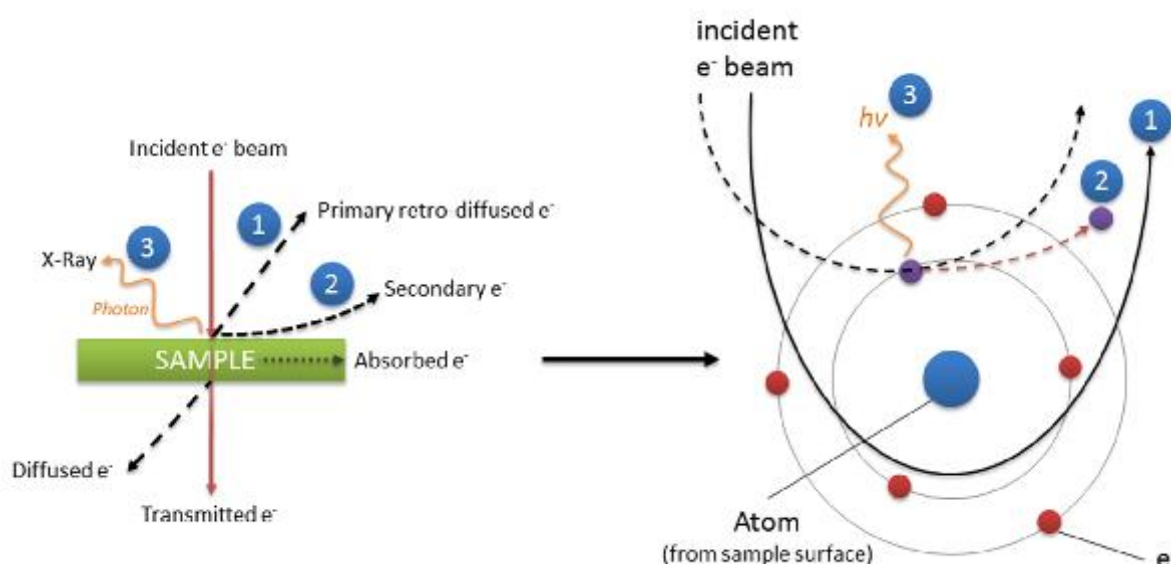


Figure 2.13: The basic principle of Scanning Electron Microscopy

2.9. Statistical analysis

Results were expressed as average \pm SD (standard deviation) or as a percentage for fractions. General linear models were applied on dataset at $\alpha = 5\%$ using the SAS-JMP v.11 Software after verification of data normality and log-transformation when necessary. A post-hoc Student test was used for significant group segregation; letters are used in tables and figures to identify them. A rank-based statistical test (Kruskal-Wallis one-way ANOVA) was also calculated with SPSS v.23 (IBM Statistics®) to strengthen confidence in GLM significance as the experiments were carried out on a small number of replicates (Crop succession: lettuce, n=10; leek, n=5. Hydropony: carrots, n=4; lettuce, n=4. Enzyme activity: peas, n=3). In peas experiment with Sb spiking and biochar, a Jonckheere-Terpstra-trends test (SPSS v.23) was also used to evidence physiological trends related with the spiking gradient of Sb (Field, 2013).

“La statistique souvent, c’est la mort... C’est la mort de l’analyse. On l’dit des fois, y’a un proverbe qui circule : y’a trois sortes de mensonges. Les p’tits mensonges, les sacrés mensonges et les statistiques.”

Cédric Villani,

Mathematician & director of the Institute Henri Poincaré, Paris

France inter Radio, Nicole Ferroni, May, 25th 2016

Dealing with the possible decrease of the scientific research allowances.

Chapter 3. HOW DO SOIL BIOFERTILIZERS AND ORGANIC AMENDMENTS INFLUENCE Cd PHYTOAVAILABILITY AND HUMAN BIOACCESSIBILITY?

Because anthropic origin was evidenced only for Pb and Sb, results concerning Cd transfer were separated from the latter elements even if they deal with the same experimental design. Hence, Chapter 3 will deal with Cd results while Chapter 4 will present Pb and Sb results.

3.1. General introduction

In this chapter, which is presented as a submitted article, the main question is to assess the impact of arbuscular mycorrhizal fungi and lombricompost (combined or not) on cadmium transfer from soil to edible plants on two contrasted peri-urban soils, in relation with human bioaccessibility of this element, because:

- Cd is one of the major contaminant found in arable soils.....(Alloway, 1995)
- AMF are naturally in symbiosis with more than 80% of the plants(Brundrett, 1991)
- They can be brought as biofertilizers(Kangwankraiphaisan et al., 2013)
- They participate in TM mobility at the soil-plant interface.....(Jarrah et al., 2014)
- Cd mobility is considered as relatively high and is affected by OM....(Beesley et al., 2010)
- Soil biophysicochemical properties are main factors of TM mobility.....(Haghir, 1974)

Accordingly, we carried out a pot experiment during ~ 1.5 year with a crop succession of two leafy vegetables commonly grown in UPA (lettuce and leek) ([Figure 3.1](#)). Although AMF commercial products exist to fertilize crops, we chose to mimic biofertilization spreading a home-made concentrated propagules solution from the tested soils (see [Chapter 2](#) for detail), as this low-cost technic could easily be transferred to UPA gardeners if the results showed remediation or stabilization potential. This modality was then named bioaugmentation (*i.e.* a biological increase of the local AMF potential) to study its impact on Cd fate.

For lettuce cultivation, we tested their growth on (1) natural soil or (2) bioaugmented soil. After this step and plant analysis, soil rested and then half of the pots received an OM amendment before leek cultivation.

Hence in the following article, the modalities on each soil were:

Lettuce		
Natural soil = Control		CTR
Bio-augmented soil		B
Leek		
Natural soil = Control		CTR
Bio-augmented soil		B
Natural soil + lombricompost		OM+
Bio-augmented soil + lombricompost		B:OM+

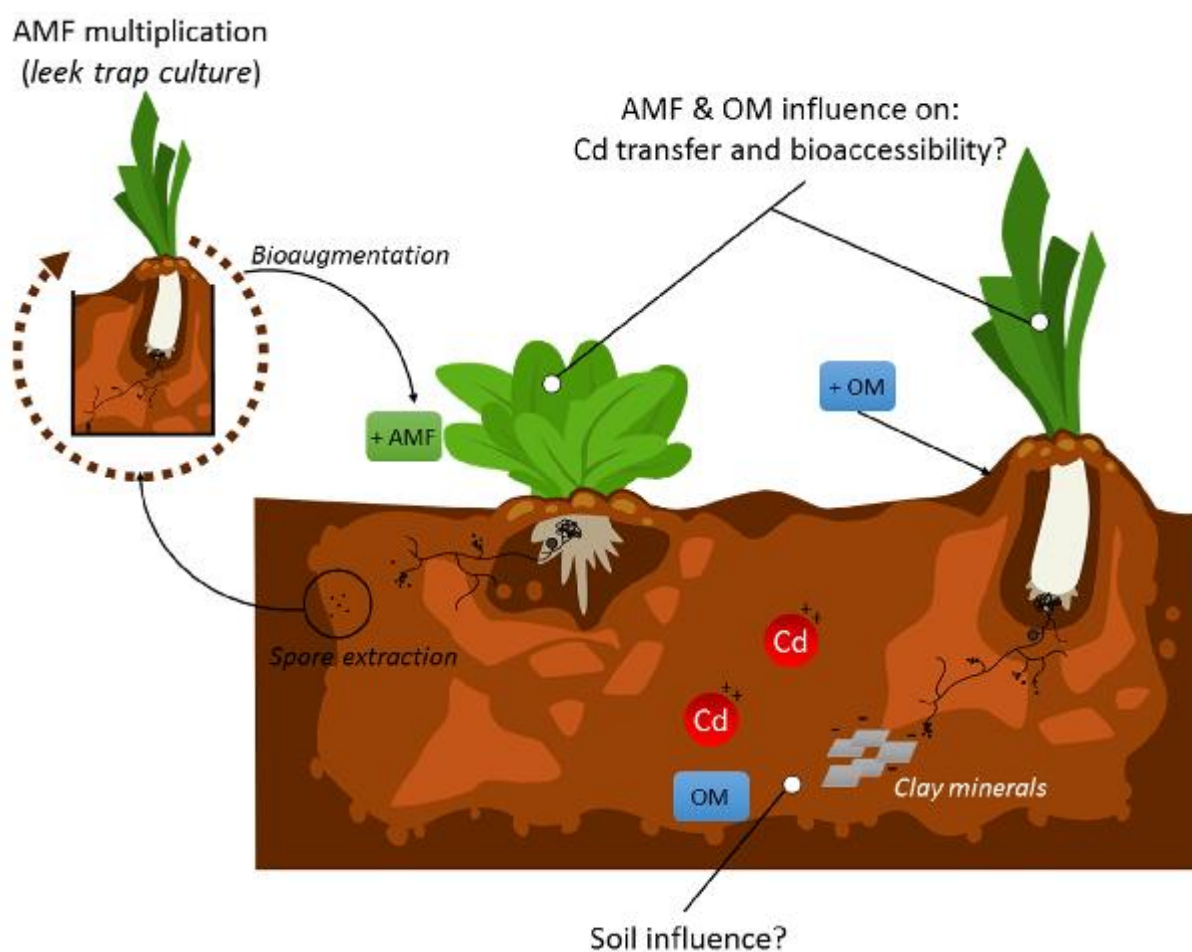


Figure 3.1: Scientific objectives and methods for Cd fate at the soil-microorganism-plant interface.

3.2. How do soil biofertilizers and organic amendments influence Cd phytoavailability and human bioaccessibility?

(Submitted to journal of hazardous materials, 19/07/2016) – Section, page, figure and table numbering were adapted to the manuscript.

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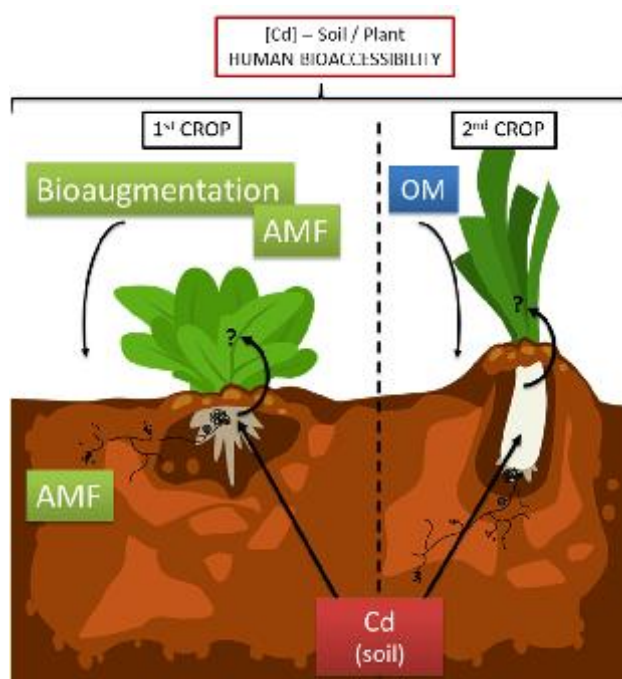
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Abstract

Urban agriculture is becoming increasingly widespread worldwide. Vegetable quality is crucial particularly in terms of persistent metal(loid) levels. Arbuscular mycorrhizal fungi (AMF) are symbiotic soil microorganisms interacting with plants and are known to participate in soil-plant metal(loid) transfer. The present study investigated, for the first time, the relationship between bio-fertilization with local AMF (*i.e.* bio-augmentation), organic matter (OM) and Cd uptake by leafy vegetables. The impact of this symbiosis on Cd human bioaccessibility was also studied. In order to simulate current gardening activities, a crop succession was carried out as follows: (1) lettuce was cultivated in pot with AMF or not; (2) the soil was rested and lombricompost was added; (3) Leek was cultivated. The Cd content was measured in soil and edible organs.



Bio-augmentation increased Cd accumulation in lettuce on a sandy-clay soil (noted BZC). OM addition appeared to decrease Cd in leek while bio-augmentation with local AMF tended to increase its accumulation. The influence of biofertilizers is complex as numerous influencing factors are involved. However, in some treatments, the Cd concentration surpassed legal thresholds in edible plants. Our findings thus highlight the importance of studying human Cd bioaccessibility in urban garden vegetables to provide coherent recommendations to gardeners.

Keywords

Urban agriculture, arbuscular mycorrhizal fungi, human bioaccessibility, sustainable food, health risk assessment

Highlights

- Cd accumulation in lettuce and leek depends on the soil physico-chemical characteristics
- Cd phytoaccumulation increased with bio-augmentation but decreased with OM
- Bioaccessible Cd is high (>85%) but is not affected by bio-augmentation or OM
- The Cd Tolerable daily intake was exceeded in representative urban conditions

3.2.1. Introduction

Metal(loid) dispersion contaminates arable land all around the world. Cadmium from phosphorus fertilizers is one of the major inorganic pollutants observed in the soil (Alloway, 1995). Cultivated urban and peri-urban land is particularly subjected to pollution from diffuse aerial deposition of contaminated particles coming from industries and traffic dust (Xiong et al., 2014b), which is another important source of Cd (Kabata-Pendias and Mukherjee, 2007). Cd shows high bioavailability compared to other pollutants (Beesley et al., 2010) (*i.e.* Cd is potentially more easily accumulated by living organisms). Due to its high toxicity, the maximal Cd value in edible plants has been legislated in several countries for years (Zorrig and El Khouni, 2013). The European directive EC1881/2006 limited Cd concentrations to 0.2 and 0.1 mg.kg⁻¹ fresh weight in leafy and stem/root vegetables. However, recent discussions from the European commission suggest a will to lower this threshold as this element is potentially highly available for plants (Nguyen et al., 2013).

The worldwide economic crisis has been driving more people to grow their own food in public, associative or kitchen gardens (Galt et al., 2014). Such places are generally set-up near roads or in the vicinity of industries or even directly in disused industrial sites (Álvarez-Ayuso et al., 2012). In these urban environments, soil and plants are therefore at risk of

contamination from airborne particles enriched with metals (Leveque et al., 2013; Shahid et al., 2013c). Because food is the major exposure route to environmental pollution for humans (Fries, 1995), metal transfer in the soil-plant-water system is often studied (Okkenhaug et al., 2011; Wan et al., 2013).

Recently, human bioaccessibility of Cd (the fraction extracted by digestive fluids in the lumen) was estimated to assess human Cd bioavailability (fraction which can pass through the intestinal barrier to bloodstream), using the *in vivo* method developed by the BioAccessability Research Group of Europe (BARGE) (Denys et al., 2012). The objective was to mimic the digestive physicochemical processes when polluted vegetables (or soil) are ingested (Wragg et al., 2011). Lettuce was shown to contain a high fraction of bioaccessible Cd, which reached 89% (Xiong et al., 2014b).

Under natural conditions, plants live in close association with other living organisms such as bacteria and fungi. Mycorrhizal symbiosis is a common growth condition for about 85% of flowering plants (Brundrett, 1991). Both organisms benefit from this mutualistic relationship: arbuscular mycorrhizal fungi (AMF) transfer nutrients (mostly phosphorus) and can enhance stress tolerance (drought, pathogens...) (Li et al., 2013; Smith and Smith, 2011). In exchange, plants provide carbon from photosynthesis to the fungus but also organic compounds in the rhizospheric soil (Smith and Smith, 2011). AMF are also known to be key actors in metal bioavailability in soil and plant absorption (Jarrah et al., 2014; Khan, 2005) through exudation of various compounds in the mycorrhizosphere. However the mechanisms involved are not yet completely understood and due to the numerous interactions observed in soil ecosystems, predicting the soil-plant transfer is complex even if some models are being developed (Lin et al., 2015). Indeed, due to the changes in pH which occur in the mycorrhizosphere when AMF excrete organic acids and protons, heavy metals can be converted into unavailable chemical species for plants (Garg and Bhandari, 2013) and they can also be bound to the fungal cell wall or glomalin (González-Chávez et al., 2004). However, in some cases when AMF solubilize inorganic phosphorus they also solubilize heavy metals, leading to possible uptake by hyphae and roots (Giasson et al., 2005).

Cd is highly bioavailable (easily extractable from soil by numerous living organisms) and accumulates in leafy vegetables (Peris et al., 2007), particularly in lettuce (Beavington, 1975; Uzu et al., 2010; Xiong et al., 2014a; Zorrig and El Khouni, 2013), a widely cultivated vegetable in urban gardens for fresh consumption (FranceAgriMer, 2013; Guitart et al., 2014). The role of AMF in Cd phytoavailability (its presence in a form that plants can solubilize and/or extract) and phytoaccumulation (its uptake and storage by plants) was previously described in the literature for different plants (Aloui et al., 2009; de Andrade et al., 2008; Rivera-Becerril et al., 2005). However, no information exists on their impact on Cd human bioaccessibility in vegetables even though these organisms are already used by

home-growers as biofertilizers (Kangwankraiphaisan et al., 2013). According to reports, two groups of soil physicochemical properties mainly govern Cd transfer between the soil solid phase and soil solution: (i) the soil solution pH, cation exchange capacity, OM and clay contents; and (ii) the soil redox potential, Fe/Al oxides and hydroxides and other metals concentrations (Kabata-Pendias and Mukherjee, 2007; Alleoni et al., 2005; Diemar et al., 2009; Ilgen and Trainor, 2012; Roper et al., 2012; Sposito, 2008; Tighe et al., 2005; Xi et al., 2010; Xu et al., 2011).

In the context of gardens, a large amount of soil organic matter (OM; currently around 5-10%) is generally observed due to gardening practices (Schwartz, 2013) and the fate of Cd in the soil is strongly influenced by its interaction with these amendments (Leveque et al., 2014; Quenea et al., 2009).

Accordingly, our goal was to assess, for representative urban garden soil conditions (high OM content, various Cd concentrations), the role of AMF and OM (both separately and combined) in Cd phytoaccumulation, compartmentation and human bioaccessibility during a crop succession involving lettuce and leek – two widely cultivated leafy vegetables in urban agriculture. Cd contents were measured in soils and plants at different scales (soil fraction, organism and organ) to examine human health risks. The Tolerable Daily intake (TDI, the amount of contaminant which is considered as acceptable to be ingested daily) was used as the threshold value and plant sanitary quality was assessed by comparing it with plant Cd contents.

3.2.2. Material and methods

3.2.2.1. Plant cultivation

STUDY SITE. To represent urban garden soil characteristics (high OM content, various but moderate Cd content), two contrasting soil types were tested (Table 3.1): a sandy soil from an urban garden in North France (Nantes (NTE): 47° 16' 1.512" N – 1° 34' 29.688" O) containing a high level of OM and average Cd content and a peri-urban sandy-clay soil (Bazoches (BZC): 48° 11' 21.375" N – 2° 3' 28.993" O) with a significant Cd contamination. Both soils were gently sieved (2 mm) to increase structural homogeneity.

Table 3.1: Soil characteristics at Bazoches (BZC, sandy clay) and Nantes (NTE, sandy). OM: total organic matter; B/NB: bio-augmentation or not. CEC: cation exchange capacity.

	pH	Initial	[Pb],	[Sb],	[Cd],	Clay,	Silt,	Sand,	CaCO ₃ ,	P ₂ O ₅	CEC
	H ₂ O	OM, %	mg.kg ⁻¹	mg.kg ⁻¹	mg.kg ⁻¹	%	%	%	%	mg.kg ⁻¹	cmol ⁺ .kg ⁻¹
BZC	8.1	3.2	412	15,4	3.0	24.0	55.3	16.7	2.0	76.7	15.85
NTE	7.0	6.0	456	2,7	0.2	14.0	28.4	57.4	0.2	380	8.71

BIO-AUGMENTATION SOLUTION. Because of its rusticity and high mycorrhization rate, organic leek was used to multiply the natural AMF inoculum from contaminated soils. Leeks were grown under a greenhouse (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). Spore extraction was performed by wet sieving (45 µm) and centrifugation (2500 rpm, 5 min) of the spore suspension in 50% glycerol (v:v). After rinsing, a spore suspension for bio-augmentation was prepared (280 sp.ml⁻¹).

LETTUCE CULTIVATION. Organic lettuce seeds (*Lactuca sativa* L., Laitue Pommée appia, Ferme de Sainte Marthe®) were sown on sterilized oildri® (20 min, 121°C) and grown under natural light at ~17°C. After three weeks, seedlings were placed in same greenhouse conditions as above to acclimatize for one week. The lettuces were then transplanted to contaminated soil in a 2 L pot, with one plant per pot (10 replicates). For the bio-augmentation condition, 9 ml of a spore suspension were added directly to the whole root system (*i.e.* approximately 2500 spores per pot). A fully randomized design was adopted to apportion the variability in greenhouse conditions. A regulated dripper line watered the pots per capillarity filling each saucer every three days, to limit spores and metal lixiviation processes.

ORGANIC MATTER ADDITION AND LEEK CULTIVATION. The lettuce plants were grown for 12 weeks before harvest. Then the soil was left in the pots for 2 months. After this period, sterilized lombricompost (organic matter NFU 44-051, Botanic®, [Supplementary materials Table 1](#)) was added to half of the replicates of each treatment to increase soil OM content of 1%. This amendment was mixed with the soil and the pots were left to stabilize for another month before planting one leek per pot (*Allium porrum* L., Poireau de Saint Victor, Ferme de Sainte Marthe®) and cultivating them for 25 weeks (5 replicates).

3.2.2.2. Plant sampling and analysis

Aerial 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 till mycorrhization rate measurement. Washing with water was chosen to mimics how gardeners usually wash their vegetables. The plant organs were oven dried (40°C/5 d), weighed and then ground with an IKA® A11 grinder.

MYCORRHIZATION RATE. Observation and counting of AMF colonization (%M) was carried out following the root intersect method (Giovannetti and Mosse, 1980) with Jet Black ink (Shaeffer®) staining (Vierheilig et al., 1998) and a X65 stereomicroscope (Olympus SZX10).

TOTAL METAL. Root and leave powder were digested in Digiprep® tubes and filtrated (0.45µm acetate membrane) following the procedure of Foucault et al (Foucault et al., 2013).

BIOACCESSIBLE FRACTION. The human bioaccessible fraction 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 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 does not extract significantly more Cd (Denys et al., 2012).

3.2.2.3. Soil sampling and analysis

Soil samples were dried (20°C, 10 d), ground with a ceramic mortar and pestle and sieved to 250 µm. Total organic matter (%OM) was assessed by calcination according to the MA-1010 procedure (CEAEQ, 2003), and pH_{H2O} was measured following to the standardized protocol NF-ISO-10390 (AFNOR, 2005); data are reported in [Table 3.1](#).

METAL CONTENT. The total soil metal content was measured according to Foucault et al (Foucault et al., 2013). EDTA and CaCl₂ chemical extractions were carried out according to the NFX-31-120 (AFNOR, 1992) and NEN-5704 (NEN 5704, 1996) norms, respectively, to assess the Cd fraction associated with OM and Cd phytoavailability. These two extraction methods are often used to highlight the phytoavailable and exchangeable metal fractions in soil (AFNOR, 1992; NEN 5704, 1996).

3.2.2.4. Cadmium analysis

Samples were analyzed using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES-Thermo® Electron IRIS-Intrepid-II) or Mass Spectrometry (ICP-MS-PerkinElmer® Agilent-7500ce). Apple leaves (SRM-1515) were used as certified material for plant analysis. In our conditions, a recovery of 82±2.5% was obtained for Cd. Two ratios were used to study soil-to-plant Cd transfer (Pérez-Sirvent et al., 2012; Xiong et al., 2014b): the Bioaccumulation Factor (BF) and the Translocation Factor (TF) which are $[Cd]_{total_plant}/[Cd]_{total_soil}$ and $[Cd]_{leaf}/[Cd]_{root}$, respectively. BF is representative of Cd phytoaccumulation and TF estimates inside-plant transfer and accumulation in organs.

3.2.2.5. Daily intake measurement as a health risk assessment tool

During health risk assessments, an estimate of the daily intake (DI, $\mu\text{g}\cdot\text{d}^{-1}$, Eq. 1) can be obtained from Cd concentrations in edible parts ($[\text{pollut}]_{\text{veg}}$, $\mu\text{g}_{\text{Cd}}\cdot\text{kg}_{\text{FW}}^{-1}$) and the daily consumption rate (DC, $\text{kg}_{\text{FW}}\cdot\text{d}^{-1}$). The latter parameter is hard to estimate because of its important inter-individual and seasonal variability. However, from urban gardener testimonials, Dumat et al (Dumat et al., 2015) estimated that the maximal consumption rate of a mix of vegetables (which usually contains lettuce and leek throughout the year) is about $300\text{g}\cdot\text{d}^{-1}$, which is consistent with the national value estimated by the European Food Safety Authority (EFSA, 2008), $342\text{ g}\cdot\text{d}^{-1}$. Lettuce consumption represents about $12\text{ g}\cdot\text{d}^{-1}$ according to a survey carried out by the CTIFL (*Centre Technique Interprofessionnel des Fruits et Legumes*) in 2010. The average human body weight (BW) was estimated to be about 62 kg (Walpole et al., 2012). In 2011, The EFSA ensured that the Tolerable Daily Intake (TDI) of Cd was $0.36\text{ }\mu\text{g}_{\text{Cd}}\cdot\text{kg}_{\text{BW}}^{-1}\cdot\text{d}^{-1}$. Hence, it is both possible to estimate if whether or not a risk exists by comparing the TDI and the ratio $\text{DI}/62$ for a fixed DC or to estimate the maximal consumption of lettuce (DC_{MAX} , $\text{g}_{\text{FW}}\cdot\text{kg}_{\text{BW}}^{-1}\cdot\text{d}^{-1}$) to stay below the TDI (Eq. 2).

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

$$\text{(Eq. 2)} \quad DC_{\text{MAX}} \leq TDI / [\text{pollut}]_{\text{veg}}$$

3.2.2.6. Statistical analysis

Results were expressed as the average $\pm\text{SD}$ (standard deviation). After verification of data normality and log-transformation when necessary, general linear models were applied on datasets 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 (lettuce, $n=10$; leek, $n=5$), 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.

3.2.3. Results

3.2.3.1. Biomass and mycorrhization

[Table 3.2](#) shows the dry biomass of the plant organs and root colonization by AMF. Addition of OM did not lead to a significant alteration in the mycorrhization rate, except in NB:OM+ leek cultivation, in which the %M significantly decreased for BZC soil, accompanied with an increased biomass. Alone, bio-augmentation did not affect plant growth in either crop compared to control while addition of OM significantly increased leek biomass.

3.2.3.2. Soil characteristics and cadmium content

The addition AMF or OM did not modify soil pH or %OM to a significant extent between the beginning and end of the experiment in BZC soil. However, in NTE soil both treatments significantly increased pH and a plant effect was also observed as pH in leek control was higher than initial pH.

After lettuce cultivation, the CaCl_2 fraction represented 0.25% and 1.5% of the total Cd in BZC and NTE soil, respectively. The bio-augmentation approach had no effect on these values ([Table 3.3](#)). After leek cultivation, the addition of OM decreased the CaCl_2 fraction in NTE soil (from 1.5% to 0.3%) but the results were not significant due to the high variability in OM- samples compared to OM+ samples. In BZC soil, OM addition had no effect on this mobile fraction. However, in NB treatments the CaCl_2 fraction increased up to 2.2% compared with the end of the lettuce cultivation while bio-augmentation maintained it at a low level (0.3%) ([Table 3.3](#)).

Cd EDTA extraction did not appear to be affected by either OM or bio-augmentation throughout the crop succession, except in NTE soil where OM led to a significant increase in its concentration compared to the control. For EDTA fraction, no significant differences among treatments were observed, but a significantly higher fraction was observed in BZC than in NTE (25% and 37% of soil total Cd respectively). This fraction increased by about 8% between lettuce and leek cultivation in all samples.

Table 3.2: Measured parameters in soil and plant. OM+/OM-: addition of lombricompost or not; B: bio-augmentation; MYC%: Mycorrhization rate; OM%: organic matter content; TF: Translocation factor. Statistical analyses were carried out between each soil and plant condition. a/b: significant groups ($\alpha=5\%$); no letters: no significance.

NTE				BZC								
LETTUCE		LEEK		LETTUCE		LEEK						
Control	B	Control	OM+	B	B:OM+	Control	B	Control	OM+	B	B:OM+	
MYC,%	65.7±3.8 a	50.1±4.5 b	39.8±5.1 b	41±1.4 b	53.6±5.9 a	56.5±0.1 a	56.2±7.1 b	67.7±4.6 a	42.7±1.2 a	28.1±1.8 b	53.3±10.2 a	49.7±3.7 a
pH	6.8±0.4 b	7.4±0.5 a	7.1±0.4 b	8.1±0.1 a	7.9±0.1 a	8±0.04 a	8.4±0.02	8.4±0.01	7.9±0.02 ab	7.9±0.01 a	7.8±0.02 b	7.9±0.03 ab
OM,%	3.2±0.3	3.2±0.1	4.4±0.1 b	5.9±0.4 a	3.9±0 b	6.1±0.5 a	3±0.03	3±0.1	5.9±0.02 b	7.5±0.3 a	6±0.05 b	7.5±0.3 a
Leaf DW, g	2.5±0.6	1.9±0.7	1.8±0.5 b	4.7±1.3 a	1.6±0.5 b	2.4±1 b	1.5±0.2	2.1±0.6	1.8±0.3 b	3.3±1.1 a	2.2±0.6 b	3.5±0.6 a
Root DW, g	0.7±0.3	0.8±0.2	0.6±0.2 b	1.7±0.6 a	0.3±0.1 b	0.7±0.4 b	0.9±0.2	0.8±0.3	0.9±0.3 b	1.4±0.3 a	1.3±0.3 a	1.6±0.2 a
Soil total	0.5±0.01 a	0.4±0.01 b	0.5±0.1 b	0.4±0.03 ab	0.4±0.02 a	0.4±0.02 ab	2.3±0.02	2.3±0.02	2.3±0.02	2.2±0.1	2.2±0.02	2.2±0.03
Soil EDTA	0.1±0.01	0.1±0.01	0.1±0.01 b	0.1±0.01 a	0.1±0.02 ab	0.1±0.01 ab	0.7±0.01	0.7±0.01	0.8±0.02	0.8±0.01	0.8±0.03	0.8±0.05
Soil CaCl ₂	0.01±0	0.01±0	0.01±0 a	0.002±0 b	0.004±0 ab	0.001±0 b	0.01±0	0.01±0	0.03±0 a	0.04±0.01 a	0.01±0 b	0.01±0 b
TF	1.4±0.4	2±0.7	0.15±0.01 a	0.11±0.01 ab	0.14±0.05 a	0.06±0.01 b	2.3±0.3	1.9±0.3	0.3±0.1 ab	0.3±0 b	0.5±0.1 a	0.4±0.1 ab
BF	3.1±0.2	3.4±0.3	0.9±0.2 bc	0.8±0.1 c	1.5±0.1 a	1.2±0.2 ab	5.2±0.1	6.1±0.2	3.1±0.2	2.4±0.4	2.7±0.3	2.6±0.3

Table 3.3: Mobile (CaCl₂ extract) and mobilizable (EDTA extract) fractions (%) of cadmium in soil. OM+/OM-: addition of lombricompost or not; B: bio-augmentation.

Statistical analyses were carried out between each soil and plant condition. a/b: significant groups($\alpha=5\%$); no letters: no significance.

	NTE						BZC					
	LETTUCE			LEEK			LETTUCE			LEEK		
	Control	B		Control	OM+	B	Control	B		Control	OM+	B
CaCl ₂ /Total												
[min-max]	[1.4-3.4]	[1.1-2.6]		[0.2-2]	[0.3-0.6]	[0.4-1.8]	[0.1-0.2]	[0.2-0.3]	[0.2-0.3]	[1.4-1.5]a	[1.4-2.2]a	[0.2-0.3]b
EDTA/Total												
[min-max]	[13.5-20.6]	[15.8-21.6]		[19.9-25.3]	[19.5-29.4]	[16.1-27.6]	[24.5-27.9]	[29-31]	[30.8-31.6]	[34.5-35.9]	[34.9-37.9]	[35.4-37.9]

3.2.3.3. Cadmium accumulation in plants and translocation

CD PHYTOACCUMULATION

Figure 3.2 shows the accumulation of Cd in both plants, at the organ scale (aerial vs root) with significant groups for total Cd in the plants highlighted (significant groups at the organ scale are shown in [supplementary material, Table 2](#)).

In lettuce, the results contrasted between soil types. A significant increase in Cd_{root} was observed for the bio-augmented (B) treatment in BZC soil but no effect was observed in NTE soil. In leek, addition of OM in BZC soil significantly decreased total plant Cd in NB samples but had no effect in B treatments. In NTE, total Cd content in the plants was significantly higher in the B modality. Results at the organ scale were slightly different: OM decreased Cd in leaves, with more contrasted results in NTE soil than BZC soil: in the latter, the only significant difference was OM+ < B while in NTE, both OM and B interacts as follow: B > Control ≥ OM+ ≥ B/OM+. Hence, a coupled effect (B:OM+) decreased Cd accumulation in leaves.

In lettuce, the addition of AMF did not affect the TF. In Leek, OM decreased TF in BZC soil while a coupled effect (B:OM+) decreased it in NTE. OM alone led to the lowest Cd accumulation in BZC soil while it appeared better to pair it with AMF bio-augmentation in NTE soil for leek cultivation.

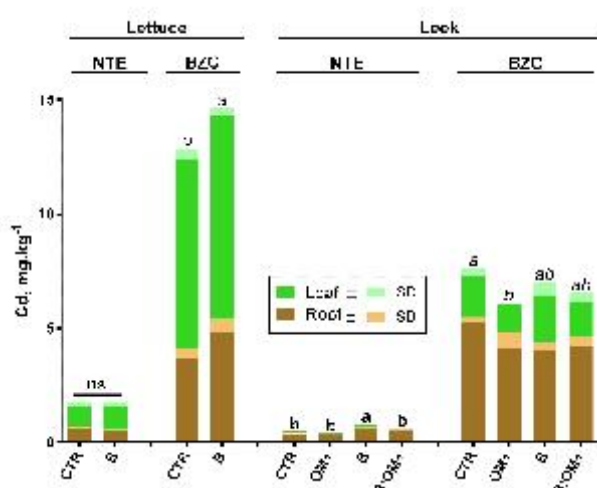


Figure 3.2: Cadmium concentration in plant organs. NTE/BZC: Nantes/Bazoches, CTR: Control, B: Bio-augmentation, OM+: Lombricompost, a/b: significant groups at $\alpha = 5\%$ for whole plant, ns: not significant. Statistical test were carried out within each soil-plant group, between the different treatments (CTR, B, OM+).

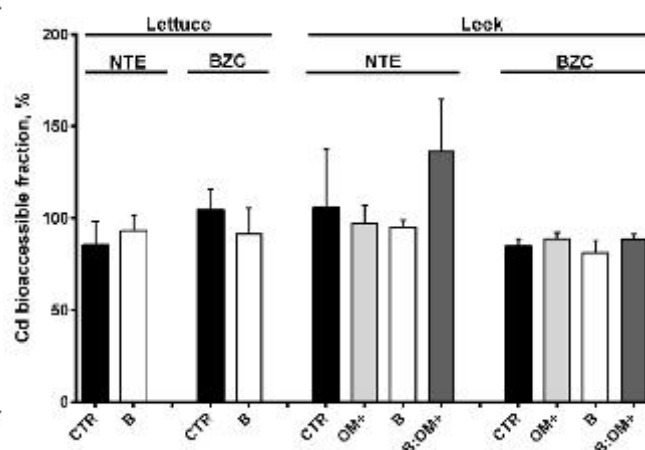


Figure 3.3: Cadmium bioaccessible fraction in edible parts. NTE/BZC: Nantes/Bazoches, CTR: Control, B: Bioaugmentation, OM+: Lombricompost.

FROM HUMAN BIOACCESSIBILITY TO HEALTH RISK ASSESSMENT THROUGH THE TDI

Human bioaccessibility was estimated for the edible part of the plants (Figure 3.3). Our results showed that a significant fraction of Cd in lettuce and leek leaves (85%) is gastric bioaccessible. OM and bio-augmentation had no effect on this fraction. Daily Intake (DI, Table 3.4) and maximal daily consumption (DC_{MAX}) were calculated using Eq.1 and Eq.2. With a consumption rate of 12 g of fresh lettuce leaves, the DI is about 3 to 30 times higher than the TDI ($0.36 \mu\text{g}_{Cd} \cdot \text{kg}_{BW}^{-1} \cdot \text{d}^{-1}$). To stay below this legal threshold, the DC_{MAX} should range between 0.4 and 4.4 $\text{g}_{FW} \cdot \text{d}^{-1}$ of lettuce. For leek, the INSEE estimates a daily consumption of about 3.6 g per person. Accordingly, the DI was generally low in NTE soil ($<0.03 \mu\text{g}_{Cd} \cdot \text{kg}_{BW}^{-1} \cdot \text{d}^{-1}$), which is lower than the TDI. In BZC soil the DI was up to two times higher than the TDI.

Table 3.4: Average cadmium Daily Intake (DI) and maximal daily consumption (DC_{MAX}) in Bazoches (BZC) and Nantes (NTE) soils. CTR: control, B: bio-augmentation, OM+: lombricompost.

	NTE						BZC					
	LETTUCE		LEEK				LETTUCE		LEEK			
	Control	B	Control	OM+	B	B:OM+	Control	B	Control	OM+	B	B:OM+
DI, $\mu\text{g}_{Cd} \cdot \text{kg}_{FW}^{-1}$	1	1.2	0.02	0.01	0.03	0.01	10	10.6	0.6	0.4	0.7	0.6
DC_{MAX} , $\text{g}_{plant} \cdot \text{d}^{-1}$	4.4	3.8	68	109	48	138	0.4	0.4	2.1	3	1.9	2.5

3.2.4. Discussion

3.2.4.1. Parameters affecting cadmium accumulation in plants

OM AMENDMENTS AFFECT CD PHYTOAVAILABILITY AND UPTAKE

Leafy vegetables accumulate Cd from the environment (Zorrig and El Khouni, 2013). Indeed, in the present study we observed Cd phytoaccumulation in leek and lettuce leaves. Lettuce accumulated significantly more Cd than leek in all treatments showing a plant-specific absorption rate. Cadmium is generally mobile in soil, with weak binding to clay, but a strong affinity to OM (Prokop et al., 2003). Its phytoavailability is considered important in comparison with other currently observed metals in urban areas such as lead (Beesley et al., 2010). However, addition of lombricompost lowered the exchangeable Cd (NTE soil). Such results are in agreement with anion sorption studies (Choi, 2006; Masset et al., 2000; Medina et al., 2010; Roth et al., 2012), showing that the presence of humic acids increases Cd adsorption by increasing the effectiveness of cation exchange capacity (CEC) through soil acidification (Haghiri, 1974; Hinsinger et al., 2003; Nye, 1981). This could explain why in BZC

soil, where the CEC is higher than in NTE, OM had no effect, as the CEC was already high enough to alleviate Cd stress. In Spinach, OM addition to soil (10-20 t.ha⁻¹ of compost) reduced Cd accumulation in shoots by up to two times (Mani et al., 2014). This is consistent with our experimental conditions and results where we found trends of less Cd in leek aerial parts when amended with OM. In a context of urban soil (intensively reworked soils), where it might be possible to have a loss of AMF and bacterial communities, the use of OM could be a useful tool to try to bind heavy metals in soils and limit their transfer in the environment.

AMF BIO-AUGMENTATION CAN AFFECT CD PHYTOACCUMULATION

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). The combined role of OM and AMF in Cd phytoaccumulation in peri-urban areas was investigated here for the first time in relation with human bioaccessibility. The effect of bio-augmentation with a concentrated spore solution varied depending on both the plant species and soil type. More Cd was root-sequestered when lettuces were bio-augmented in the Cd contaminated sandy-clay (BZC) soil while no visible effect was observed in the non-contaminated sandy soil (NTE). Bio-augmentation (B) during leek cultivated in BZC soil had no effect on leaf accumulation or TF while for those cultivated in NTE soil, leaf accumulation increased with B when no OM was added. Our results are consistent with data obtained in Cd spiking studies (Aloui, 2009). In this thesis, the author found that mycorrhized plants accumulate more Cd than non-mycorrhized plants, through the activation of stress alleviation metabolic pathways. Furthermore, isolated metal-tolerant AMF (*Funneliformis mosseae*) was shown to passively adsorb up to 0.5 mg_{Cd}.mg⁻¹_{soil} (Joner et al., 2000). The authors also highlight other Cd stress alleviation mechanisms such as synthesis of organic acids (malic, oxalic, and citric) lowering the mycorrhizospheric pH, leading to either an increased mobility or a precipitation of Cd (Garg and Bhandari, 2013). However, opposing results were also previously obtained with mycorrhized tobacco plants (Janoušková et al., 2005) and thus fungal uptake appears to depend on the fungal strain (Liu et al., 2014).

Bio-augmentation with local strains of AMF (in natural soil already containing a microbial community) specifically decreased the Cd mobile fraction (CaCl₂ extraction) in leek cultivated in BZC soil and to our knowledge this is the first report of such observation. This could be due to a positive interaction between leek and some fungal strains in the bio-augmentation solution as the Cd mobile fraction was 10 times higher for the leek control (natural AMF present in the soil) (Table 3.3). Concerning the mobilizable fraction (EDTA) which represents the organic and carbonate bound fraction, an *in vitro* speciation experiment showed that AMF can solubilize a non-available form of cadmium (CdCO₃) through acidification of the mycorrhizosphere (Giasson et al., 2005). We observed a trend of the Cd_{EDTA} fraction

increasing slightly (+1.5%) by bio-augmentation in the sandy-clay soil which is consistent with this soil having high level of carbonates from the alteration of a rich calcareous mother rock (Cecchi et al., 2008) where Cd could be bound. However, this change of phytoavailability due to bio-augmentation did not increase plant Cd content in that soil. This could be due to: (i) the presence of glomalin, a largely studied fungal protein exudate from AMF, which can bind high amounts of Cd and other pollutants in soil (González-Chávez et al., 2004) or (ii) a difference in the fungal and bacterial community as the fate heavy metals at the soil-plant interface was previously shown to vary with the fungal species (Hassan et al., 2013; Zhang et al., 2015).

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) as they can increase TM mobility in soil. However, under natural conditions the soil flora and fauna would be much more complex, so these results should be strengthened either with field work or at least in more complex systems. For example, a recent study focusing on the combined or independent role of AMF and earthworms reported that the latter might also affect Cd speciation, even more than AMF (Aghababaei et al., 2014).

3.2.4.2. Health risk assessment

HUMAN BIOACCESSIBILITY OF LETTUCE IS HIGH

Human bioaccessibility of Cd in lettuce and leek was not affected by AMF bio-augmentation or OM addition 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 lettuce and leek cultivated in urban gardens contained up to 0.7 mg.kg⁻¹_{DW} Cd, with about 80% bioaccessibility (Mombo et al., 2016). 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.

Finally, due to the lack of certified material for the BARGE method, we could only compare one another soils or plants to draw valuable scientific conclusion. The development of certified materials for these methods would allow the focus to be placed on bioaccessible concentrations, with the final aim to define more representative legal thresholds for metals in edibles rather than total concentration per kg dry weight, as it is currently the case.

LEGAL THRESHOLDS AND LEAFY VEGETABLE CONSUMPTION

Urban areas have been intensively studied during the last decade because of economic needs and the growing and interest of such places for growing food with a low ecological footprint (Martellozzo et al., 2014). For lettuce cultivated in contaminated peri-urban soils, Cd

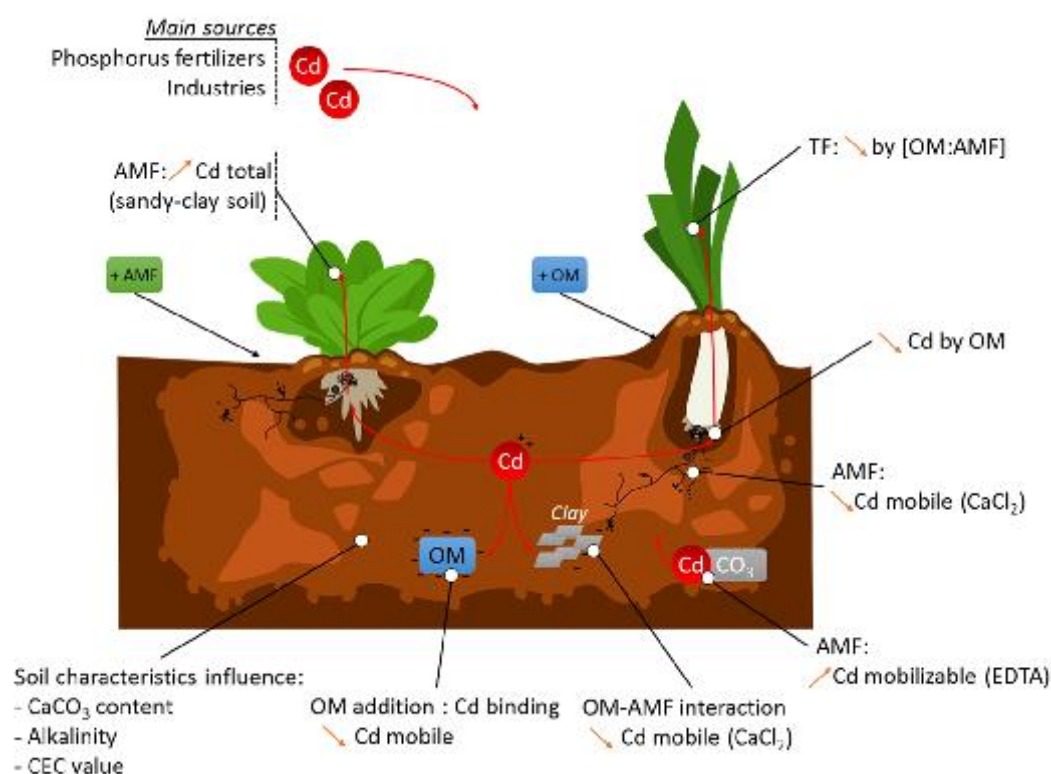
accumulation led to a very low maximal consumption ratio ($<1 \text{ g}_{\text{FW}}.\text{d}^{-1}$). In the case of leek cultivated in a low contaminated sandy soil (NTE), an acceptable accumulation rate was measured ($<\text{TDI}$) and this value decreased with OM addition, while in BZC soil, the maximal consumption rate should not exceed $2.5 \text{ g}_{\text{FW}}.\text{d}^{-1}$. Because Cd human bioaccessibility is almost 90%, even considering this fraction and not the total Cd concentration, the DC_{MAX} stays low. Warming et al (Warming et al., 2015) estimated the human risk through the Hazard Quotient (HQ), in which only a - hard to estimate - fraction (f) of local products is consumed: $\text{HQ}=(\text{DI}\times\text{f})/\text{TDI}$. Indeed, in a case of recreational production, the home-grown fraction could be low, diluting the potential local pollution in the whole diet. The most relevant parameters governing Cd accumulation are (ordered by significance): crop species > presence of plantings forming a barrier between roadside and crop > number of vehicles per day in the nearest road > crop planting organization > distance to the nearest road (Säumel et al., 2012). In addition, lettuce cultivars can also behave differently (Michalska and Asp, 2001). The cultivar we grew (Quelio) was not tested in the former study but seemed to behave the same as Oresto, with a higher accumulation of Cd in leaves than in roots. The cultivar taxa seems therefore to be more relevant than the species taxa for health risk assessments (Wang et al., 2007). Phytoaccumulation also appears to be site-specific (Hough et al., 2004) but also season and fertilizer dependent (Florijn et al., 1992; Tyksiński and Kurdubska, 2005). Hence, according to our results, together with current knowledge on leafy vegetable Cd accumulation (Xiong et al., 2014b; Michalska and Asp, 2001; Tyksiński and Kurdubska, 2005; Zhang et al., 2013), it can be concluded that these crops should be avoided (from a health point of view) in the case of urban and peri-urban areas facing pollution. However, a good selection of low-accumulator cultivars could reduce the risks when grown on low or medium contaminated soil, as it 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 and special growing conditions could be chosen such as pots, greenhouses or hydroponics.

3.2.5. Conclusions and perspectives

This study aimed to investigate, for the first time, the influence of arbuscular mycorrhizal fungi on Cd phytoaccumulation in leafy vegetables in relation to its human bioaccessibility in edible parts. As a summary of the actual state of knowledge on Cd uptake by plants, we propose the following [Figure 3.4](#)¹. First, we found that neither OM nor AMF influenced the bioaccessible fraction of Cd. Then, the plant species appeared to play a major role in Cd accumulation as lettuce accumulated more than leek in both soils. Soil characteristics such

¹ Leek drawing adapted from Freepik

as CaCO_3 content, alkalinity and CEC value also affected both the mobile and mobilizable fractions (CaCl_2 and EDTA). These variations influenced Cd accumulation in the plant with higher phytoaccumulation in a sandy-clay soil than in a sandy soil. Lombricompost addition as an OM amendment decreased the Cd mobile fraction by increasing soil negative surfaces but it had no visible effect on Cd accumulation in plants. We showed here for the first time that bio-augmentation with local AMF propagules also decreased the Cd mobile fraction but only after leek cultivation which might be either a long term effect or a combined plant-fungal effect. Finally, a coupled effect of OM and bio-augmentation can decrease both mobile and mobilizable Cd but also the root to leaf translocation factor. The complexity of understanding the mechanisms of Cd binding and uptake at the soil-fungal-plant interface highlights the importance of studying these microorganisms. Indeed, gardeners use such biofertilizers, but can also face metal contamination through various media as Cd is used in industrial processes and phosphorus fertilizers. Because urban areas are also subjected to significant aerial particle contamination, these results would also need to be linked with investigations on aerial exposure routes in order to highlight the whole contamination process and provide clear recommendations to urban gardeners.



3.2.6. Acknowledgment

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3.2.8. Supplementary data

Table 1 (SM): Chemical composition of lombricompost.
Lombricomposted horse a/o cattle manure, organic matter NFU 44-051, Botanic®.

% Dry Matter	48%
% Organic matter	20%
N total	1% v/v
N organic	0.9%
P ₂ O ₅ total	0.7%
C/N	10

Table 2 (SM): Detail of significant groups at $\alpha = 5\%$ for Cd concentration in plant organs (detailed in Figure 1).

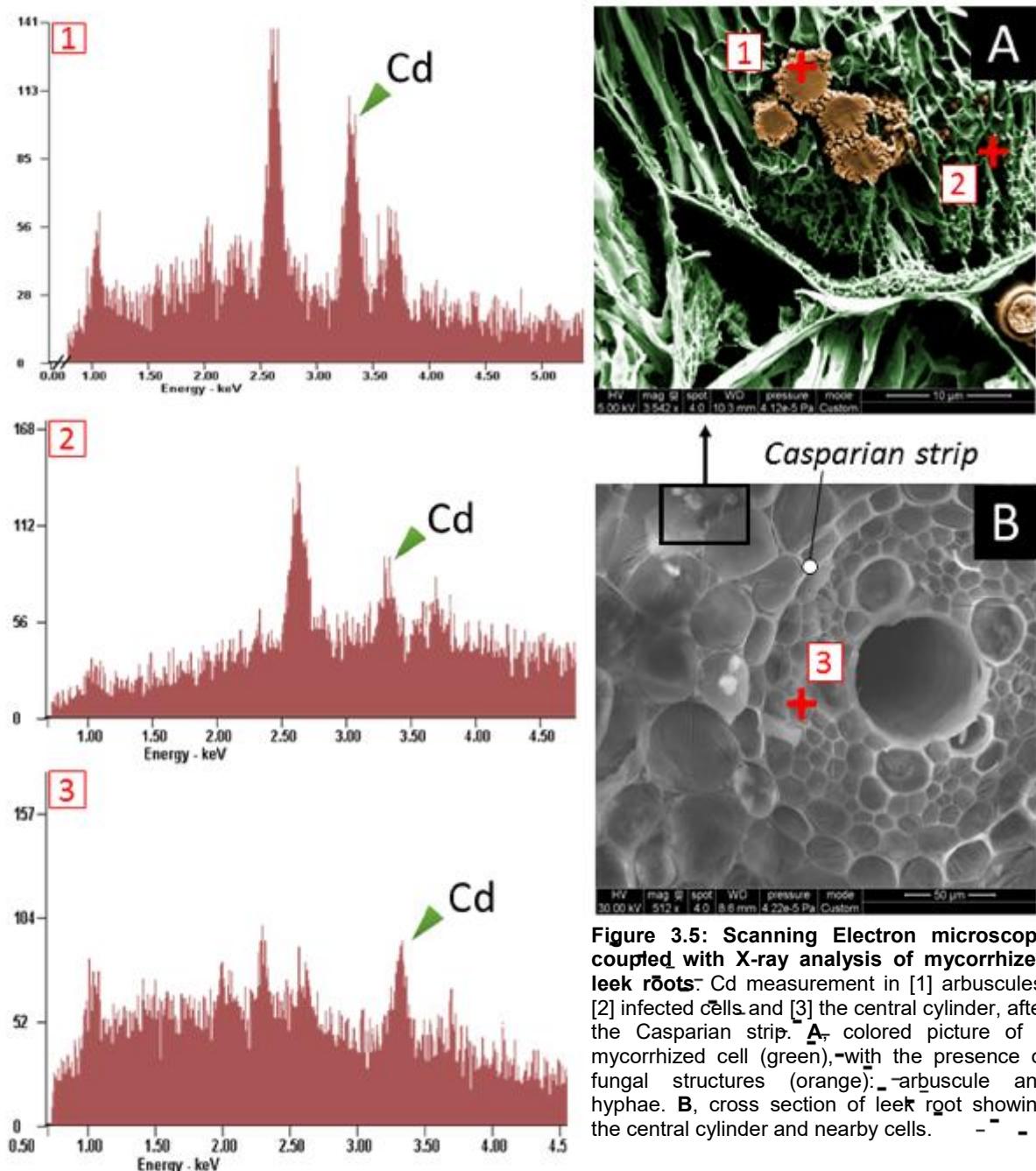
[Cd]	NTE						BZC					
	LETTUCE		LEEK				LETTUCE		LEEK			
	Control	B	Control	OM+	B	B:OM+	Control	B	Control	OM+	B	B:OM+
Plant	ns	ns	b	b	a	b	b	a	a	b	ab	ab
Leaf	ns	ns	b	bc	a	c	ns	ns	ab	b	a	ab
Root	ns	ns	bc	c	a	ab	b	a	a	b	b	ab

End of submitted article.

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3.3. Additional results

3.3.1. Cd compartmentation in plant



Scanning electron microscopy observations of leek root tissues were performed to study Cd compartmentation and transfer within plant. As shown in [Figure 3.5](#), Cd (green arrow) was detected both in fungal arbuscules within cells [\[1\]](#) and colonized cells [\[2\]](#). When measures were performed in cells from the central cylinder ([Figure 3.5-B](#)), Cd was still detected almost at the same intensity [\[3\]](#).

These localization results were consistent with Cd content in plant, where we found that Cd was partly translocated from roots to leaves (in lettuce more than in leek). These results were also in agreement with the fact that Cd is known to be internalized from the apoplast into the symplast through Zn, Fe or Ca transporters. Moreover, Cd uptake by plants has been shown to be dependent on root architecture (Laporte et al., 2014, 2013), which might explain the plant-specific absorption pattern as leek and lettuce root architecture were different.

3.3.2. Cd bioaccessibility in soil

Cd human bioaccessibility was measured in soil samples to evaluate human health risks in case of soil particles ingestion (Table 3.5). Cd bioaccessible fraction in soil did not change in a large extent along the experiment, meaning that no plant effect was observed. Organic matter addition seemed to increase this fraction (significantly in NTE soil but only a tendency in BZC soil) while bioaugmentation had no effect on it.

Table 3.5: Soil bioaccessible fraction of Cd.

It is expressed as the ratio between bioaccessible concentration and total concentration of Cd in soil. Letters indicates significant groups at $\alpha < 5\%$, no letters indicates no significance.

Plant	Soil	Treatment	% Cd
T0	NTE <i>b</i>		30.6 ± 0.7
	BZC <i>a</i>		62.1 ± 0.6
LETTUCE	NTE <i>b</i>	Control	26.8 ± 1.5
		B	25 ± 2.3
	BZC <i>a</i>	Control	63 ± 1.1
		B	64.1 ± 1.5
LEEK	NTE <i>b</i>	Control	21.6 ± 5.1 <i>c</i>
		+OM	36.8 ± 7 <i>a</i>
		B	28.2 ± 6.4 <i>abc</i>
		B :+OM	35.4 ± 3.4 <i>ab</i>
	BZC <i>a</i>	Control	66.5 ± 2.2 <i>ab</i>
		+OM	68.2 ± 0.9 <i>a</i>
		B	64.3 ± 1.4 <i>b</i>
		B :+OM	65.5 ± 1.5 <i>b</i>

Furthermore, we evidenced here a soil effect, as Cd bioaccessible fraction was about 2 times higher in BZC soil than in NTE soil. However, the differences in Cd bioaccessible fraction ought to be explained not by contaminant origin but by soil characteristics and/or Cd speciation because geogenic origin of Cd in NTE soil was not confirmed. The significant positive Pearson correlation (Table 3.6) between soil Cd bioaccessible fraction, soil characteristics and Cd_{EDTA} fraction confirmed this hypothesis. In NTE soil, Cd bioaccessible concentration and Cd_{EDTA} concentration were almost equal (~0.1 ppm) suggesting that the bioaccessible Cd might be all the phytoavailable Cd. However, in BZC soil the bioaccessible Cd concentration was about 1.5 times higher than the phytoavailable concentration. Consequently, in this soil another soil fraction, which is not extracted by EDTA, might be bioaccessible.

Table 3.6 Two tails Pearson correlation matrix between bioaccessible fraction of Cd, mobile fractions of Cd, and soil parameters (pH, OM content). ** significant at $\alpha=1\%$

		pH	OM	% Cd CaCl ₂	% Cd EDTA
% Cd bioaccessible	Pearson corr.	0.584**	0.583**	-0.097	0.939**
	<i>Pvalue</i>	<0.001	<0.001	0.510	<0.001

3.4. General conclusion

The objective of the present chapter was to assess the impact of two biological inputs (AMF and lombricompost) that could potentially be used in urban and periurban agriculture on cadmium transfer from soil to leafy vegetables depending on soil characteristics, and their effect on human bioaccessibility. The main results can be summarized as follow:

- Cadmium human bioaccessibility was almost total in leafy vegetables,
- Neither AMF (bioaugmentation) nor OM addition modified Cd human bioaccessibility in plant, but OM addition increased soil bioaccessibility,
- Associated with leek, AMF decreased Cd phytoavailable fraction in sandy clay soil but had no effect on sandy soil (where OM decreased this fraction),
- AMF increased Cd in lettuce root on a sandy clay soil but had no effect on a sandy soil,
- AMF and OM addition had important effect on mobile Cd fraction (EDTA extraction) while we evidenced a plant effect ($\sim +8\%$ increase in leek cultivation),
- In Leek, OM addition in sandy clay soil decreased significantly plant total Cd in natural conditions but had no effect in bioaugmented assays,
- In the sandy soil, AMF increased total Cd in plant. A coupled effect with OM lowered Cd in edible part, which present a good potential when facing Cd moderate contamination,
- If one had to choose between lettuce and leek, he should rather grow leek, as lettuce accumulated significantly more Cd in both soils. However, our outcomes suggested to avoid leafy vegetable on contaminated soil when possible.

As a misfortune never comes alone, contaminations in soils are rarely monospecific, meaning that these effects of OM and AMF treatments on Cd mobility and uptake might be different on other TM. As presented in [Chapter 2](#), the studied soils were also highly contaminated with Pb (>400 ppm) and moderately with Sb ($\sim 2\text{--}15$ ppm) with a proven anthropogenic or geogenic origin (sandy clay soil and sandy soil respectively) for these two elements. Consequently, similar analysis were performed regarding Pb and Sb transfer with a special focus on AMF community impact on TM transfer as it was not included in the present article ([Chapter 4](#)).

Chapter 4. INFLUENCE OF ARBUSCULAR MYCORRHIZAL FUNGI AND ORGANIC MATTER ON Pb AND Sb ACCUMULATION AND BIOACCESSIBILITY IN PLANTS

4.1. General introduction

This fifth chapter is the preparation of a future article entitled *Pb and Sb accumulation influenced by arbuscular mycorrhizal fungi community and organic matter in a crop succession (Lactuca sativa L. → Allium porrum L.)*. It is based on the same crop succession experiment as presented in [Chapter 3](#). The objectives were similar, *i.e.* to study the roles of AMF and lombricompost (combined or not) on metal(loid) (TM) transfer from soil to edible plants on two contrasted peri-urban soils in relation with human bioaccessibility of these elements. Indeed, as explained in [Chapter 3](#), the available literature shows that:

- OM can affect TM mobility.....(Beesley et al., 2010)
- Soil physicochemical properties are main factors of TM mobility.....(Haghiri, 1974)
- AMF are naturally in symbiosis with more than 80% of terrestrial plants..(Brundrett, 1991)
- Some strains are already sold as biofertilizers.....(Kangwankraiphaisan et al., 2013)
- They are known to participate in TM mobility in soil.....(Jarrah et al., 2014)
- Their relationship with TM is at least species dependent.....(Hassan et al., 2013)

Pb is present almost in every soil due to its important use in industrial processes and its long term use in lead gasoline (Robbins et al., 2010; Hu et al., 2014). As presented in [Chapter 1](#), its accumulation in living organisms is a potential source of diseases. [The second part of Chapter 1](#) reviewed in detail the fate of Sb, an emerging pollutant (Krachler et al., 2005; Smichowski, 2008), at the soil-plant interface and highlighted a lack of knowledge regarding its behavior with AMF. The present chapter thereby focused on the influence of AMF and OM addition in Pb and Sb phytoaccumulation and their risks for human health as summarized in [Figure 4.1](#).

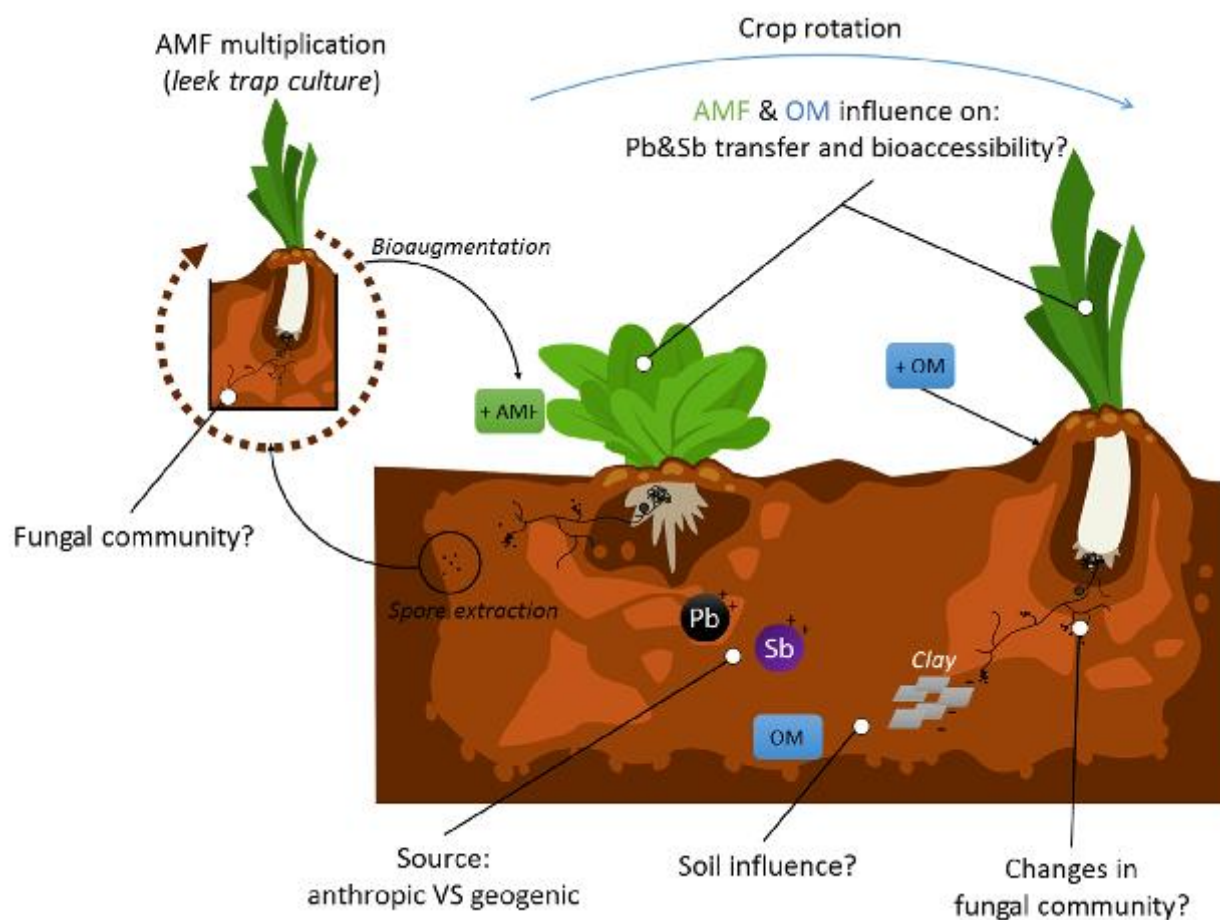


Figure 4.1: Scientific objectives for studying the fate of Pb and Sb at the 'soil-microorganism-plant' interface.

Our main results showed that:

- Sb and Pb accumulation is plant dependent, with higher accumulation in lettuce than in leek.
- Sb and Pb mobility depends on their origin, with higher mobility of elements from anthropogenic sources than geogenic ones.
- Bioaugmentation and organic matter decreased Pb and Sb mobility in soil but also AMF diversity associated with plants.
- Changes in AMF community might participate in Pb and Sb mobility.
- Bioaugmentation influenced Pb and Sb bioaccumulation factors.
- Soil bioaccessible fraction of Pb and Sb is contaminant origin dependent and is correlated to their mobile and mobilizable fractions (CaCl_2 and EDTA extracts)
- For the first time, we report a plant-dependent effect on soil bioaccessibility of Pb and Sb.

4.2. Pb and Sb accumulation influenced by arbuscular mycorrhizal fungi community and organic matter in a crop succession (*Lactuca sativa* L. → *Allium porrum* L.).

Article in final phase before submission

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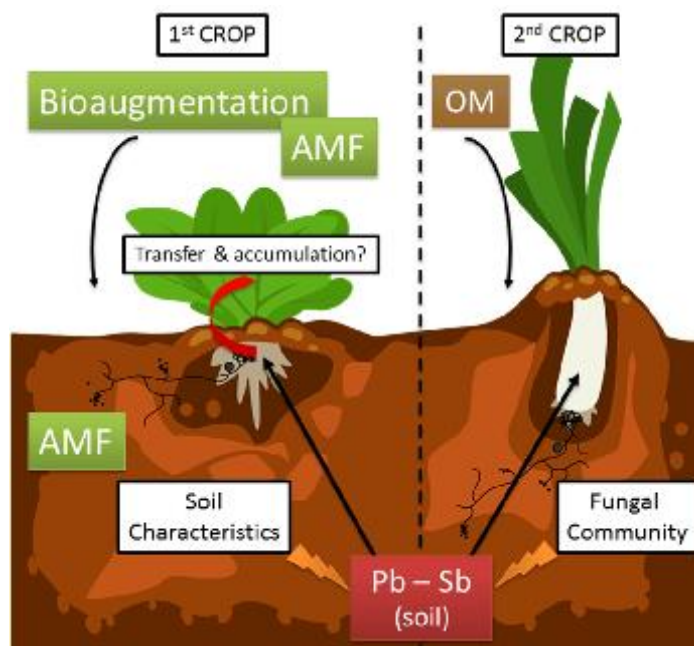
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ABSTRACT

The role of arbuscular mycorrhizal fungi (AMF) and organic matter (lombricompost) in lead (Pb) and antimony (Sb) uptake by lettuce (*Lactuca sativa* L.) and Leek (*Allium porrum* L.) consecutively grown in contaminated soils was investigated. Plants were subjected both to geogenic and anthropogenic metal(loid) (TM) sources, at similar Pb concentrations but different Sb concentrations, to assess the influence of pollutant origin. A crop succession was carried out as follow: (1)



lettuce cultivation with two conditions tested: natural soil (CTR) and bio-augmented (with the addition of a concentrated spore solution, referred as B); (2) Soil resting and addition of OM in half replicates of each modality; (3) Leek cultivation. After each harvest, plants and soils were analyzed to compare Sb and Pb accumulation in edible parts. Fungal communities were identified before harvesting leeks by Illumina MiSeq analysis and metal compartmentation was studied by electron microscopy in plants tissues. Bio-augmentation showed a significant (but opposite depending on the contaminant origin) effect on TM mobile/mobilizable fractions in soil and on the bioaccumulation factor of Pb and Sb in lettuce. In both soils, bio-augmentation and organic matter addition generated a loss of diversity in favor of *Rhizophagus irregularis* which could explain the changes of metal transfer at the soil-AMF-plant interface.

KEYWORDS

Antimony, lead, arbuscular mycorrhizal fungi, human bioaccessibility, organic matter, urban agriculture.

HIGHLIGHTS

- Sb and Pb accumulation are plant and soil origin dependent
- Sb behavior with arbuscular mycorrhizal fungi is investigated for the first time
- Bio-augmentation and organic matter influence Pb and Sb mobility in soil
- Bio-augmentation influence Pb and Sb bioaccumulation factors
- Bio-augmentation and organic matter decreased AMF diversity associated with plants
- Changes in AMF community might participate in Pb and Sb mobility

4.2.1. Introduction

Important metal(loid) (TM) contaminations are induced all around the world through various historical and actual human activities. Such soil (and water) contamination is a major environmental issue and urban soils are currently polluted with lead (Pb) worldwide (Hu et al., 2014) and more recently with antimony (Krachler et al., 2005; Smichowski, 2008). During the last decades, vehicle exhausts from leaded gasoline combustion were a major Pb contamination source (Robbins et al., 2010), nowadays, smelter areas are important ones (Zhuang et al., 2009). Antimony (Sb) is a metalloid which normally occurs as 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 during the last 3 decades, mainly from anthropogenic sources (Krachler et al., 2005). Smelting, mining and its presence in fire retardant mixtures are the most important actual Sb sources. However, during the last decades, its use in plant protection products manufacture and sewage sludge (Edwards et al., 1995; Wagner et al., 2003) led to a large-scale contamination of agricultural lands. Since a few years, Sb has been used in brake linings, lubricant and battery manufacture (Fujiwara et al., 2011; Wiseman et al., 2013), leading to an increased risk nearby communication paths through road traffic dust.

Although the worldwide economic crisis has driven more people to grow their own food in public, associative or kitchen gardens (Galt et al., 2014), such places are generally set-up in roads and industries vicinity or even directly in disused and not always cured industrial sites (Álvarez-Ayuso et al., 2012). In these urban environments, soils and plants are consequently subjected to a significant contamination risk through airborne particles enriched with TM (Leveque et al., 2013; Shahid et al., 2013). Because food is the major exposition pathway for human to environmental pollutants (Fries, 1995), TM transfer in the soil-plant-water system is often studied (Okkenhaug et al., 2011; Wan et al., 2013).

Mycorrhizal symbiosis is the normal condition for about 85% of flowering plants (Brundrett, 1991). Both organisms benefit from this mutualistic exchange: arbuscular mycorrhizal fungi (AMF) transfer nutrients (mostly phosphorus) and can enhance tolerance to stress (drought, pathogens...) (Li et al., 2013; Smith and Smith, 2011), plants give back organic compounds to the fungus (Smith and Smith, 2011). AMF are also known to be key actors in TM bioavailability in soils and plants absorption (Jarrah et al., 2014; Khan, 2005), while gardeners can already use them as bio-fertilizers (Kangwankraiphaisan et al., 2013). Pb behavior in presence of mycorrhized plant has been described (Jarrah et al., 2014) but has not yet been studied in leek, which is a widely cultivated vegetable (FranceAgriMer, 2013). Under TM stress, lettuce has been shown to accumulate high level of Pb, particularly in its leaves (Beavington, 1975; Uzu et al., 2010; Xiong et al., 2014a). As shown by Pierart et al. (2015), data about the relationship between Sb and AMF are very scattered. Different studies revealed Pb and Sb toxicity for both the environment and human beings (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. But, soil physico-chemical properties (organic matter, pH, Fe/Al oxides and hydroxides amounts...) and soil biological activity (rhizosphere, drilosphere¹...) can affect these parameters (Diemar et al., 2009; Ilgen and Trainor, 2012; Roper et al., 2012; Tighe et al., 2005; Xi et al., 2010; Xu et al., 2011) as AMF can also influence them.

¹ Fraction of the soil which is influenced by earthworms

In that context, our work aims to evaluate the role of bio-augmentation and organic matter amendment in lettuce and leek accumulation of Pb and Sb depending on their origin (anthropic vs geogenic), two leafy vegetables commonly grown in urban gardens for fresh and cooked consumption (Guitart et al., 2014). Pb and Sb content analyses were performed in different compartments (soil, plant) and at different scales (soil fraction, organism, organ) with the adapted extraction procedure (Okkenhaug et al., 2011). Finally, because fungal community could influence TM fate at the soil-plant interface, we estimated their composition by high throughput sequencing (illumina Miseq®) focusing on a commonly studied ribosomal region, the Internal Transcribed Spacer (ITS) (Jansa et al., 2002; Schoch et al., 2012).

4.2.2. Material and methods

4.2.2.1. Plant cultivation

STUDY SITE. Two peri-urban soils were studied for their similar Pb content but different contamination history (Table 4.1): an urban garden sandy soil (NTE: 47°16'1.512"N – 1°34'29.688"O) presenting a high geochemical Pb anomaly and a moderate Sb anomaly and a peri-urban sandy-clay soil (BZC: 48°11'21.375"N – 2°3'28.993"O) in which Pb and Sb were proven to come from a recent anthropic contamination (Cecchi et al., 2008; Leveque, 2014).

Table 4.1: Bazoches (BZC) and Nantes (NTE) soil characteristics.

	pH	Initial H ₂ O	[Pb], mg.kg ⁻¹	[Sb], mg.kg ⁻¹	[Cd], mg.kg ⁻¹	Clay, %	Silt, %	Sand, %	CaCO ₃ , %	P ₂ O ₅ , mg.kg ⁻¹	CEC cmol ⁺ .kg ⁻¹
BZC	8.1	3.2	412	15,4	3.0	24.0	55.3	16.7	2.0	76.7	15.85
NTE	7.0	6.0	456	2,7	0.2	14.0	28.4	57.4	0.2	380	8.71

LETTUCE BREEDING AND TRANSPLANTATION. Organic lettuce seeds (*Lactuca sativa* L., Laitue Pommée appia, Ferme de Sainte Marthe®) were sown on sterilized oildri® (20 min, 121 °C) and grown under natural light at ~17°C. After 3 weeks growing, seedlings were placed in a greenhouse (artificial lights turned on if brightness < 2800 lx, 18/20 °C day/night – 50% relative humidity) for 1 week acclimation. Lettuces were transplanted on 2mm sieved contaminated soils, each in a 2 L pot. For the bio-augmentation condition, 9 ml of a spore suspension were added directly on the whole root system. A fully randomized design was adopted to apportion on each modality the eventual greenhouse climatic heterogeneity. A regulated dripper line watered the pots per capillarity filling each saucer every three days to limit spores and metal lixiviation processes.

BIO-AUGMENTATION SOLUTION. Organic leeks were grown to multiply the natural AMF inoculum from contaminated soils because of its rusticity and good mycorrhizal potential. Same greenhouse conditions were used to grow plants in pots filled with a mix of both soils and sterilized oildri® (1:1:6/v:v:v). Spore extraction was performed by wet sieving (45 µm) and centrifugation (2500 rpm, 5 min) of spore suspension on 50% glycerol (v:v). After rinsing, spore suspension was prepared for bio-augmentation treatments.

ORGANIC MATTER ADDITION AND LEEK CULTIVATION. After lettuces cultivation for 12 weeks, plants were harvested and each pot kept its soil and rested for 2 months. Then, sterilized lombricompost (organic matter NFU 44-051, Botanic®) was mixed with soil in half of the replicates of each modality, in order to increase soil OM content of 1%. The pots rested for stabilization during another month before leeks (*Allium porrum* L., Poireau de Saint Victor, Ferme de Sainte Marthe®) sowing and cultivation (25 weeks).

4.2.2.2. Sampling and analysis

Aerial parts were cut and rinsed with deionized water to remove any soil or atmospheric particle. Root fragments were sampled, rinsed with deionized water and stored at 4°C for mycorrhization rate measurement and DNA sequencing. Plant organs were oven dried (40°C/5 d), weighted 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.

MYCORRHIZATION RATE. Observation and counting of AMF colonization (%M) was carried out following the root intersect method (Giovannetti and Mosse, 1980) with Jet Black ink (Shaeffer®) coloration (Vierheilig et al., 1998) and a ×65 stereomicroscope (Olympus SZX10).

METAL CONTENT. Leaves, roots and soil powders were digested in Digiprep® tubes following Foucault et al procedure (Foucault et al., 2013) and then filtrated with a 0.45 µm membrane. The CaCl₂ and EDTA fractions are currently used as pertinent predictors of respectively easily and medium phytoavailable metal(loid) fractions in soil (Meers et al., 2007) (i.e. exchangeable and phytoavailable fractions). The analysis were performed following the NFX-31-120 (AFNOR, 1992) and NEN-5704 (NEN 5704, 1996) norms respectively to assess Pb and Sb fractions associated with OM and their phytoavailability.

ORGANIC MATTER & pH. Total organic matter (%OM) was assessed by calcination according to the MA-1010 procedure (CEAEQ, 2003), and pH_{H2O} was measured following the standardized protocol NF-ISO-10390 (AFNOR, 2005); other soil physicochemical parameters were measured by INRA (Arras, France) for NTE soil and by Cecchi (Cecchi, 2008) for BZC soil. Data are reported in Table 4.1.

4.2.2.3. Metal analysis

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 samples analysis. In our conditions, Pb and Sb recovery were respectively ~78% and ~67%, which is in the range of previous analysis under similar acid digestion, as reported in Table 4.2. To study TM transfer from soil to plant, two ratios were calculated: the bioaccumulation Factor (BF) and the translocation factor (TF) which are respectively $[TM]_{total_plant} / [TM]_{total_soil}$ and $[TM]_{leaf} / [TM]_{root}$. Therefore, BF is representative of metalloid phytoaccumulation and TF evidences the inside-plant transfer and organs storage.

Table 4.2: Metalloid recovery rate. Average \pm Standard error, %

Element	Recovery rate, %	
	Apple leave (SRM-1515)	(IAEA-336) (Agnan, 2013)
Pb	78 \pm 0.6	81 \pm 9
Sb	67 \pm 2.4	66 \pm 20

4.2.2.4. Statistical analysis

Results were expressed as average \pm SD (standard deviation). 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 (lettuce, n=10; leek, 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.

4.2.2.5. Metal(loid) 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) permitted to evaluate Pb and Sb localization in plant cells and fungal structure of 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). Last steps consisted of (1) sublimation (10 min) to vapor the water and (2) platinum covering (5-10 nm).

4.2.2.6. Fungal and bacterial community identification

As the response of a host plant could depends both on the environment and the fungal community in symbiosis (Tonin et al., 2001), it is essential to estimate this community and study its variation between treatments. For that, we used new generation sequencing (ngs) approach with Illumina MiSeq analysis.

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 brought with propagule solutions in bioaugmented modalities, microbial DNA was also extracted from spore solution using the DNeasy® Plant Mini kit (Quiagen) with a pre-step as: (1) spores extraction; (2) Water take-out through deposition of the spore solution on a sterile membrane and vacuum depression; (3) Spore crushing in Fastprep 2ml tubes containing silica fragments and spheres by vortexing 5 times (15 sec each) the tubes after liquid nitrogen freezing. DNA extracts were finally diluted 10 × in DNA-free water to limit PCR inhibition.

PRIMER CHOICE. Fungi amplicons library were obtained with ITS3ngs4-ITS4ngs primer pair on the ITS2 region (Internal transcribed spacer) as these primers gave perfect matching for Glomeromycota (Tedersoo et al., 2014) (CATCGATGAAGAACGTAG - TCCTSCGCTTATTGATATGC). The primer pair was barcoded with a unique (sample-specific) error-correcting 10-12 bases barcode for Illumina MiSeq.

AMPLIFICATION CYCLE. For each modality, three independent samples were amplified and finally combined to lower potential early round PCR errors. For each sample, the amplification was performed twice, in 25 µl reaction volume, 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.µl⁻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 H₂O. The amplification cycles were carried out following the KAPA 3G protocol.

After the amplification, the duplicates were pooled to obtain 50 µl for each sample.

SEQUENCING MIX PREPARATION. Samples were sent to 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.

SEQUENCE ANALYSIS. MiSeq sequences were analyzed with MothurGUI (V 1.0.0) for pairing, trimming and sequence cleaning and Usearch (V 8.1.1861) for OTU clustering with chimera removal, OTU abundance and taxonomy (using the database Unite+INSD V7 – 31/01/2016 and additional blast on NCBI to improve assignation of non-assigned taxa when possible) (Edgar, 2013).

4.2.3. Results

The experiment consisted in a lettuce-leek crop succession on contaminated soils (with anthropic or geogenic Pb and Sb) in which AMF and OM were brought to assess their effect on the mobility and transfer of Pb and Sb between soil and plant. After each harvest, plants and soils were analyzed to compare Sb and Pb accumulation in edible parts. Leek roots were used to study fungal community and metal compartmentation.

4.2.3.1. Changes in soil TM mobility

Concerning soil, total TM content in soil were not significantly affected by OM or AMF addition at the global scale, but some trends were observed. Indeed, bio-augmentation tended to decrease Pb concentration in both soils after lettuce cultivation which could be due to either an increased transfer to plants or a change of TM speciation to a form preferentially bounded to the residual soil fraction. After leek crops, AMF addition alone may decrease Pb in NTE soil (sandy soil), but only when coupled with OM in BZC soil (sandy clay soil). Sb also seemed decreased in both soils after lettuce crop with AMF addition. However, after leek cultivation, its decrease was only measured in NTE soil with a coupled effect of AMF and OM while no effect was observed in BZC soil.

Evolution of Pb and Sb mobile fraction (CaCl₂)

Mobile fractions of Pb and Sb were assessed by CaCl₂ extraction. These fractions, considered as easily phytoavailable (*i.e.* exchangeable), are presented in [Table 4.3](#).

In lettuce cultivation, neither AMF nor OM addition influenced mobile fractions of Pb and Sb.

In leek cultivation, results were more contrasted for each metal and soil: for Pb, OM addition alone had no influence in both soils while a combined treatment with AMF and OM decreased significantly [Pb]_{CaCl₂} and %Pb_{CaCl₂}. In BZC soil, bio-augmentation alone led to a greater decrease of [Pb]_{CaCl₂} (and a similar decrease of %Pb_{CaCl₂}) than when combined with OM. For Sb, no effect was observed in BZC soil. In NTE soil, bio-augmentation had no influence while OM addition increased significantly both %Sb_{CaCl₂} and [Sb]_{CaCl₂}. Finally, in both soils, leek cultivation increased Pb and Sb mobility (concentration and fraction, [Table 4.3](#)) compared to lettuce cultivation.

Table 4.3: Lead (Pb) and Antimony (Sb) content in Bazochoes (BZC) and Nantes (NTE) soils after each crop cultivation. CTR: control, B: bio-augmented, OM+: organic matter. The % column represents the ratio between EDTA (or CaCl₂) extract and total metal in soil. Statistical analyses were performed within each soil and treatment for each parameter. Letters indicates significant groups at $\alpha=5\%$. No letters indicates no significance.

	Soil Total				EDTA extraction				CaCl ₂ extraction			
	Pb	Sb	%	Pb	Sb	%	Pb	Sb	Pb	Sb	%	%
Lettuce	CTR	380±46.8	2.5±0.1	30.3±1.1	8.1±1	0.01±0.01	0.5±0.2	0.1±0.1	0.03±0.02	0.004±0.0003	0.2±0.01	
	B	371.4±43.3	2.3±0.1	30.2±1.4	8.2±0.8	0.02±0.01	0.7±0.3	0.1±0.02	0.02±0.004	0.004±0.001	0.2±0.02	
BZC	CTR	410.6±3.2	14.6±0.7	160.7±3	39.1±0.9	0.5±0.01	3.2±0.2	0.004±0.004	0.001±0.001	0.1±0.003	1±0.1	
	B	404.7±5	14.1±0.4	161±0.8	39.8±0.4	0.5±0.01	3.3±0.05	0.01±0.007	0.002±0.002	0.1±0.01	1±0.1	
NTE	CTR	389.7±36.7	2.5±0.2	39.8±0.7	10.3±0.9	0.16±0.01	14.9±11.1	0.1±0.1	0.02±0.01	0.01±0.001	0.3±0.1	b
	OM+	301.8±45.8	2.1±0.1	29.2±2.7	10±2.4	0.18±0.01	8.8±1	0.02±0.005	ab	0.01±0.003	a	0.9±0.2
	B	299.1±8.6	2.3±0.1	32.7±2.1	10.9±0.7	0.18±0.01	7.8±1	0.04±0.05	ab	0.01±0.02	a	0.3±0.1
	B:OM+	334.4±54.3	2±0.1	29.4±1.1	9±1.6	0.24±0.002	21.1±12.6	0.002±0.002	b	0.001±0.001	b	0.5±0.2
BZC	CTR	402.4±7.3	10.1±0.5	170.6±2.6	42.4±1.3	0.7±0.03	7.2±0.3	0.2±0.01	a	0.04±0.002	b	1.7±0.04
	OM+	385.2±4.6	10.1±0.7	154.5±3.7	40.1±1.4	0.6±0.05	6.4±0.8	0.2±0.03	a	0.1±0.01	a	1.8±0.4
	B	395.4±6.9	10.4±0.6	169.7±2.6	42.9±0.4	0.8±0.04	7.2±0.4	0.01±0.001	c	0.001±0.0004	c	1.7±0.3
	B:OM+	383.5±7.1	10.4±0.6	150.1±10.1	39.2±3	0.7±0.04	6.4±0.6	0.03±0.01	b	0.01±0.001	c	1.4±0.1

Table 4.4: Soil physicochemical data (pH, %OM) and plant (leek or lettuce) parameters: mycorrhization rate-%MYC, biomasses, translocation and bioaccumulation factors TF and BF respectively. CTR: control, B: bio-augmented, OM+: organic matter. Statistical analyses were performed within each soil and treatment for each parameter. Letters indicates significant groups at $\alpha=5\%$. No letters indicates no significance. *max means no Sb found in roots, implying a full root-to-leave transfer.

	%MYC	pH	%OM	Leaf DW, g	Root DW, g	TF Pb	BF Pb	TF Sb	BF Sb
Lettuce	CTR	65.7±3.8	a	6.8±0.4	b	3.2±0.3	0.13±0.05	0.11±0.03	a
	B	50.1±4.5	b	7.4±0.5	a	3.2±0.1	0.31±0.11	0.04±0.003	b
BZC	CTR	56.2±7.1	b	8.4±0.02	3±0.03	3±0.1	0.11±0.03	0.09±0.01	b
	B	67.7±4.6	a	8.4±0.01	3±0.1	3±0.1	0.08±0.02	0.13±0.01	a
NTE	CTR	39.8±5.1	b	7.1±0.4	b	4.4±0.1	0.02±0.004	0.05±0.02	max*
	OM+	41±1.4	b	8.1±0.1	a	5.9±0.4	0.03±0.03	0.06±0.02	max
	B	53.6±5.9	a	7.9±0.1	a	3.9±0.6	0.02±0.02	0.06±0.02	max
	B:OM+	56.5±0.1	a	8±0.04	a	6.1±0.5	0.01±0.004	0.045±0.01	max
Leek	CTR	42.7±1.2	a	7.9±0.02	ab	5.9±0.02	0.025±0.004	0.041±0.002	max
	OM+	28.1±1.8	b	7.9±0.01	a	7.5±0.3	0.034±0.005	0.05±0.01	max
	B	53.3±10.2	a	7.8±0.02	b	6±0.05	0.038±0.01	0.045±0.01	max
	B:OM+	49.7±3.7	a	7.9±0.03	ab	7.5±0.3	0.04±0.015	0.041±0.011	max

Mobilizable fraction of Pb and Sb (EDTA extraction)

The mobilizable fraction of TM corresponds to the fraction bounded to clay and carbonates, which could be available for plants through solubilization. The results of this analysis are reported in [Table 4.3](#). A plant effect was observed as Pb mobilizable concentration was higher after leek cultivation than after lettuce cultivation.

In both soils, AMF had no influence under lettuce cultivation. However, for the following results, only the concentrations were impacted (no significant effects were measured on Pb or Sb mobilizable fractions).

For Pb, OM addition (alone or combined with bio-augmentation) decreased significantly $[Pb]_{EDTA}$ (Pvalue < 0.0001). Bio-augmentation alone only decreased $[Pb]_{EDTA}$ in NTE soil. Moreover, these effects of bio-augmentation and OM addition alleviated the leek plant effect described earlier. Actually, $[Pb]_{EDTA}$ in these treatments after leek cultivation was similar to $[Pb]_{EDTA}$ after lettuce cultivation. For Sb, a significant increase of $[Sb]_{EDTA}$ occurred in NTE soil when a combined treatment was applied (B:OM+). In BZC soil only tendencies were observed compared to the control (CTR): Bio-augmentation tended to increase $[Sb]_{EDTA}$ while OM treatments (with or without AMF) seemed to decrease $[Sb]_{EDTA}$.

4.2.3.2. AMF and OM addition effects on plant growth and accumulation

Plant development is impacted by OM and bio-augmentation

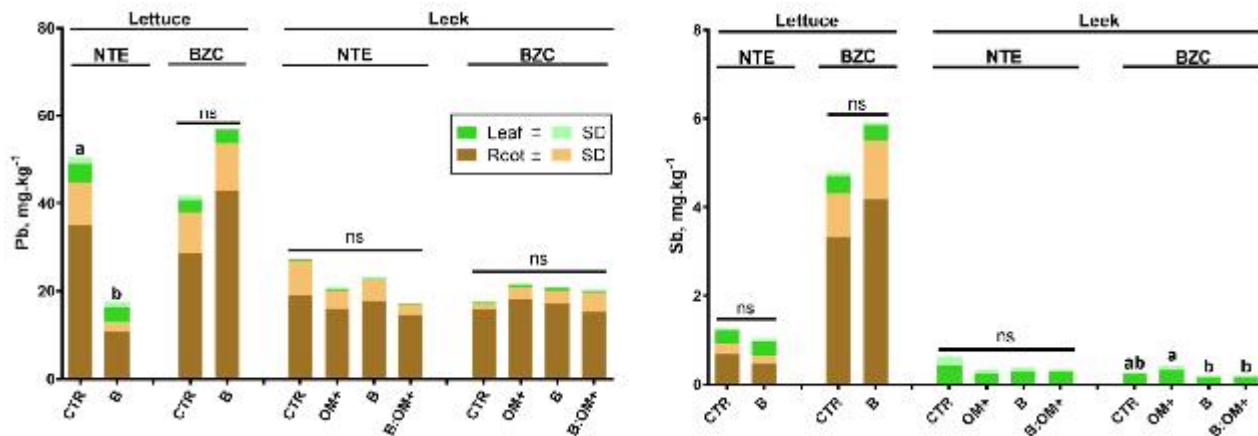
Plant aerial biomass of lettuce and leek are reported in [Table 4.4](#). For lettuce, these parameters presented no significant difference between treatments. Leek biomass significantly increased in both soils with OM addition while bio-augmentation had no effect.

Phytoaccumulation of lead and antimony

After each crop, TM concentrations were measured in plant organs and data are reported in [Figure 4.2](#). In this figure, statistical information refer to analyses performed at the plant scale to simplify the reading. Statistical analyses at the organ scale were also performed but are not fully reported here.

In lettuce grown on NTE soil, bio-augmentation significantly decreased Pb concentration (only tendency for Sb) in the whole plant, through a significant decrease of root accumulation. On the opposite, addition of AMF in BZC soil tended to increase root accumulation of both Pb and Sb. In both soils, no effect was observed on leaf accumulation. In leek, Pb accumulation was not significantly affected by the treatments at the plant scale in both soils, but OM addition seemed to decrease Pb in NTE soil.

However, at the organ scale, both bio-augmentation and OM (separately) significantly increased Pb content in leaves of leeks cultivated in BZC soil (Pvalue = 0.029 and 0.047 respectively). In NTE soil, no significant effect was observed between treatments and control.



Regarding Pb Translocation Factor from root to leaves (TF_{Pb}) which results are reported in [Table 4.4](#), the response varied between soil and plant. First, no effect was observed in lettuce cultivated in BZC soil while a trend of increased TF_{Pb} is observed in NTE bioaugmented soils. In leek cultivation, results seemed opposite with no effect of treatments in NTE soil, but a trend of increased TF_{Pb} in BZC bioaugmented soils. Finally, TF_{Pb} was significantly higher in lettuce than in leek (about 3 and 10 times in BZC and NTE soils respectively).

Regarding Sb phytoaccumulation in leek, no effect was observed in NTE soil. However, in BZC soil it seemed to increase with OM but to decrease with bio-augmentation and significant difference was measured between these modalities (OM > B). No Sb was found in root organs in both soils (values below detection limit) while Sb we measured in leaves. This means that TF_{Sb} was maximal in leek. In lettuce, TF_{Sb} wasn't significantly impacted by OM or AMF addition.

Bioaccumulation Factor (BF) results are reported in [Table 4.4](#). They represent the plant capacity to accumulate TM from soils. In our conditions, this ratio was significantly affected by AMF or OM addition as follow:

BF_{Pb} significantly decreased ($\div 3$) in NTE soil but increased in BZC soil during lettuce cultivation while it did not change in leek cultivation. BF_{Sb} evolved differently depending on soil

contamination origin. Indeed, none of the treatments changed this ratio in NTE soil, while a plant-dependent response occurred in BZC soil: in lettuce, bio-augmentation increased significantly BF_{Sb} whereas, it seemed to decrease in leek. Organic matter addition induced also a trend of an increase of BF_{Sb} (with a final BF_{Sb} significantly higher than the ratio in bioaugmented modalities).

Pb and Sb compartmentation in plants

After growth on polluted soils, Scanning electron microscopy observations of leek root tissues were performed to study Pb and Sb compartmentation and transfer within plant. As shown in [Figure 4.3](#), TM (blue and orange arrow for Pb and Sb respectively) were detected both in fungal arbuscules within cells [1] and colonized cells [2]. When measures were performed in cells from the central cylinder [3] ([Figure 4.3-B](#)), Pb was still detected with a lower intensity while Sb was merged in background noise. These localization results are consistent with low TF_{Pb} from roots to leaves observed in leek.

Fungal community in bio-augmentation solution

MiSeq analysis allowed identifying parts of the fungi present in the bio-augmentation solution. Among 1134 OTU (Operational Taxonomic Unit) identified in the bio-augmentation solution, 384 OTU belonged to Glomeromycota Phylum. Although some OTU were weakly assigned (only to the gender or the family), we found a majority of *R. irregularis* and unidentified Glomerales, as summarized in [Table 4.5](#).

Table 4.5: Relative abundance of fungi from Glomeromycota Phylum in the bio-augmentation solution

Assigned OTU	Relative abundance, %
Glomerales sp	33.46
<i>Rhizophagus irregularis</i>	21.09
<i>Rhizophagus</i> like	36.85
<i>Funneliformis mosseae</i>	1.67
<i>Septoglomus viscosum</i>	0.04
Claroideoglomeraceae sp	4.82
Diversisporales sp	1.06
Diversisporaceae sp	0.32
<i>Diversispora celata</i>	0.38
<i>Diversispora epigeae</i>	0.28
<i>Ambispora gerdemannii</i>	0.02

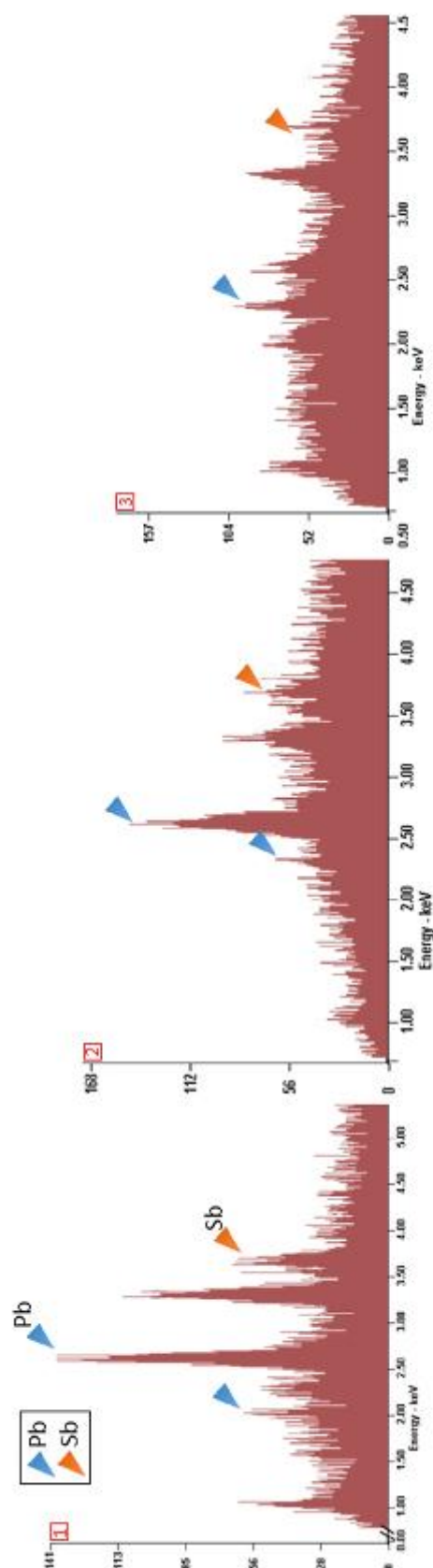
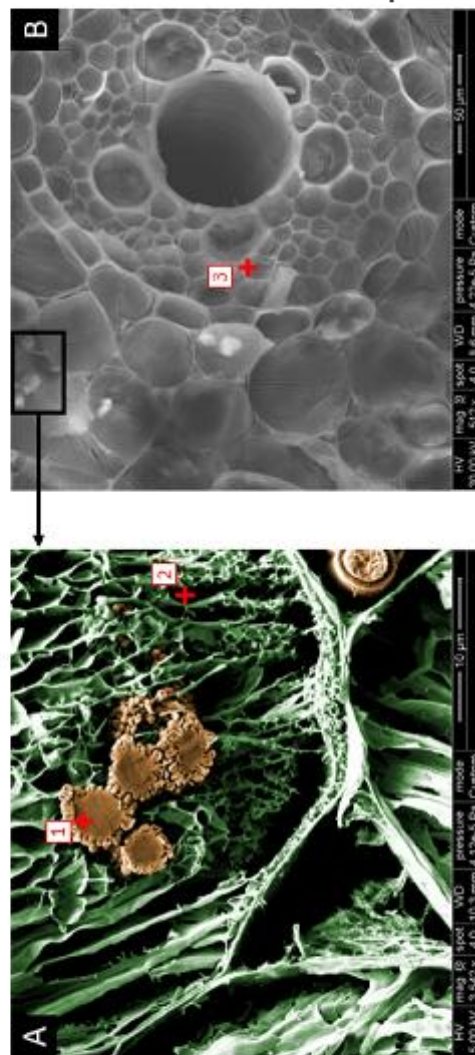


Figure 4.3: Scanning Electron microscopy coupled with X-ray analysis of mycorrhizal leek roots. Pb and Sb measurement in [1] arbuscules, [2] infected cells and [3] the central cylinder, after the Casparian strip. A, colored picture of a mycorrhizal cell (green), with the presence of fungal structures (orange): arbuscule and hyphae. B, cross section of leek root showing the central cylinder and nearby cells.



Fungal community in soil

High throughput sequencing and metagenomics analysis in leek roots from each modality generated about 13200 OTU, all modalities together. After taxonomy assignation we found in average: *Rhizophagus irregularis* 64.8%, *Rhizophagus* sp 19.5%, Xylariaceae sp 4.7%, Ascomycota sp 3.6%, Glomeraceae sp 3.3%, Nectriaceae sp 3.3%, *Funneliformis mosseae* 0.6%, *Alternaria infectoria* 0.3%, and *Incertae sedis* 0.1%. However, their relative abundance varied between treatments, as reported in Figure 4.4, while no difference were observed in terms of mycorrhization rate. Indeed, we observed a higher diversity in NTE_CTR compared to BZC_CTR, with the presence of *F. mosseae* and a proportion of unidentified Glomeraceae while BZC soil contained almost only *Rhizophagus* species. Bio-augmentation led to a loss of diversity in both soils (about 90% of *R. irregularis* in BZC_B and disappearance of *F. mosseae* in NTE_B soil). However, bio-augmentation in NTE soil also increased the proportion of unassigned Glomeraceae. Organic matter addition increased the proportion of *R. irregularis* in both soil, but much drastically in NTE soil (96%). When combined, OM and B addition led to the same results as only B treatment in BZC soil. In NTE, it gave similar results as OM addition (73% *R. irregularis* and loss of Glomeraceae). Hence, alone or combined, OM and B treatments caused a loss of diversity in fungal community associated with leek roots, favoring *Rhizophagus irregularis*.

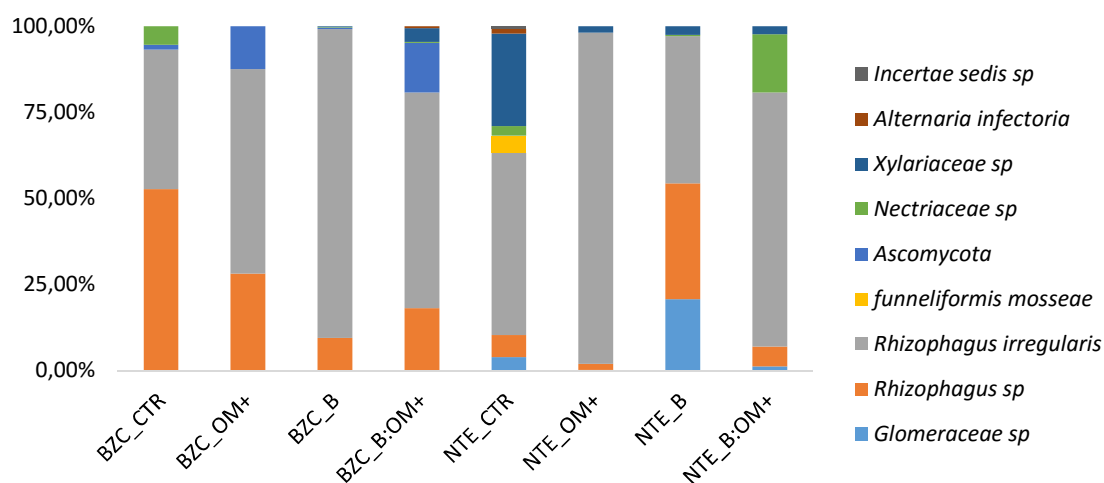


Figure 4.4: Relative abundance of fungi in different treatments.

CTR: control, B: bio-augmented, OM+: organic matter.

4.2.4. Discussion

4.2.4.1. Both lead and antimony phytoavailability and uptake are affected by OM

In our experiment, NTE soil presented a natural anomaly in Pb and Sb, coming from the alteration of the Pb-rich bedrock while BZC soil has been contaminated for years by atmospheric Pb/Sb rich particles. Studies focused on industrial Pb contaminated soils showed that TM chemical speciation and origin strongly affects their phytoavailability (Cecchi, 2008). Hence, in accordance with this study, our results indicate that mobilizable Pb (EDTA extract) is about 30% higher in BZC soil than in NTE soil.

Then, as soil organic matter has been frequently associated with high sorption capacity for metals (Quenea et al., 2009), we tested here the influence of lombricompost on Pb and Sb mobility on these two contrasted periurban soils. For years, the scientific community intended to evaluate the efficiency of different amendments such as P-rich substances or organic matter such as composts on TM immobilization in soils and most of them used partial chemical extraction to estimate this efficiency (CaCl_2 or $\text{Ca}(\text{NO}_3)_2$ for example) (Ruttens et al., 2006). The main difficulty is that these mechanisms seem soil and amendment dependent (Geebelen et al., 2003; Kumpiene et al., 2008; Shahid et al., 2012). Such variation is consistent with our results. Indeed, OM addition had contrasted effect depending on both soil/contaminant origin and considered TM: (1) Alone, it had no effect on $\text{Pb}_{\text{CaCl}_2}$ and $\text{Sb}_{\text{CaCl}_2}$ on BZC soil while in NTE soil it increased $\text{Sb}_{\text{CaCl}_2}$ and tended to decrease $\text{Pb}_{\text{CaCl}_2}$. (2) it decreased Pb_{EDTA} in both soils but had no significant effect on Sb_{EDTA} . When combined with bio-augmentation, OM addition decreased $\text{Pb}_{\text{CaCl}_2}$ in both soils. Consequently, a risk could remain to use these composts as amendments in urban and peri urban soils potentially contaminated with low or medium TM concentrations because they could solubilize hazardous metal concentrations to plants which would have grown clean otherwise (Murray et al., 2011).

These variations in TM mobility can affect plant uptake (and health risk as a consequence) and modelling approach has been developed to estimate plant accumulation using CaCl_2 extracts (Uzu et al., 2009) with a linear relationship between $[\text{Pb}]_{\text{leave}}$ (or $[\text{Pb}]_{\text{root}}$) and $[\text{Pb}]_{\text{CaCl}_2}$ for lettuce. However, contrary to the former authors, we found no such correlation in our different treatments. Same goes for $[\text{Pb}]_{\text{EDTA}}$ and for Sb contents in both lettuce and leek, even in the control where the equilibriums were not affected by OM or B addition.

4.2.4.2. Changes in TM mobility and uptake due to plant and AMF

The plant case

When cultivated in urban areas, vegetables showed different pattern of Pb accumulation, depending on the soil and the plant species (Säumel et al., 2012) consistently with the observed differences between lettuce and leek accumulation in the present study. The authors mentioned that the difference in TM accumulation seemed more plant species (and cultivar) dependent than plant type (leafy, root...) dependent even if it was usually accepted that the accumulation rate is as follow: fruit < root vegetable < leafy vegetable. Michalska and Asp (Michalska and Asp, 2001) found that phytoaccumulation variations of Pb could also occur at the cultivar scale for lettuces. Although their study was carried out in hydroponic conditions on different cultivars, we found similar pattern of root accumulation. In their conditions, TF_{Pb} was very low (0.005 to 0.2 depending on the cultivar) compared with our results where 0.2 is the average TF_{Pb} in both soils. This difference of TF could come from the variation of experimental condition.

In a less contaminated area (~200 ppm anthropic Pb), Mombo et al. showed that leek and lettuce cultivated in urban garden accumulated twice less Pb than in BZC soil (in terms of BF) (Mombo et al., 2016). The high accumulation in our conditions could be explained by the experiment duration (5 months) and the pot cultivation. Indeed, plants grew their roots in the same Pb/Sb-rich soil all along the experiment in pot cultivation, while in natural conditions the root system could grow deeper in less contaminated soil horizons (anthropic contamination usually present a decreasing pattern of TM content from surface to deeper soil). However, Pb soil content could also explain these variations of accumulation.

Sb accumulation in edible plants is highly variable, but some leafy vegetable such as spinach can be hyperaccumulators (Pierart et al., 2015). However, available literature focused on lettuce and leek accumulation patterns is scattered (Kouimtzis et al., 1992). The authors showed that Sb accumulated generally more in lettuce (~1 mg.kg_{DW}⁻¹) than in leek (~0.2 mg.kg_{DW}⁻¹), which is consistent with our results (lettuce>leek). However, our range of concentrations was higher, but this could be explained by our experimental conditions as described for Pb. Regarding these accumulations, emerging Sb contamination should be carefully monitored in urban and peri-urban areas where new projects of urban agriculture are being considered.

Finally, even if lombricompost addition did not significantly influenced Pb and Sb accumulation in plants, attention ought to be paid on (1) the physicochemical mechanisms which depend greatly on the compost composition and the contaminant considered (Ruttens et al., 2006; Murray et al., 2011; Karami et al., 2011) and (2) depending on the compost origin, it can contain TM which will accumulate in cultivated soils through repeated application (Madrid et al., 2007).

The fungal case

Along with plants, microorganisms from the rhizosphere can exude organic ligands and affect soil pH (Hinsinger et al., 2003; Wang et al., 2006), leading to a change of TM mobility (Lin et al., 2004). However, the mechanisms of metal stress alleviation are numerous, and fungal species dependent (Gu et al., 2013; Hassan et al., 2013; Wei et al., 2016).

We observed differences in the fungal diversity between BZC and NTE soils. The higher diversity observed in the soil facing geogenic contamination (NTE) is consistent with the tolerance of AMF strains such as *F. mosseae* to TM contaminated soils (Hassan et al., 2011). Indeed, in this area (NTE), TM have been present for decades, allowing fungal communities to adapt to the contamination. On the opposite, a recent contamination with TM, as in BZC can alter the fungal community (Hassan et al., 2011; Sun et al., 2016).

Commercial biofertilizers are generally monospecific, with an efficient fungal strain for mycorrhization and phosphorus acquisition (leading *in fine* to potential increased yields). However, as shown by our bio-augmentation solution, a loss of diversity can occur even by taking a multiple strain solution as fertilizer, favoring *Rhizophagus* species (*irregularis* and sp). Same loss of diversity occurred with OM treatments, which has also been reported in soil receiving organic manures (Chen et al., 2016).

On the opposite, a study focused on the effect of composts on soil microbial diversity showed no loss of diversity of AMF fungi in soil through spore counting and morphological identification (Viti et al., 2010). The difference could come from the difference of approach (morphological identification versus next generation sequencing), or it could show that even if the soil spore diversity remains, the fungi in symbiosis are more monospecific (which could be due to a loss of colonization potential for some sensitive species).

More recently, a long term field study (8 years) based on cloning and genome sequencing showed an increase of AMF diversity associated with plants when organic amendments were applied (Montiel-Rozas et al., 2016) suggesting that (1) long term exposure could alleviate the loss of diversity observed in short term and (2) the diversity observed in pot experiment is biased and doesn't represent the whole fungal diversity (Sýkorová et al., 2007).

Rhizophagus irregularis has been shown to enhance metal (Cd) transfer from low contaminated soil to plant (Hassan et al., 2013). However, the disappearance of *F. mosseae* in NTE soil (all modalities compared to control) did not led to an increase of Pb in plants but a trend of decrease for both Pb and Sb in leek. This could highlight that TM uptake or binding by fungi is metal dependent. Making the hypothesis that the fungal community found in leek CTR and B treatments were representative of what it could have been during lettuce cultivation, we observed a significant decrease of Pb in NTE bioaugmented soil, which is consistent with the trend observed in leek plants. This could suggest that among the Glomeraceae and *Rhizophagus* sp which increased with bio-augmentation treatment, one (or more) might be good candidate for phyto-stabilization of metal in soil. Unfortunately, we were not able to identify precisely this species due to scattered data in ITS AMF databases (Schoch et al., 2014).

Finally, these technics are generating a revolution in community analysis thanks to the depth of analysis and the quantity of sequences amplified. It was already known that ribosomal ITS regions were variable among fungal kingdom (Nilsson et al., 2008; Simon and Weiß, 2008), this region still remains a good DNA barcode marker (Schoch et al., 2012). It is estimated that the diversity of AMF could reach 1600 species (Öpik et al., 2013; Pagano et al., 2016; Sudová et al., 2015), underlying the necessity to develop studies to isolate and describe more species of these organisms, as they can be used as bio-inoculants both in agriculture and contaminated areas for both phytoextraction or phytostabilization. The main bolt of these approaches comes from the lack of precise databases. Indeed, while these technics reveal an extreme diversity as shown by the number of OTU identified (about 1600 OTU per sample in leek roots; 348 OTU in the spore solution of which half of them were identified as *Rhizophagus irregularis* like), most of the sequences were only identified at the family or gender level (Table 4.5 and Figure 4.4) while their functional diversity facing TM contamination is at least species or strain specific (Chen et al., 2007; Hassan et al., 2013).

4.2.5. Conclusion

Our results indicate that soil characteristics and particularly Pb and Sb origin (anthropic or geogenic) influenced strongly their mobility as we observed differences in results obtained in each soil. Pb and Sb mobile fractions were generally higher in the anthropic soil, where the contamination is recent and more bioavailable than the naturally TM rich soil where Pb and Sb might be in more stable forms. However, these differences did not influence plant uptake as it was similar in average between the two studied soils.

Lombricompost addition significantly decreased Pb mobility in soil and had a lower effect on Sb mobility but these effects were not visible on plant BF. Such amendment has to be sustained with care as it sorely affects fungal diversity in soil.

Bio-augmentation could be a suitable technic to propose to gardeners: (i) to favor phosphorus phytoavailability and reduce fertilizers addition (currently enriched with Cd); (ii) to limit Pb and Sb accumulation in plants compared to lombricompost addition. However, its efficiency seems to be both plant and soil dependent. Moreover, the developed fungal community in the bio-augmentation solution and the changes in fungal community in soil when applied would need to be carefully monitored as some fungal strains might enhance metal transfer to plants. Moreover, our study focused on fungal community in soil, but bacterial communities also need to be considered as they are major constituents of the mycorrhizosphere in solubilization processes and plant development.

Finally, our study focused on soil to plant transfer of contaminants and showed that bioaccumulation and translocation factors of Pb and Sb were low for both plants. Nevertheless, contaminated particles from anthropic sources are also major sources of TM phytoaccumulation for leafy vegetable through direct deposition on edible organs (Xiong et al., 2014a), which doesn't make these crops advised in polluted urban areas.

4.2.6. Acknowledgment

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--- End of article in preparation ---

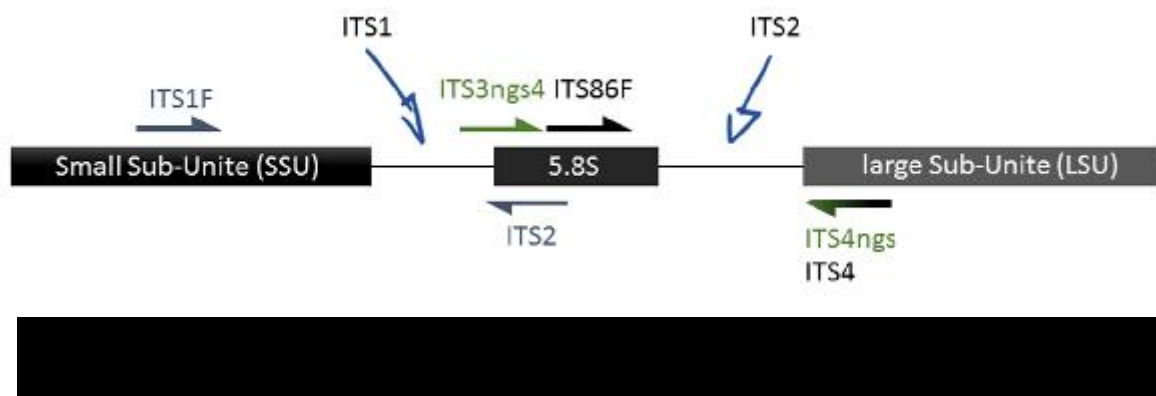
4.3. Additional results and discussion

The former article focused on total concentrations of Pb and Sb in plants and the influence of OM addition and fungal community associated with crops. Results have been discussed, but some additional measurements were carried out for which interesting information were obtained. This section is aimed to present and discuss various results concerning fungal community development and human bioaccessibility.

4.3.1. Fungal community analysis

4.3.1.1. Which region of the genome do we study?

Arbuscular mycorrhizal fungi community investigation is a tricky discipline in which numerous authors have been working for years (Sanders et al., 1996, 1997). Genetic analysis implies the use of conserved regions of the genome between living organisms, but still enough variable to distinguish individuals. AMF genetic is complex as they are polynuclear organisms and present a high allelic variability. The nuclear ribosomal internal transcribed spacer (ITS) region has been shown to be a reliable universal DNA barcode marker for fungi (Schoch et al., 2012). This region is part of the nucleotide sequences in ribosomal genes, a series of multicopy genes organized in tandem along the genome as summarized in [Figure 4.5](#). Each of these genes encodes for three subunits (18S[SSU], 5.8S and 28S[LSU]) separated one another by an Internal non-transcribed region (ITS). An Inter Genic Spacer (IGS) separates the genes themselves. ITS and IGS are variable regions which mutate more frequently than the three conserved regions (18S, 5.8S, 28S) making the former a better indicator of closely related genomes.



Since a few years, high throughput sequencing technics and meta-barcoding analysis have been developed with a great potential for ecological study of communities; the Illumina MiSeq is one of them. Due to the very large number of sequences amplified in comparison with classical technics such as Sanger, the depth of analysis has been improved up to the possibility to distinguish allelic diversity among individuals. This technology is very efficient for bacterial community identification as they possess well documented databases based on fewer variability regions of the genome. However, despite this tool is extremely powerful, the case of AMF is more complex because of (1) the high variability of ITS regions in these fungi; (2) the technological biases (sequence recognition and amplification heterogeneity due to variability in primer pairing for example); (3) the lack of complete databases already adapted to the results.

4.3.1.2. Fungal community development and analysis

Facing the difficulties freshly developed, it is recommended to study artificial communities along with environmental samples to evaluate the variation of recovery and identification of each individual depending on the primer chosen (Tedersoo et al., 2014). Accordingly, an artificial community was prepared with 18 fungal isolates identified through classical sequencing method (Sanger sequencing), 4 isolates from pure cultures to be identified, 1 plant sample (leek leaf). These individuals were amplified and sequenced with Illumina MiSeq both alone and in equi-volume mix (1/23° of each sample) with the procedure as developed in [Chapter 2](#).

The interest of adding unidentified isolates was multiple: it permitted to extend the community to strengthen the analysis and to evaluate the efficiency of MiSeq technology to identify individual genotypes in an artificial mix. The plant DNA sample was included to estimate the specificity of primer pairs to fungi, as plants also possess similar ITS regions, and to amplify other fungal endophytes from leaves. Three replicates were prepared for each sample. [Table 4.6](#) summarizes the artificial community. This community was studied with three primer pairs which already gave good results for AMF identification (Beeck et al., 2014; Tedersoo et al., 2015) to compare their efficiency in our conditions. Their sequences were presented in [Chapter 2](#) and their pairing localization is reported in [Figure 4.5](#). To simplify the writing (and the reading!), for the following chapter the primer pairs will be named from the region they amplified:

$$\begin{aligned}\text{ITS1F-ITS2} &= \text{ITS1} \\ \text{ITS86F-ITS4} &= \text{ITS2} \\ \text{ITS3ngs4-ITS4ngs} &= \text{ITS2ngs}\end{aligned}$$

Table 4.6: Artificial community designed for Illumina Miseq analysis

Sample	Strain	Species
AC1	DAOM197198	<i>Rhizophagus irregularis</i>
AC 2	DAOM197198	<i>Rhizophagus irregularis</i>
AC 3	COL-01	<i>Rhizophagus irregularis like</i>
AC 4	AMB06-11	<i>Unidentified isolate</i>
AC 5	AMB07-7	<i>Funneliformis mosseae</i>
AC 6	AMB04-3	<i>Unidentified isolate</i>
AC 7	AMB02-11	<i>Unidentified isolate</i>
AC 8	AMBAIA08-2b2	<i>Funneliformis caledonium</i>
AC 9	AMBAIA07-10	<i>Funneliformis mosseae</i>
AC 10	AMB02-5	<i>Funneliformis caledonium</i>
AC 11	AMB04-6	<i>Funneliformis mosseae</i>
AC 12	AMB01-14	<i>Funneliformis caledonium</i>
AC 13	AMB04-2	<i>Funneliformis mosseae</i>
AC 14	MUCL43204	<i>Rhizophagus irregularis like</i>
AC 15	AMBAIA10-2b3	<i>Funneliformis caledonius</i>
AC 16	AMB05-3	<i>Funneliformis caledonius</i>
AC 17	AMB04-1	<i>Unidentified isolate</i>
AC 18	AMB01-9	<i>Glomus fragilistratum</i>
AC 19	AMBAIA10-1j2	<i>Glomus fragilistratum</i>
AC 20	DAOM194757	<i>Gigaspora rosea</i>
AC 21	Leek leaf	<i>Allium ampeloprasum</i>
AC 22	BEG147	<i>Diversispora epigae</i>
AC 23	Canadian strain	<i>Rhizophagus clarus</i>

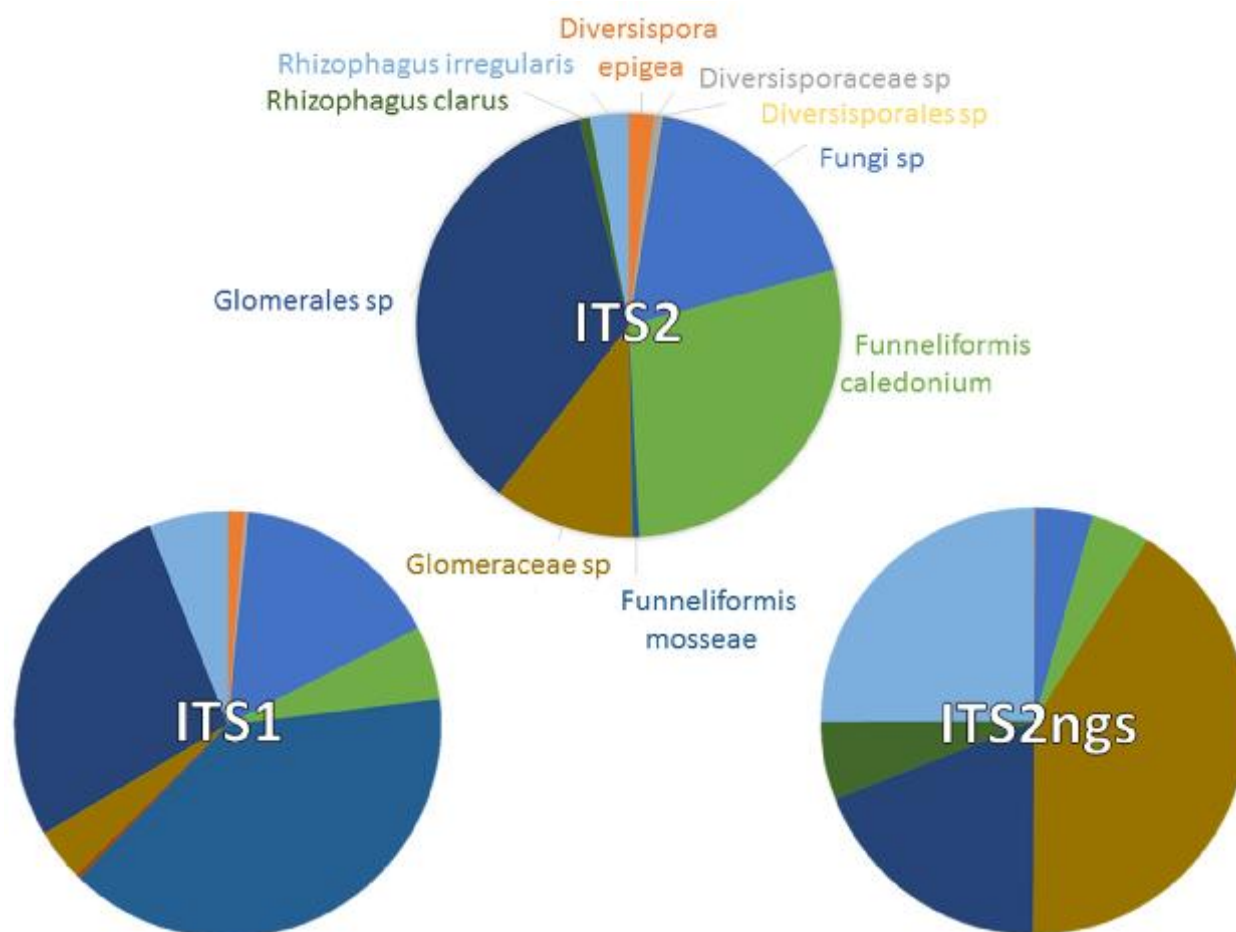
As explained earlier, high throughput sequencing generates very large dataset (1.111.470 DNA sequences for one environmental sample for example). Within these sequences, some are actually technological artifacts, such as chimeras (amplified sequences which are not representative of the genome targeted area, but generally composed of two combined fragments of different sequences²) and short fragments (from uncompleted PCR for example) meaning the set of data has to be cleaned before analysis. The following pages gather the whole procedure we developed to clean and analyze our datasets. The pipelines and the parameters associated were presented in [Chapter 2](#). This whole procedure (Mothur+Usearch+Seaview softwares) allows to evaluate relative abundance of each species/gender and their evolution with experimental treatments.

Our goals were to assess if the bioaugmentation solution or organic matter addition could generate a shift in soil fungal community. Moreover, as the soils are different, their natural community probably differs, but in which proportion? Are they more metal accumulators in one soil or another?

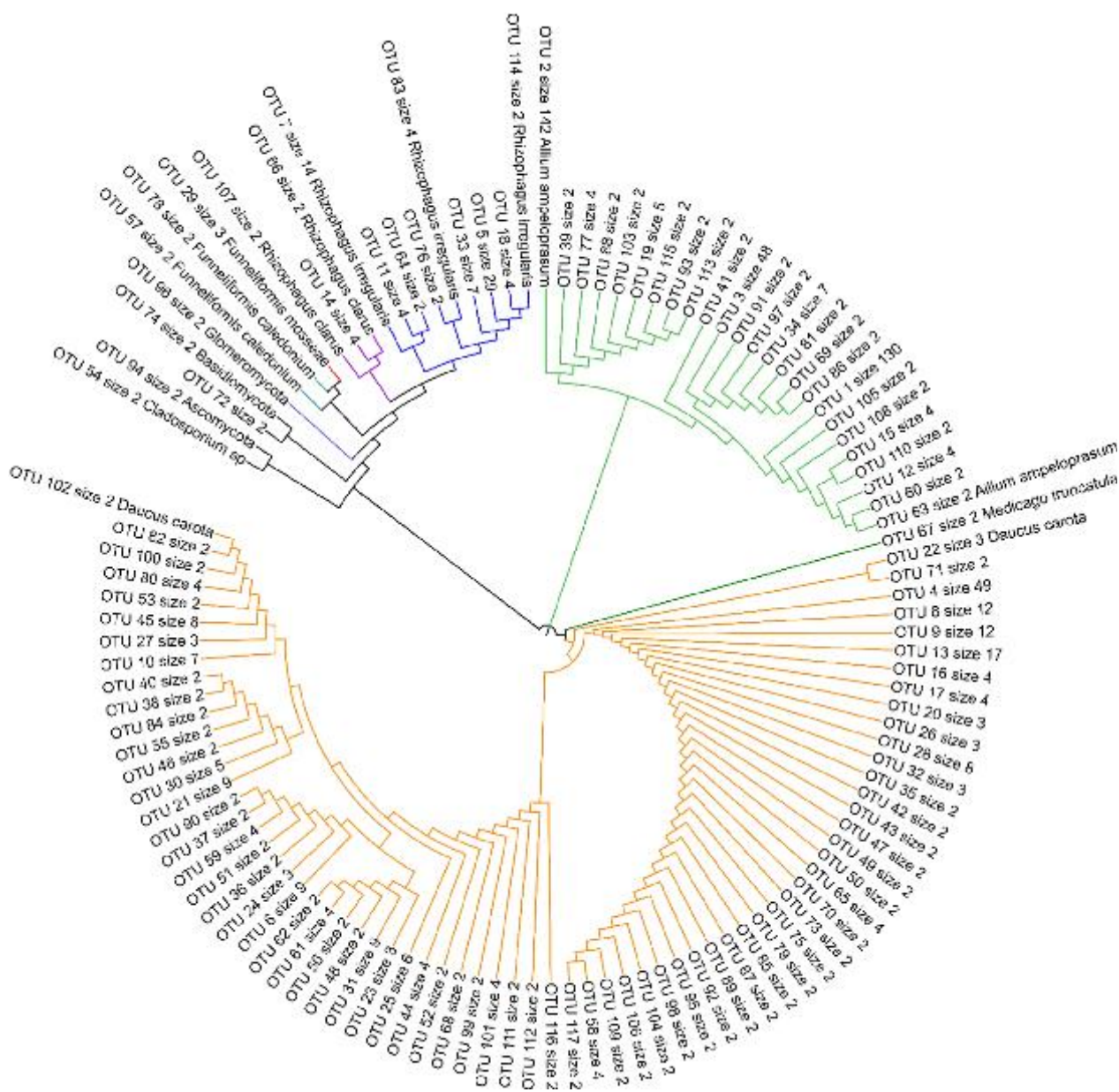
² See link for chimera description: http://drive5.com/usearch/manual/chimera_formation.html

4.3.1.3. Artificial fungal community results

Based on the former procedure, we compared three primer pairs on the artificial community. The best primer was finally very difficult to select because each one presented advantages and drawbacks as presented in Figure 4.6: for example, ITS1 detected well *Funneliformis mosseae* but poorly *Funneliformis caledonium* while the opposite was found for ITS2 and ITS2ngs. To the contrary, *Rhizophagus clarus* was detected by ITS2ngs but poorly by the others. Generally, all primer pairs detected *Rhizophagus irregularis* (but ITS2ngs showed better results compared to others which underestimate it). ITS2ngs was more specific to AMF as the plant sample presented much less diversity of other fungi (Ascomycota). Although ITS2ngs badly detected *Funneliformis mosseae* with the first assignation through Usearch, the phylogenic tree approach allowed correlating some of the dimly assigned OTU (glomerales sp) to *Funneliformis mosseae* as presented earlier in Chapter 2 - Figure 2.12. Hence, we decided to focus on the primer pair ITS3ngs4-ITS4ngs, knowing its weaknesses.



To go further in the description of the taxa, Seaview was used to align sequences with Muscle procedure and generate neighbor-joining phylogenetic tree (bioNJ protocol with 100 bootstrap). Then, trees were edited with the online ITOL tool (Letunic and Bork, 2016) as presented in Figure 4.7.



As explained in [Chapter 2](#), such representation allows to identify OTU which were not assigned with Usearch. We can see that even ITS2ngs primers amplified host plants DNA (*Daucus carota* and *Allium ampeloprasum*). This can be due to the sampling procedure in which root fragments were also taken. Among Glomeromycota, we showed an interesting diversity of *Rhizophagus irregularis*, with 6 unidentified OTU. The growing use of these new generation sequencing technologies will allow to strengthen the existing databases and therefor the reliability of the assignments.

4.3.2. Antimony and Lead bioaccessibility and human health risks

As presented in [Chapter 1](#), explained in [Chapter 2](#) and demonstrated in [Chapter 3](#) for cadmium analysis, human bioaccessibility measurement following the UBM permits to evaluate more precisely the human health risks in case of TM consumption by calculating the bioaccessible fraction of TM during digestion. These measurements were performed in edible organs (with the same procedure as presented in [Chapter 3](#) for Cd), and also in soil samples as it can potentially be an important source of exposure for children through hand to mouth contacts and more generally for gardeners if vegetables are badly washed after harvest. Consequently, we present here the results of bioaccessible fraction in soils and edible organs for Pb and Sb to complete the conclusions obtained for Cd.

4.3.2.1. Human bioaccessibility of Pb and Sb in edibles

The human bioaccessible fraction of Pb and Sb was measured in edible organs to assess human health risk of eating these crops and results are reported in [Table 4.7](#). The results indicate soil, plant, and treatment effect on the bioaccessible fraction:

Concerning the soil effect, we found a significantly higher bioaccessibility of Pb in lettuces cultivated in BZC soil compared to NTE soil ($\times 2.2$), in both CTR and bioaugmented treatments. The elevated bioaccessible fraction in plant cultivated in BZC soil is consistent, and in the same range with previous measurements on the same study site (Xiong et al., 2014a). However, the low bioaccessibility found for Pb in lettuces cultivated in NTE soil is reported for the first time. This suggests that [contaminant origin could influence metal bioaccessibility](#) in plant, even if total concentrations were not impacted (as shown in [section 4.2](#)). In other terms, the chemical form absorbed might be as easily taken up from soil, but was probably stored in plant in a low bioaccessible form.

However, as said earlier, **this mechanism might be plant dependent** (maybe with biotransformations of metal speciation in plants). Indeed, on leek crops, no significant differences were observed between soils for both Pb and Sb. Moreover, while Pb bioaccessibility was generally lower in leek than in lettuce cultivated in BZC soil (-14%), it increased in leeks cultivated in NTE soil ($\times 1.7$) even though total Pb in leek leaves was lower than in lettuce ones. In the case of Sb, its bioaccessible concentration was under the detection limit (<LD) in lettuce so the bioaccessible fraction could not be calculated while it ranged between 15 and 33% in leek crops even if total concentrations of Sb were similar in both crops.

Finally, OM and B treatments did not influence significantly Pb bioaccessible fraction in edible organs (Table 4.7) but had a significant effect on Sb bioaccessible fraction. Indeed, bioaugmentation increased %Sb bioaccessible in both soils (significantly in NTE soil and tendency in BZC soil), and OM had no effect in NTE soil. In BZC soil however, it significantly decreased Sb bioaccessible fraction in leeks. Consequently, these results suggest that the compartmentation mechanisms ought to be plant specific, but can be influenced by amendments, leading to variations in bioaccessibility of pollutants. These soil and plant effects might be considered in human health risk assessment, especially in conditions where the bioaccessible fraction could increase. From one side these complex effects reduce the possibility to simply discuss and extrapolate these phenomenons, but from another side they open opportunities to reduce bioavailability of pollutants for humans through special food diet as the bioaccessibility differs between plants.

Table 4.7: Human bioaccessible fraction of Pb and Sb in leaves. It is expressed as the ratio between bioaccessible concentration and total concentration of TM. <DL: under detection limit. Letters indicates significant groups at $\alpha < 5\%$, no letters indicates no significance.

Plant	Soil	Treatment	% Pb	% Sb
LETTUCE	NTE b	CTR	37.1 \pm 9.2	< DL
		B	35.8 \pm 8.4	< DL
	BZC a	CTR	86.5 \pm 15.9	< DL
		B	79.3 \pm 23.4	< DL
LEEK	NTE	CTR	65.7 \pm 9.3	20.6 \pm 6 b
		OM+	54.6 \pm 15.8	20.1 \pm 1.6 b
		B	53.6 \pm 14.5	33.2 \pm 4.7 a
		B :OM+	74.4 \pm 15	21 \pm 2 b
	BZC	CTR	66.4 \pm 7.5	19.8 \pm 4.1 <i>ab</i>
		OM+	59 \pm 12.2	15 \pm 1.5 <i>b</i>
		B	56.4 \pm 6.9	25.5 \pm 5.4 <i>a</i>
		B :OM+	56.6 \pm 11.2	22.9 \pm 1.6 <i>ab</i>

4.3.2.2. Human bioaccessibility of Pb and Sb in soil

Comparing bioaccessible fractions in the two soils after lettuce cultivation, significant differences were obtained between them as summarized in [Table 4.8](#). Values of soil Pb bioaccessibility in BZC were in the same range of variations than previously found in this area (Leveque, 2014).

For both Pb and Sb, bioaccessible fraction was significantly higher in BZC soil than in NTE soil. This suggests that soil characteristics and/or contaminant origin influenced its bioaccessibility, which is consistent with Leveque hypothesis (Leveque, 2014), certainly because of a difference of chemical speciation and soil binding.

Table 4.8: Soil bioaccessible fraction of Pb and Sb is expressed as the ratio between bioaccessible concentration and total concentration of TM. Letters indicates significant groups at $\alpha < 5\%$, no letters indicates no significance.

Plant	Soil	Treatment	% Pb	% Sb
T0	NTE <i>b</i>		15 ± 0.1	0.1 ± 0.001
	BZC <i>a</i>		74.8 ± 0.8	4.1 ± 0.04
LETTUCE	NTE <i>b</i>	CTR	13.8 ± 1.1	0.2 ± 0.3
		B	14.4 ± 2	0.1 ± 0.1
	BZC <i>a</i>	CTR	74 ± 1	4.4 ± 0.2
		B	73.8 ± 2	4.2 ± 0.2
LEEK	NTE <i>b</i>	CTR	17.8 ± 0.6	1.1 ± 0.4
		OM+	22.2 ± 5.3	1.2 ± 0.1
		B	21.7 ± 1.4	1.1 ± 0.1
		B :OM+	17.9 ± 2.1	1.2 ± 0.1
	BZC <i>a</i>	CTR	77.5 ± 1.7	9.7 ± 1.1
		OM+	75.3 ± 1.3	10 ± 0.8
		B	77.4 ± 1.4	10.2 ± 0.4
		B :OM+	76.5 ± 2.4	9.1 ± 0.5

Indeed, high Pb bioaccessibility has been shown to be correlated with highly soluble Pb chemical forms such as lead bound carbonates and oxides (Bannon et al., 2009; Leveque, 2014), while in mine soils, low bioaccessible forms might be associated with phosphates, sulfates, or sulfides (Schröder et al., 2004). Our results are consistent with these hypotheses as shown by the significant correlation between Pb/Sb bioaccessible fractions and Pb/Sb CaCl₂ and EDTA fractions as presented in Table 4.9.

Table 4.9: Two tails Pearson correlation matrix between bioaccessible fraction of TM (Pb and Sb) and soil parameters (pH, OM content).*: significant at $\alpha=5\%$; **: significant at $\alpha=1\%$

		OM	% Pb bioaccessible	% Sb bioaccessible	% Pb EDTA	% Sb EDTA	% Pb CaCl ₂	% Sb CaCl ₂
pH	Pearson corr.	0.400**	0.447**	0.436**	0.461**	-0.155	0.022	0.364*
	<i>Pvalue</i>	0.005	0.002	0.002	0.001	0.292	0.884	0.011
OM	Pearson corr.		0.507**	0.552**	0.474**	-0.223	0.062	0.483**
	<i>Pvalue</i>		<0.001	<0.001	0.001	0.128	0.674	0.001
% Pb bioaccessible	Pearson corr.			0.989**	0.992**	-0.330*	0.348*	0.866**
	<i>Pvalue</i>			<0.001	<0.001	0.023	0.017	<0.001
% Sb bioaccessible	Pearson corr.				0.981**	-0.328*	0.298*	0.850**
	<i>Pvalue</i>				<0.001	0.026	0.044	<0.001
% Pb EDTA	Pearson corr.					-0.320*	0.356*	0.848**
	<i>Pvalue</i>					0.027	0.013	<0.001
% Sb EDTA	Pearson corr.						-0.116	-0.317*
	<i>Pvalue</i>						0.432	0.028
% Pb CaCl ₂	Pearson corr.							0.370**
	<i>Pvalue</i>							0.010

Furthermore, Pb bioaccessibility in soil seemed more correlated to Pb_{EDTA} fraction than Pb_{CaCl₂} fraction, while Sb bioaccessibility was strongly correlated to Sb_{CaCl₂} and negatively correlated to Sb_{EDTA}. This suggests different behavior of Sb compared to Pb with soil matrix. Moreover, some studies found correlations between the metalloid bioaccessible fractions and OM and clay contents (Poggio et al., 2009) but these correlations seem not to be always evidenced (Wang et al., 2003; Leveque, 2014). In our conditions, a good correlation seems to link Pb and Sb bioaccessible fractions and soil physicochemical properties (OM and pH).

Moreover, to our knowledge, we found here for the first time that human bioaccessibility of soil is influenced by bio-augmented plant. We observed no significant difference between the beginning of the experiment (T0) and after lettuce cultivation in the two soils, for Pb and Sb. However, after leek cultivation, bioaccessibility of Pb significantly increased in NTE soil (approximately +40%) and bioaccessibility of Sb increased in both soils ($\times 7.7$ in NTE soil and $\times 2.3$ in BZC soil approximately) and this increase occurred in all the treatments. Therefore, leek plant and its mycorrhizosphere might have exudated chemical compounds able to convert Pb and Sb in a bioaccessible form. As presented in the former [section 4.2](#), we also found that plants can influence mobility of Pb and Sb (EDTA and CaCl_2 extracts), which is consistent with the fact that bioaccessible fractions of Pb and Sb were correlated with EDTA and CaCl_2 fractions.

Interestingly, while we found that Pb and Sb bioaccessible fractions were positively correlated to soil OM content, the addition of lombricompost had no effect on these fractions in both soils. Further investigation is thereby needed to clarify these results before being able to give straight recommendations to gardeners. Indeed, the interactions between scientific research and field activities of gardeners is complex to organize but very important in terms of “Science and Society” synergetic exchanges.

4.4. General conclusion

The present study showed that:

- Soil characteristics and particularly TM origin (anthropic or geogenic) influenced strongly their mobility and bioaccessibility as we observed differences in results obtained in each soil. Pb and Sb mobile fractions and bioaccessible fractions were generally higher in the anthropic soil, where the contamination is recent and eventually more mobile than in the naturally TM rich soil, where Pb and Sb might be in a more stable form. These differences in bioaccessibility seem to be correlated to Pb and Sb mobility in soil and their solubility (availability in soil solution), which could indicate that their chemical speciation influence their bioaccessibility. However, as shown in section 5.2, these differences did not influence plant uptake.
- Lombricompost (OM) addition significantly decreased Pb mobility and had a lower effect on Sb mobility, but these effects were not observed in plant accumulation. Moreover, OM addition seemed to decrease Pb and Sb bioaccessible fractions in soil, but with contrasted and poorly significant results. Furthermore, OM addition decreased AMF diversity associated with leek roots, so this amendment has to be sustained with care. As the latter effect is OM-origin dependent, further research is needed to characterize the effects of the most commonly used OM amendments in urban gardens.
- Bioaugmentation might not be a suitable tool to propose to gardeners to limit Pb and Sb accumulation in plants. Indeed, its efficiency seemed to be both plant and soil dependent and its influence on AMF community associated with leek highlights the need to study precisely the role of each main species in TM solubilization and plant uptake. With the actual state of knowledge, this technic could be proposed in phytoremediation studies focused on non-edible plants.
- Because rhizospheric bacterial communities are known to influence TM mobility, they would also need to be identified to evaluate their role in the obtained results.
- The gaps in results interpretation highlight the complexity of the interactions at the 'soil-microorganism-plant' interface. Therefore, a simplified model could be developed to explore more precisely the influence of AMF and Sb chemical species on its accumulation by edible plants. With that aim, we developed an approach in hydroponic conditions with AMF and Sb spiking to limit soil influence on Sb transfer to plants.

Chapter 5. USING A SIMPLIFIED HYDROPONIC APPROACH TO STUDY THE ROLE OF ARBUSCULAR MYCORRHIZAL FUNGI IN VEGETABLE Sb UPTAKE

5.1. General introduction

The results of [Chapter 4](#), concerning Sb fate at the soil-microorganism-plant interface when AMF or lombricompost were added to two contrasted peri-urban soils, showed that AMF might play a role in Sb phytoaccumulation. This is not the same trend for other trace elements such as Pb or Cd, for which literature already reported the complex role of AMF in their transfer to plants.

However, it had been poorly studied for an emerging pollutant such as antimony, as reported in part 1.3 of [Chapter 1](#). The experimental protocol developed in the former chapter to study the role of contaminant origin (anthropic vs geogenic) gave interesting results, but the complexity of the mechanisms involved (due to soil physicochemical properties) justified the interest to study less complex ecosystems to lower interactions.

To do so, the role of AMF in Sb uptake by lettuce (*Lactuca sativa* L.) and carrot (*Daucus carota* L.), separately grown in artificial substrate (Sorbix®) spiked with different Sb chemical species was studied. Plants were grown under hydroponic condition and half of the modalities received a concentrated spores solution (350 sp.ml⁻¹) isolated from the leek trap cultures presented in [Chapter 2](#) to obtain mycorrhized (+MYC) and non-mycorrhized (control, noted CTR) plants. Three weeks before harvest, plants were spiked with 10 mg.l⁻¹ of either Sb₂O₃ or KSbO-tartrate (KSb). Total and human bioaccessible Sb fractions content were measured to evaluate the role of AMF in Sb transfer and bioavailability depending on plant and Sb species as summarized in [Figure 5.1](#).

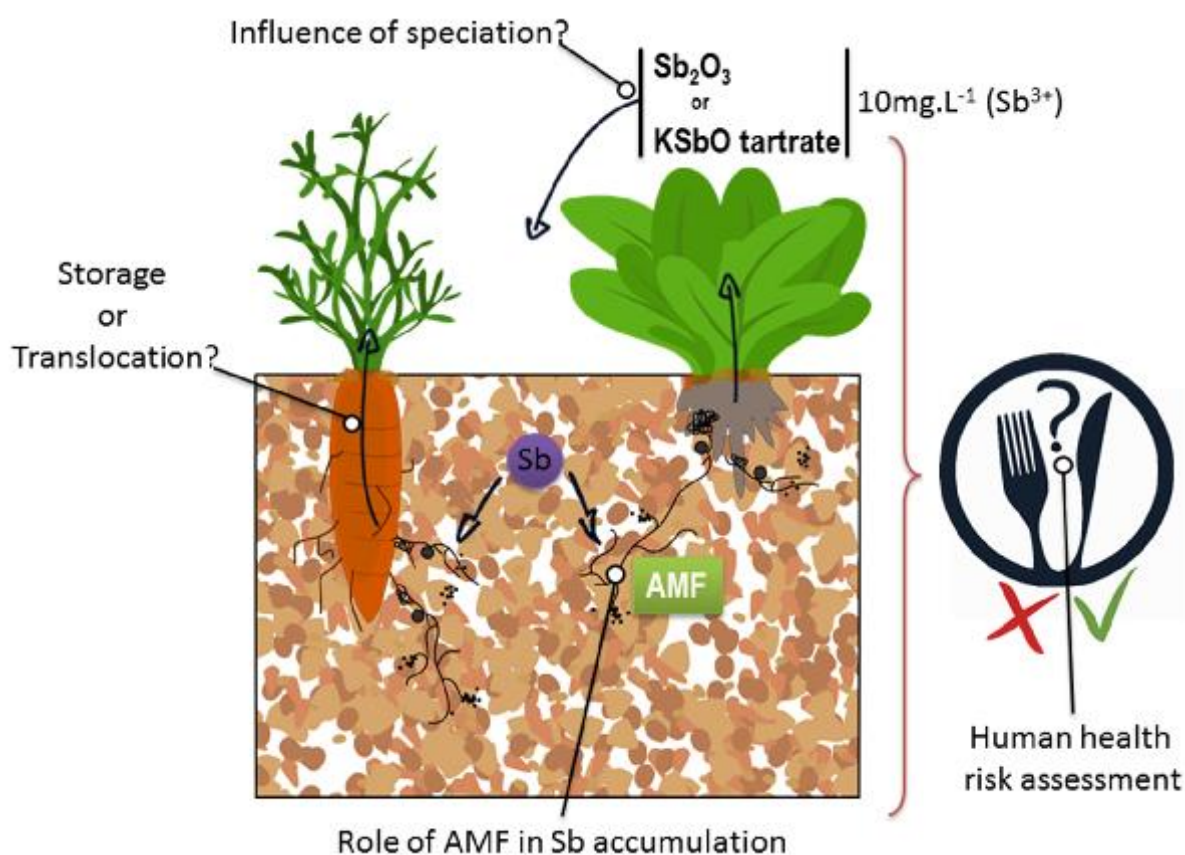


Figure 5.1: Objectives of the hydroponic experiment regarding AMF influence on Sb transfer to plants

To summarize the results, after 3 weeks growing on Sb spiked Sorbix, the presence of AMF significantly increased Sb accumulation in carrot (leaves, roots and edible parts) with higher accumulation in roots, regardless of the initial Sb chemical speciation. In lettuce, changes of Sb accumulation in organs was significantly affected by Sb chemical species (increased in roots with Sb₂O₃, but decreased with KSb). The present results highlighted a possible risk of Sb transfer from soil to edible plants cultivated on soil when containing AMF propagules or when added as biofertilizers.

Such conclusions still need validation in further field trials. However, the influence of biofertilizers on pollutant should be considered in future health risk assessment about urban gardening.

5.2. Experimental design summary

All the methods are detailed in [Chapter 2](#); the [Figure 5.2](#) summarizes the experimental design set up to study the role of AMF bioaugmentation in the transfer and storage of different Sb species in vegetables cultivated in hydroponic conditions.

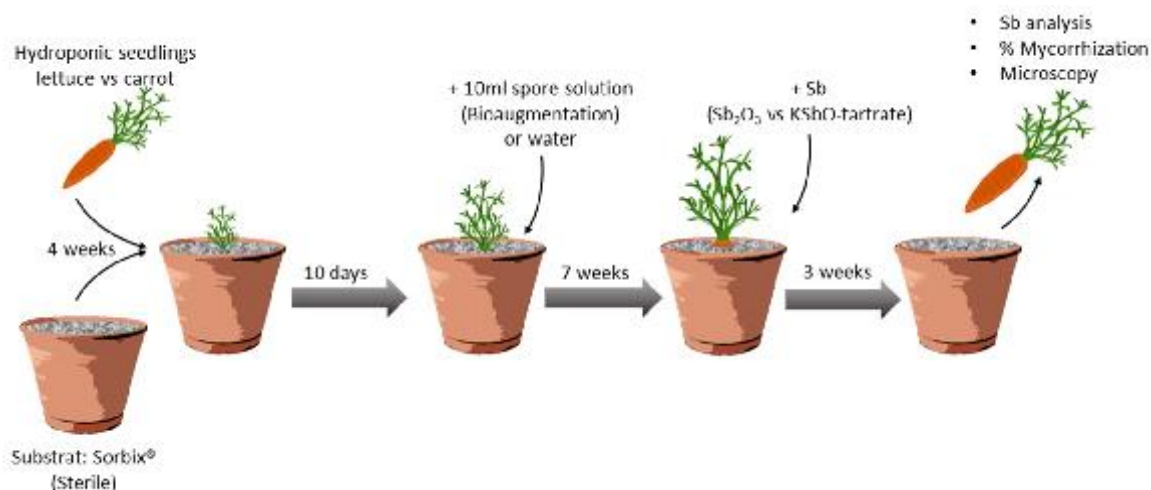


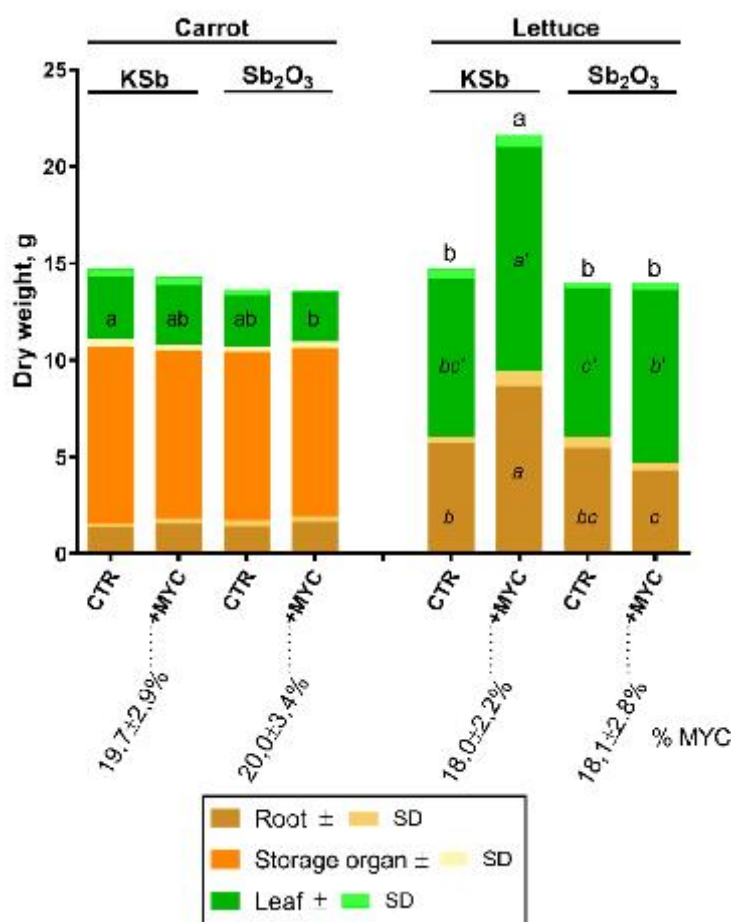
Figure 5.2: Experimental design for hydroponic carrot cultivation treated with Sb

5.3. Results and discussion

5.3.1. Sb didn't affect plant growth or mycorrhizal rate

Regardless of the Sb species used to spike the substrate, the control treatments (CTR) were non-mycorrhized, while the mycorrhization rate of MYC+ treatments ranged between 16 and 23% with no significant difference between modalities as reported in [Figure 5.3](#). Such value is low compared to plants cultivated in soil for which the mycorrhizal rate can reach more than 70% (Cozzolino et al., 2016). Other study using Sorbix as a substrate also reported a low mycorrhization rate (~23%) (Wang et al., 2015).

Lettuces and carrots grew well in all modalities, with no visible phytotoxicity effect of Sb species, or deficiencies due to hydroponic conditions. Generally, their total biomass (in each plant sub-group) was not different among modalities as shown in [Figure 5.3](#), except for lettuce in the treatment [KSb; +MYC] for which the total biomass was significantly higher than the others, both for roots and leaves. The interaction between Sb₂O₃ and MYC also enhanced biomass of leaves but was not significant in roots. In carrots, roots and storage organs (edible part) grew equally in each treatment. At the organ scale (root / storage organ / leaf), mycorrhizal treatment decreased significantly leaf biomass.

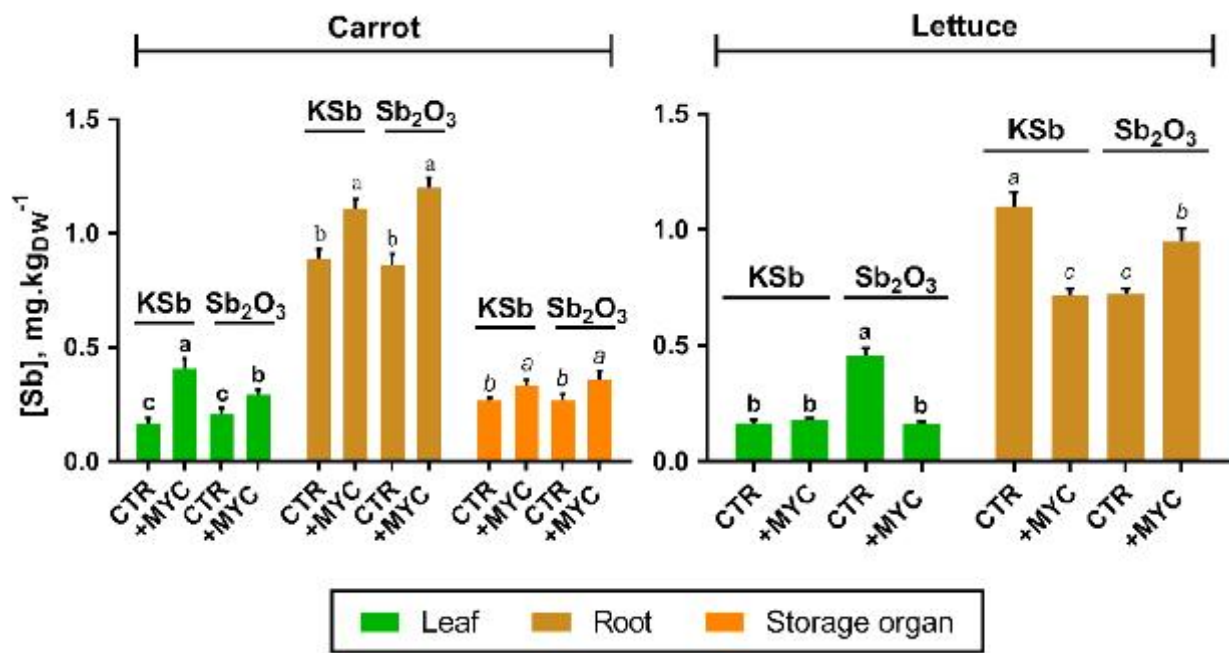


5.3.2. Mycorrhization and Sb Speciation influenced its accumulation

The total Sb concentrations in lettuce and carrots are presented in Figure 5.4. Sb concentration was low in every modality and ranged between 1-2 mg.kg_{DW}⁻¹, with a preferential accumulation in roots than in leaves for all plants. That trend was previously observed with potassium hexahydroxoantimonate-KSb(OH)₆ when spiked at high concentrations (500-1000 mg.kg⁻¹) on Bermuda grass (*Cynodon dactylon*), mycorrhized with *Funneliformis mosseae* (Wei et al., 2016). The Sb content in leaves are also consistent with the study of Hammel et al (Hammel et al., 2000), who found that carrots cultivated in Sb contaminated soil (~160 ppm total, ~0,25 ppm mobile) accumulated up to 0.8 and 0.03 mg_{Sb}.kg⁻¹_{DW} in leaves and storage organ respectively, but gave no information about the rest of the root system. Anyway, such concentrations can be considered as low in comparison with Sb accumulation in other edibles (Pierart et al., 2015).

In carrot plants, inoculation with AMF led to a significant increase of Sb accumulation in the whole plant, with higher concentrations in roots than in other organs. No differences were observed between Sb species, except in leaves. Indeed, the combined treatment [KSb:+MYC] led to the highest concentration (~0.4 ppm), which is ~30% and 50% more than [Sb₂O₃:+MYC] and CTR treatments respectively. This increase with KSb could be due to an elementary competition with an increase of potassium absorption by AMF as it has been shown in tomatoes crops (Abdel Latef and Chaoxing, 2011).

In the case of lettuce, results are more complex. Indeed, AMF decreased Sb accumulation in roots from plants treated with KSb (but increased in plant treated with Sb₂O₃). In leaves, the mycorrhization showed no effect in KSb treatment, but significantly decreased Sb in Sb₂O₃ spiked plants (+2). This latter result is interesting as Sb₂O₃ is frequently measured in urban areas (Smichowski, 2008).



In this experiment, MiSeq analysis was not carried out on root samples. Consequently, it's still difficult to analyze fully the influence of fungal species on these results, while it has already been shown that TM uptake was AMF species dependent (Hassan et al., 2013) as discussed in [Chapter 4](#).

However, the solution used for +MYC treatment was prepared from the same leek trap crop than the one used in [Chapter 3](#) and [Chapter 4](#). Accordingly, we know the relative abundance of the species that were brought (which are reminded in [Chapter 4-Table 4.5](#)). On that base, the major inoculant was *Rhizophagus irregularis*. However, *Funneliformis mosseae* strains, which are known to enhance Sb uptake (Wei et al., 2016), were also present. Hence, the differences observed between plants could come from a plant effect or a difference in fungal community associated with plant roots.

Furthermore, in the study of Cozzolino et al (Cozzolino et al., 2010), inoculation with a commercial *Rhizophagus irregularis* preparation led to a decrease of arsenic in lettuce which is not the case in our conditions for Sb even if these metalloids are considered to behave similarly in soil-plant systems (Casiot et al., 2007; Wilson et al., 2010). Other parameters such as soil nature could differ between our study and these previous experiments: Sb might behave differently than As or another fungal species could be associated to lettuce roots.

(Chapter 4 – Table 4.5): Relative abundance of fungi from Glomeromycota Phylum in the bioaugmentation solution

Assigned OTU	Relative abundance, %
Glomerales sp	33.46
<i>Rhizophagus irregularis</i>	21.09
Rhizophagus like	36.85
<i>Funneliformis mosseae</i>	1.67
<i>Septoglomus viscosum</i>	0.04
Claroideoglomeraceae sp	4.82
Diversisporales sp	1.06
Diversisporaceae sp	0.32
<i>Diversispora celata</i>	0.38
<i>Diversispora epigeae</i>	0.28
<i>Ambispora gerdemannii</i>	0.02

5.3.3. Translocation and bioaccumulation factors are affected by AMF

As described in Chapter 2, two indicators are commonly calculated to study the risk of TM accumulation along food chain: The translocation Factor (TF), representing the risk of transfer from root to leaves and the bioaccumulation factor (BF), which is the ratio between TM content in the whole plant and its concentration in soil. The latter factor permits to evaluate the risk of accumulation depending on soil contamination.

Results of TF and BF were reported in Table 5.1. In our conditions, we observed a significant effect of both AMF and Sb speciation. Indeed, in carrot plants, AMF increased twice TF in KSb treatment, but had no effect on Sb₂O₃ treatment (*i.e.* Sb speciation effect). In lettuces treated with KSb, AMF also increased TF. However, when spiked with Sb₂O₃, AMF plant presented a TF three-time lower than non-mycorrhized ones.

Table 5.1: Translocation factor (TF) and bioaccumulation factor (BF) in different edible plants.

Data represent mean \pm SD. Statistical analysis was performed separately in carrot and lettuce, for TF and BF. Letters: significant groups

	KSb		Sb ₂ O ₃	
	CARROT			
	CTR	MYC+	CTR	MYC+
TF	0.15 ± 0.03 (b)	0.28 ± 0.04 (a)	0.19 ± 0.03 (b)	0.19 ± 0.01 (b)
BF	0.13 ± 0.01 (b)	0.19 ± 0.01 (a)	0.13 ± 0.001 (b)	0.19 ± 0.01 (a)
	LETTUCE			
	CTR	MYC+	CTR	MYC+
TF	0.15 ± 0.01 (c)	0.25 ± 0.02 (b)	0.63 ± 0.06 (a)	0.17 ± 0.02 (c)
BF	0.13 ± 0.01 (a)	0.09 ± 0.003 (c)	0.12 ± 0.002 (ab)	0.11 ± 0.01 (b)

Focusing on carrot plants, MYC+ modalities presented a higher BF than CTR, regardless of Sb chemical species. This means that the interaction between carrot and AMF led to a higher uptake and storage of Sb than non-mycorrhized plants. Such increase of TF and its value are highly consistent with the results of Wei et al (Wei et al., 2016) where the authors showed that Bermuda grass in symbiosis with *Funneliformis mosseae* had a higher Sb content, TF, and BF. In the same study, the authors spiked with Sb^V showing that AMF might be able to lower the reduction of antimony in plant (from Sb^V to Sb^{III}), decreasing thereby its toxicity (Smichowski, 2008). Here, various Sb^{III} forms (oxidative state) were used, meaning that Sb phytotoxicity might be higher in our case. Sb speciation should be performed to conclude on Sb plant storage forms.

Furthermore, the significant decrease of BF in lettuce (KSb:+MYC) treatments suggests the complex interaction between plant species, AMF community and TM speciation. Sb speciation measurements should be performed to conclude on the storage forms of Sb.

5.3.4. Human bioaccessibility is affected by AMF and Sb species

Human bioaccessibility has been measured in edible organs of carrot and lettuce (edible root and leaf respectively) with the unified Barge method described in [Chapter 2 \(Table 5.2\)](#). As evidenced in [Chapter 4](#), Sb bioaccessibility is generally low, with values depending of the plant species, Sb species and mycorrhization. Indeed, Sb bioaccessibility is generally higher in carrot than in lettuce, but with higher variability (SD). Mycorrhization had no effect on Sb bioaccessibility in carrot. In lettuce, we showed here for the first time that mycorrhization significantly increased Sb bioaccessibility in lettuce leaves in the case of Sb₂O₃ treatment. Such result strengthen the findings obtained in [Chapter 4](#) in which bioaugmentation was shown to increase significantly Sb bioaccessibility under certain conditions. This can suggest a change of Sb chemical species when absorbed by AMF. However, as this phenomenon was not observed in all treatments, various hypothesis can be proposed:

- Does the fungal community differs between plants & Sb treatment?
- Dose the biotransformation of Sb occurs in the fungus or in plants?
- What would happen with other Sb species?

Table 5.2: bioaccessible fraction of Sb in edible organs.

Data represent mean \pm SD. Statistical analysis was performed separately in carrot and lettuce, for each Sb species. Letters: significant groups

Carrot				Lettuce leaf			
KSbO-t		Sb ₂ O ₃		KSbO-t		Sb ₂ O ₃	
MYC-	MYC+	MYC-	MYC+	MYC-	MYC+	MYC-	MYC+
19 \pm 6,5	12,6 \pm 6,3	12,5 \pm 3	9,5 \pm 1,3	8,2 \pm 2,2	4,4 \pm 1,6	2,2 \pm 1,2 (b)	8,9 \pm 3,7 (a)

5.4. Conclusions and perspectives

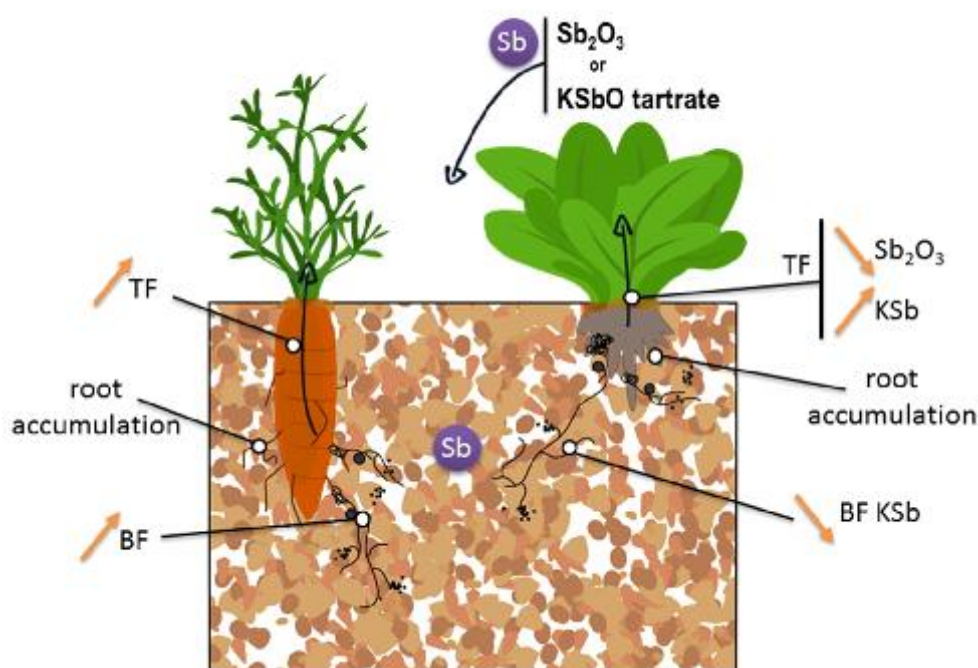


Figure 5.5: Main results of the influence of AMF on Sb transfer depending on plant and Sb speciation

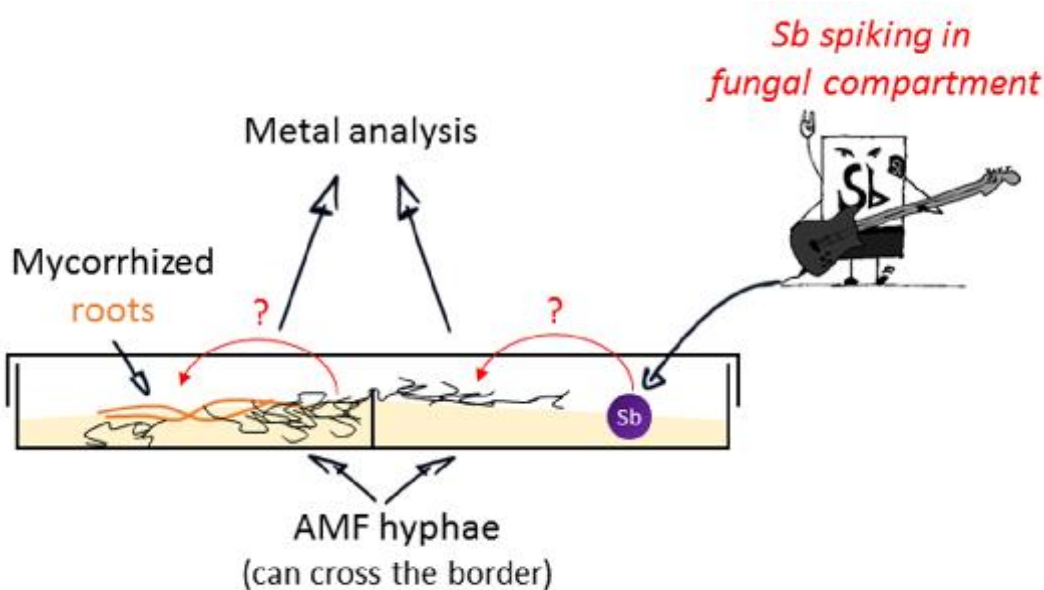
The aim of the present chapter was to simplify the experimental design to evidence the role of arbuscular mycorrhizal fungi in the transfer of antimony species from solution to edible plants. Indeed, as described in [Chapter 1](#), the role of AMF in Sb transfer is poorly known. Recent studies of Wei et al (Wei et al., 2016, 2015) highlighted the growing interest of these biofertilizers and the need to fill that gap. In [Chapter 4](#), we showed that with contaminated soil (anthropic or geogenic Sb origin), AMF seem to influence Sb mobility. However, the results were complex to discuss, so we intended a simplified approach with hydroponic leafy and roots vegetables, spiked with both Sb and AMF to avoid the multiple interactions with soil matrix.

Here, we found that a mix of fungal species isolated from a Pb-Sb contaminated soil led to a variable range of response depending on Sb chemical species and plant species ([Figure 5.5](#)). Results were more homogeneous in carrot plants, in which Sb uptake increased in all +MYC treatments, regardless of its chemical form. In lettuce, the Sb chemical form seemed to influence its accumulation.

Furthermore, AMF enhanced both the translocation factor and the bioaccumulation factor, except in lettuces spiked with Sb_2O_3 in which we observed a significant decrease of TF. As the spore solution contained a mix of AMF species, and soil bacteria, it would be interesting to study the fungal and bacterial communities in symbiosis with each plant species. This could permit to identify the main fungal species responsible of Sb uptake by plants or Sb binding in soil to develop management strategies in contaminated urban areas and beyond.

Facing these sanitary risks, the absence of legal threshold for Sb in food remains of high concern while its toxicity is well known (Sundar and Chakravarty, 2010).

Then, when plants are in symbiosis, the proportion of inorganic element transported by roots and AMF can vary. It is therefore necessary to evaluate the proportion of Sb that AMF hyphae bind, take up and transfer to plant. To do so, the use of *in vitro* hairy root system in double compartment as described in Figure 5.6 could be of great interest for phytoremediation studies (Agostini et al., 2013; Ludwig-Müller et al., 2014).



Chapter 6. PHYTOACCUMULATION OF SB AND SOIL ENZYME ACTIVITIES: EFFECTS OF ORGANIC WASTES AND BIOCHAR

6.1. Introduction

The former [Chapter 3](#), [4](#) and [5](#) aimed to study the role of arbuscular mycorrhizal fungi (AMF) and organic matter (OM) amendment on the transfer of metal(loid)s (TM) at the soil-microorganisms-plant interface. The results highlighted part of the complexity of the interactions occurring between soil matrix, AMF and TM but other major soil living organisms can also affect TM mobility and fate. Making an exhaustive study of the role of each living organisms on TM mobility in soil is almost impossible, so the focus is generally performed on major soil actors. For example, while this thesis focused on AMF, Leveque (Leveque, 2014) studied the role of earthworms in TM mobility, bioaccessibility and toxicity, but interactions seem to exist between earthworms and AMF regarding TM mobility (Aghababaei et al., 2014). Other studies focused specifically on bacterial community and their interactions with TM (An and Kim, 2009; Shi et al., 2013).

Consequently, we intended to approach the interaction between TM (specifically antimony) and the biological components of the soil system through a global point of view with soil enzymatic activity measurement. Impact of TM on soil biological traits has been extensively investigated using microfauna, mesofauna and macrofauna; involving both laboratory and field approaches (Bello et al., 2014). However, how these environmental contaminants may interact with soil enzyme activities has been comparatively less studied.

Because soil enzyme activities are key catalysts in the biogeochemical cycles of nutrients, environmental stressors such as pollution, changes in OM and land management are serious hazards to soil quality (Puglisi et al., 2006; Pan et al., 2010; Ahmad et al., 2014; Kim et al., 2014; Lessard et al., 2014; Sanchez-Hernandez et al., under review). Recently, biochar has become a promising low-cost and environmental-friendly technology for recovering low-productive soils. Biochar also plays an important role as an absorbent material in soil pollution, reducing thereby contaminant bioavailability in soil. This retention capability has been proven with several heavy metals, including antimony (Pan et al., 2010; Ahmad et al., 2014). Nevertheless, sorption efficiency of biochar is highly dependent on its surface area, micro-porosity, and hydrophobicity. These parameters are in turn dependent on the raw material used to prepare it (e.g., wood residues, crops residues, organic wastes, grape seeds...) (Ahmad et al., 2014; Kim et al., 2014). Here, we used spent coffee ground as raw

material because it is a low-cost waste produced worldwide in important quantities. Moreover, current research is needed to develop innovative and sustainable ways to increase its valorization (Campos-Vega et al., 2015).

Consequently, this chapter shows a microcosm experiment performed to attend the following aims:

- The interest of charred Spent Coffee Ground (SCGc) for microbial development
- The impact of both SCG and SCGc on extracellular soil enzyme activities
- The impact of SCG and SCGc on plant growth and Sb uptake
- The effect of Sb concentrations on the activity of selected extracellular soil enzymes

Accordingly, we set-up a pot experiment using Pea (*Pisum sativum* L.) as a model plant to study the interactions between antimony and SCG/SCGc on both extracellular soil enzyme activities and soil-to-plant Sb transfer and phytoaccumulation (Figure 6.1).

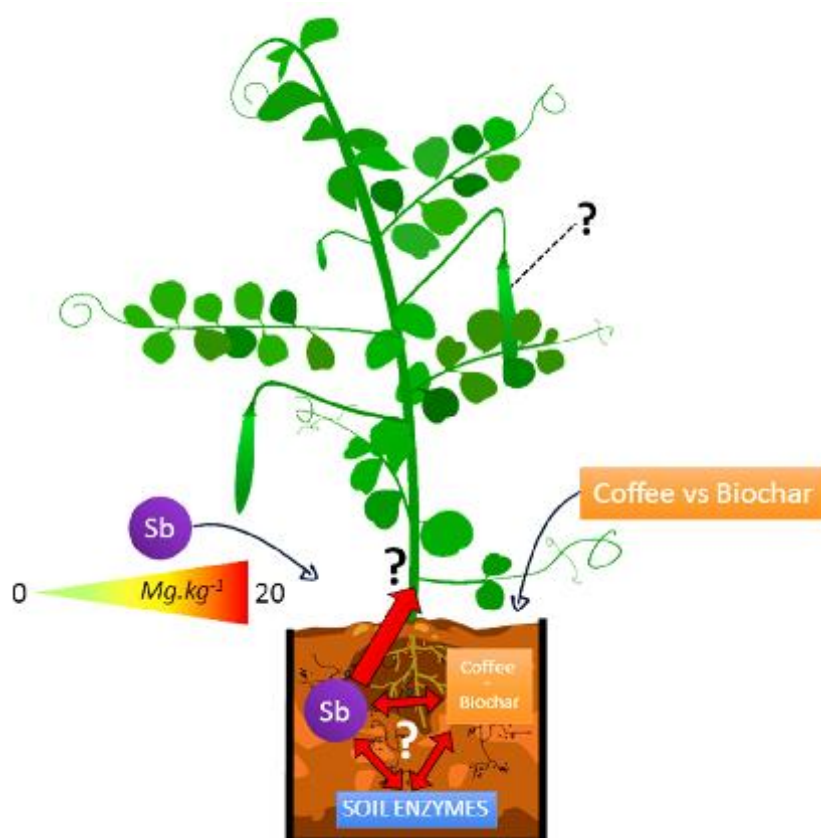


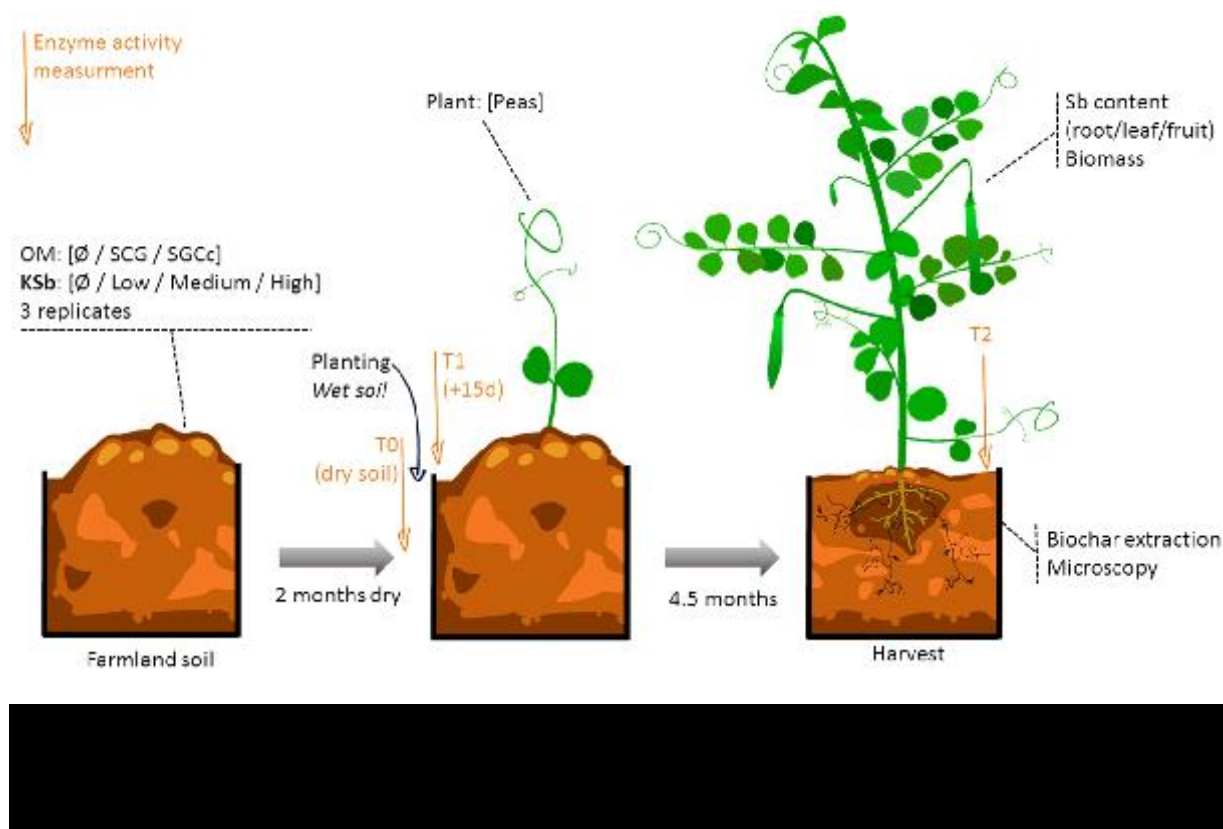
Figure 6.1: Conceptual scheme illustrating the main objectives of the Sb–biochar–soil enzymes experiment.

The following experiment as long as soil enzyme assays were carried out in the Ecotoxicology Laboratory (Environmental Science and Biochemistry Faculty, University of Castilla-La Mancha, Toledo, Spain) in collaboration and under the supervision of Pr. Juan Carlos Sanchez-Hernandez with loud heavy metal music to focus.



6.2. Experimental design

Methodology used in this microcosm experiment has been already described in [Chapter 2](#). The [Figure 6.2](#) summarizes the experimental set up to study the effect of Sb, spent coffee ground (SCG) and its biochar (SCGc) on soil enzyme activities at three different periods T0 = dry soil, T1 = 15d (seedling), and T2 = harvest.



The principal results were as follow:

- Spent Coffee ground biochar (SCGc) was well colonized by AMF and soil bacteria, supporting its potential as a microfauna-stimulating material and a source of nutrients. This type of biochar revealed a promising amendment for recovering degraded soils.
- SCGc conserved microbial enzymatic activity after sterilization, meaning that biochar offered shelter to microorganisms, protecting them from environmental stressors such as high temperatures or desiccation. These outcomes stimulated future research aimed to activate SCGc with selected microorganisms able to solubilize or stabilize TM, so helping in the phytoremediation or phytostabilization processes.

- Soil enzyme activities were strongly changed: Sb spiking increased significantly the IBRv2 index. However, this effect seemed to be partly concentration dependent, with higher influence at low Sb concentration. These results highlighted the potential use of the IBRv2 index to develop soil standard quality values.
- SCGc buffered Sb toxicity at the end of the experiment compared to SCG. However, some enzymes were still affected by Sb contamination (carboxylesterase, urease, and acid phosphatase).

6.3. Results and discussion

Note: One of the difficulties of using the IBRv2 index is that the study which developed the IBRv2 index did not give information about how to set properly the zero (i.e. which treatment would be set as zero for the basal line?). Indeed, it is possible to (i) Select the [T0-ØSb-No OM] condition as a control for all other points; (ii) choose a time-mobile control by using the $[T_i\text{-No Sb-No OM}]_{i=0-1-2}$ condition; (iii) use each [no OM] condition as a control for each Sb dose and time. Here we decided here to use the second method to study the results at each time in terms of variation compared to a natural soil (no Sb, no OM), even if in this control the enzyme activities could have evolved along the experiment.

6.3.1. Influence of Sb, SCG and SCGc on soil enzyme activities

In this experiment, the effect of antimony on eight soil enzyme activities, which were representative of carbon, nitrogen and phosphorus biogeochemical cycles, was evaluated in the presence and absence of two organic amendments (spent coffee grounds - SCG and its biochar SCGc) (Figure 6.2). Such effect was examined at three sampling times, so the number of interactions was high (8 enzymes \times 3 times \times 3 conditions of OM \times 4 Sb concentrations). Because of the complexity of studying all possible interactions, several authors suggest to use numerical indexes for a more accurate and simple interpretation of enzyme-based results (Puglisi et al., 2006; Lessard et al., 2014; Sanchez-Hernandez et al., under review).

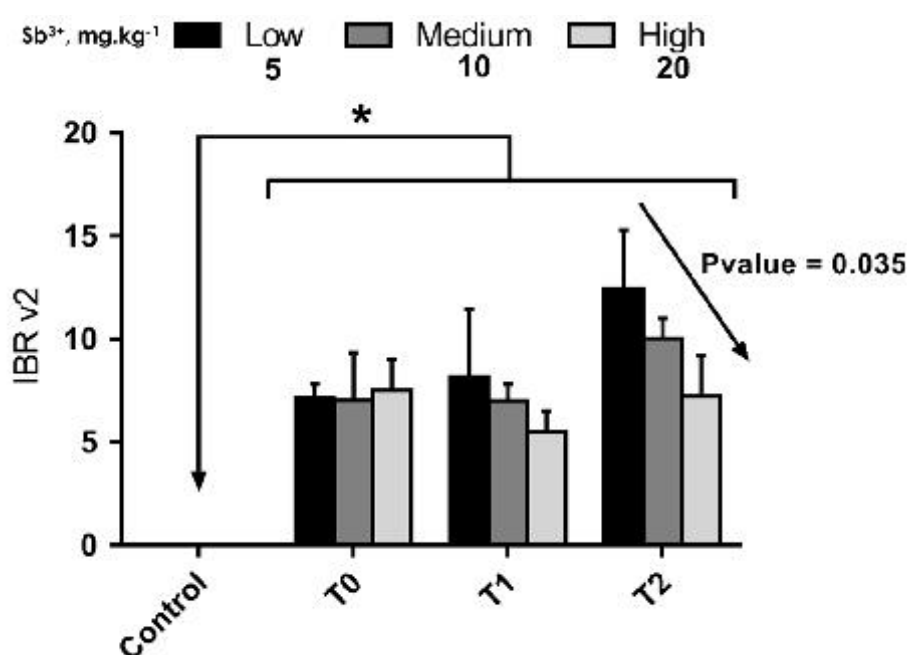
Therefore, we calculated four mathematical indexes (GM, WM, T-SQI and IBRv2), as detailed in [Chapter 2](#). In the present chapter, we only present the results of the IBRv2 index to facilitate the reading and understanding of the multiple factors interacting on soil enzyme activities. Additional results for the other indexes are presented in supplementary material ([section 6.5, Figure 6.11](#)).

6.3.1.1. Concentration- and time-specific responses of soil enzyme activities to Sb exposure

In this section, results are only provided for control soils (SCG- and SCGc-free soils), which were spiked with serial Sb concentrations. Responses of soil enzyme activities were assessed by the IBRv2 index ([Figure 6.3](#)).

There was a significant increase of the IBRv2 values in the Sb-treated soils ($P < 0.05$, Kruskal-Wallis test), regardless of Sb concentration ([Figure 6.3](#)). However, at short-term exposure (T0 and T1), the range of variation was similar (IBRv2 ≈ 8) with no significant effect of Sb dose.

Contrariwise, there was an inverse relationship between IBRv2 values and Sb concentrations at T1 even if it is not significant (P value = 0.149, Jonckheere-Terpstra trend test). This trend was consistent (P value = 0.035, same test) at the moment of harvesting (T2). Accordingly, we observed a trend of increasing IBR with time in low and medium Sb treatment (more evident at low Sb concentrations) while at high Sb dose, the IBRv2 remained unchanged along the experiment.

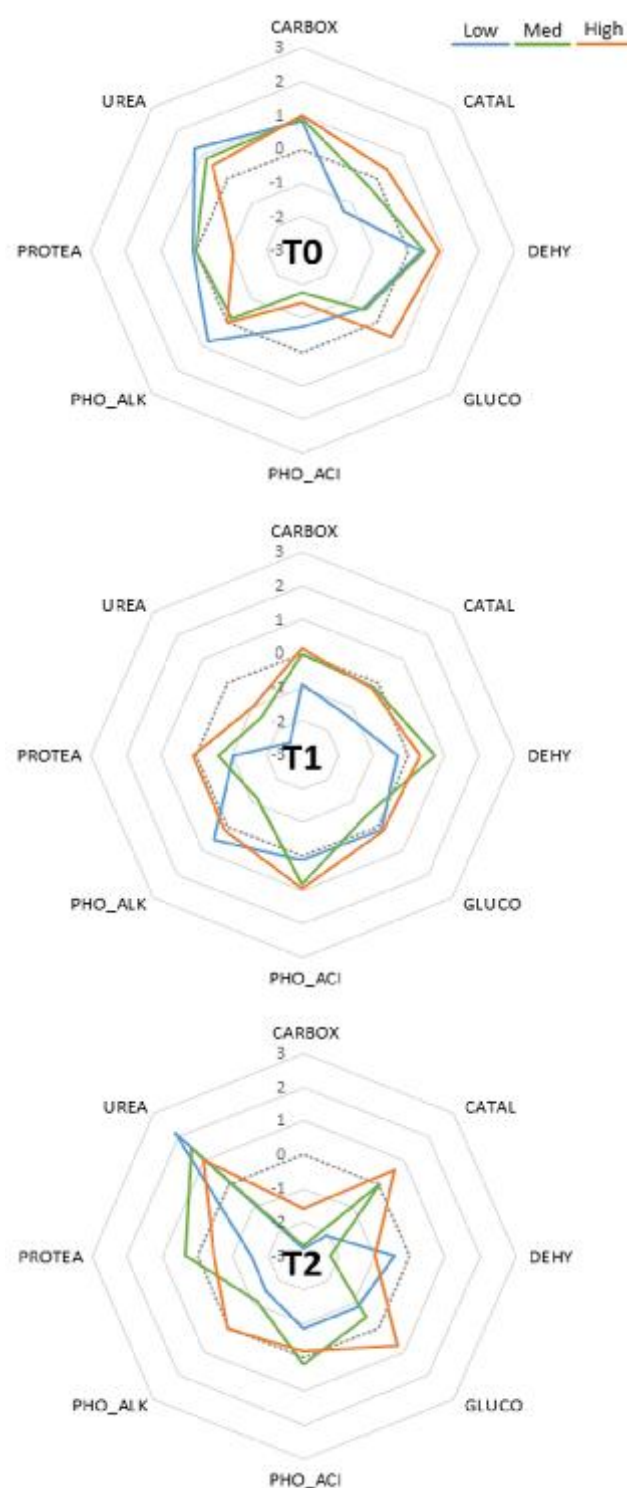


These results leave open several hypotheses related to Sb toxicity in soil. One of them could be the threshold toxicity of Sb, so higher concentrations of this metalloid did not affect negatively the selected soil enzymes in this study. A high capability of Sb to bind organo-mineral complexes may be a plausible reasons to explain why at T0 no differences where observed between doses.

A second hypothesis may be the rhizosphere, which is expected to be well-developed in the moment of harvesting. This highly-functional and active environment may reduce the impact of Sb on soil enzyme activities because of an enhanced microbial activity and biomass.

Furthermore, Sb is not toxic to soil microorganisms at the exposure levels tested in this study, as extracellular enzyme activities (with a microbial origin) were not affected negatively by Sb.

Regarding each enzyme activity (Figure 6.4), we observed that the carboxylesterase was positively impacted by Sb at T0, but with no dose-dependent response while other enzymes such as the catalase presented a dose-dependent response (inhibition at low Sb, no effect at medium Sb and activation at high Sb).



Moreover, the urease activity increased with an opposite dose response (higher activity with lower Sb). This short term increase of N-cycle enzymes was also found in another experiment where soil urease activity increased up to 168% after 3 days of exposure of potassium antimony tartrate (An and Kim, 2009).

After 15 days (T1), Sb toxicity on enzymatic activity was partly buffered in high Sb treatments (all the enzymes were closer to the basal line). However, contrary to its positive activity at T0, urease was strongly inhibited at T1, still with an opposite dose dependent response. Protease and catalase were also strongly inhibited in low Sb treatment. Under TM stress, reactive oxygen species are produced by living organisms (Jiang et al., 2016). These molecules are generally converted in H_2O_2 by superoxide dismutase. The catalase enzyme is an oxido-reductase which is known to use H_2O_2 as substrate. Accordingly, its activity can evidence antioxidant metabolism under stress condition.

In the case of Sb stress, few literature is available on antioxidant enzymes. Pan et al (Pan et al., 2010) showed that under Sb contamination, the catalase of maize leaves increased to alleviate the stress. In our conditions, soil catalase was inhibited at low Sb dose and a small increase was observed at high Sb dose. This dose dependent response of antioxidant enzymes was also found with rice α -amylase facing Sb contamination (He and Yang, 1999).

At T2, results were more contrasted. First, as shown in [Figure 6.3](#), the IBRv2 of each Sb treatment was higher than at T1, suggesting a 'long term' effect of antimony on soil life. Then, the dose dependent buffering effect is still measured as the Jonckheere-Terpstra test showed a significant opposite dose dependent decrease of the IBRv2. Surprisingly, the urease activity was positive, with the same pattern than in T0 (while it was inhibited at T1), suggesting a complex pattern of response of this enzyme, making it a difficult tool for Sb contamination study (An and Kim, 2009). The dehydrogenase, which was positive at T0 and started to decrease at T1, showed a loss of activity at T2 for all Sb concentrations. This was previously described in soil spiked with 1 to 50 mg.kg⁻¹ KSb-tartrate where *Brassica rapa* L. were cultivated (Xiao et al., 2015). However, the authors also found a decreased of urease activity while we only found it at T1.

Dehydrogenase has been shown to be a good indicator of soil microbial activity (Trevors, 1984). Its variations suggested a toxic effect of Sb on soil microbial community. An and Kim (An and Kim, 2009) also found that dehydrogenase was a potentially good early indicator of Sb contamination in soil, along with urease. These results are in agreement with the long term decrease of β -glucosidase and acid phosphatase activities observed in our conditions, while these two enzymes are excreted by microorganisms and have a major role in their nutrition.

These temporal variations have already been evidenced in enzyme response to TM stress. Indeed, microorganisms can have an adaptation period facing a new metal contamination (Baran et al., 2004). Then, an increase of microbial community tolerance to chemicals can occur. This highlights the need to measure enzyme activity after long-term exposure, to let time to the microorganisms to adapt their metabolism to the stress (Moreno et al., 2002). This period of adaptation might be a good explanation of the differences observed between T0, T1 and T2. Indeed, in T2 the Sb dose dependent response was significant, which can be due to a higher activation of stress alleviation metabolism in high Sb treatment. Finally, we tested here only one Sb chemical species (potassium antimony tartrate) because of its good solubility. However, enzymatic response to TM is chemical species dependent (Chaperon and Sauvé, 2008; Wang et al., 2010) so further investigation is needed on other chemical species of Sb more susceptible to be found in soil (antimony oxides and sulfides for example).

6.3.1.2. Interactions between Sb and SCG/SCGc treatments

EFFECTS ON BIOMASS

Pea plants grew well under all treatments, with non-significant difference between them as summarized in [Table 6.1](#). No measurable effect of biochar (SCGc) were observed compared with the control soil, regardless of the Sb dose. The only difference was found in the Sb-free spent coffee ground (SCG) treatment. In this modality, plant biomass significantly decreased compared with Sb-free SCGc and Sb-free control treatments. Such phytotoxicity of SCG has also been described by some studies (Kim et al., 2014; Hardgrove and Livesley, 2016). Peas cultivated with SCGc had generally more cloves (*i.e.* higher peas yield), except with medium Sb treatment in which the plants were still flowering and had very few cloves. However, the small number of replicates limits these analysis.

Table 6.1: Pea plant aerial biomass, g. Letters indicates significant groups at $\alpha=5\%$. No letters indicates no significance.

Treatment	Sb	Aerial biomass, g
Control	NO	52 ± 3.5
	LOW	61.4 ± 8.7
	MEDIUM	74.6 ± 2.6
	HIGH	58.4 ± 17.3
SCG	NO	35.5 ± 2.7 b
	LOW	66.7 ± 4.9 a
	MEDIUM	52.6 ± 15 a
	HIGH	52.5 ± 10.6 a
SCGc	NO	67.7 ± 6.9
	LOW	59.9 ± 2.5
	MEDIUM	60.8 ± 12.1
	HIGH	74.5 ± 3.1

EFFECTS ON SOIL ENZYME ACTIVITIES

SCG and SCGc applications in soil had different effect on the overall enzymatic activity (integrated by the IBRv2 index) depending on Sb concentration (Table 6.2).

Table 6.2: Variations of the IBRv2 index in control, SCG, and SCGc treatments according to Sb concentrations and sampling period (T0, T1 & T2). A lower IBR indicates a more similar global soil enzyme activity to control soil.

Treatment	Sb	IBRv2 T0	IBRv2 T1	IBRv2 T2
Control	LOW	7.1 ± 0.7	8.2 ± 3.3	12.4 ± 2.9
	MEDIUM	7 ± 2.3	7 ± 0.8	10 ± 1
	HIGH	7.5 ± 1.5	5.5 ± 1	7.2 ± 2
SCG	NO	6.6 ± 0.8 b	6.5 ± 1.2 b	8 ± 0.6 ab
	LOW	6.5 ± 1.3 b	11.8 ± 3.1 a	10.4 ± 1.8 a
	MEDIUM	8.3 ± 0.2 a	8 ± 1.4 ab	6.9 ± 0.8 b
	HIGH	6.8 ± 0.3 ab	7.7 ± 0.6 ab	7.2 ± 1.4 b
SCGc	NO	8.9 ± 1.4	8.2 ± 0.9	8.8 ± 0.6
	LOW	8.5 ± 1.5	8.6 ± 1.5	6.6 ± 1.6
	MEDIUM	12 ± 1.9	9.4 ± 2.5	7.3 ± 0.8
	HIGH	9.3 ± 2.5	8 ± 0.8	6.2 ± 1.7

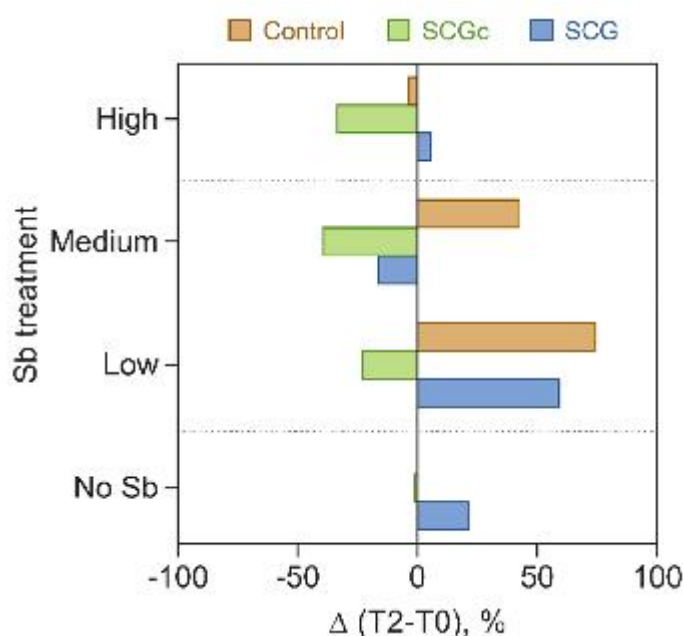
Indeed, Sb dose had no significant influence on the IBRv2 outcomes from the SCGc treatment, whatever the time considered., except at medium Sb dose where the IBRv2 was significantly higher than at lower Sb doses (Pvalue <0.05).

On the opposite, Sb exposure had a significant impact on the IBRv2 output of the SCG treatment: the medium Sb dose increased the global variation of activity at short-term exposure, while at longer exposure time (T2) the highest variation of activity was found for the low Sb dose. Compared to the control soil, the IBRv2 value at T0 for the SCGc treatment was higher in all Sb exposure levels whereas SCG-amended soils had an almost similar or a little lower enzyme activities.

The $\Delta T2-T0$ (Figure 6.5) highlighted the possible stress alleviation by microorganisms on long term exposure. Indeed, if the IBRv2 outcomes decreased between T0 and T2, it would mean that the global effect of Sb/SCG/SCGc on soil enzyme activities decreased. In the control soil, Sb effect has been described in section 6.3.2.1.

With SCG application, time-dependent changes in the IBRv2 values seemed not to be associated to Sb dose. Indeed, the IBR increased between T0 and T2 in the groups No Sb, Low Sb and High Sb with the highest increase (+59%) in low Sb treatment while it decreased with medium Sb treatment.

With SCGc application, the IBR decreased in all modalities with a marked trend of threshold response (higher decrease with medium and high Sb compared to low Sb), suggesting a beneficial effect of SCGc on soil enzyme resilience under Sb stress.



However, even if the IBRv2 generally decreased with SCGc addition, some variations among enzymes were measured, which is only visible with the A factor from the IBRv2 calculation, as presented in Figure 6.5. This is one of the interests of the IBRv2 compared to other integrative numerical indexes (such as the Gmean or the WM indexes). Indeed, the sunray-plot representation allows a fast visualization of the impact of the environmental stress (*i.e.* Sb or SCG) on each enzyme activity (Nannipieri et al., 2002; Sanchez-Hernandez et al., under review).

As described in the former section, temporal variations can occur in enzyme response to TM stress because microorganisms can have an adaptation period facing a new metal contamination (Baran et al., 2004). Accordingly, we focused here on the results at the end of the experiment (T2), [Figure 6.6](#):

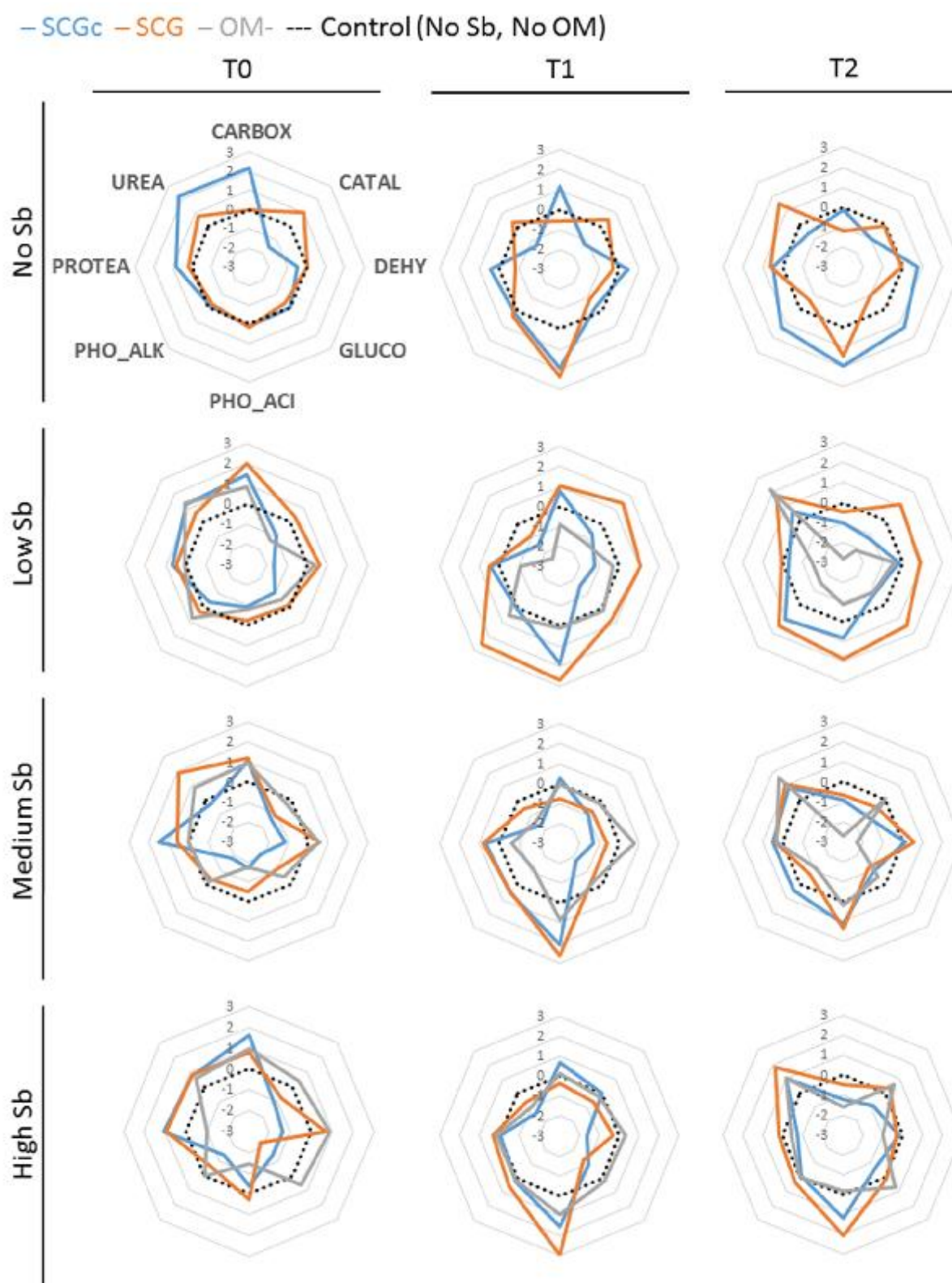
Carboxylesterase activity decreased in all treatments, with no dose response. This enzyme seems to be a suitable biomarker of organophosphate pesticide contamination (Sanchez-Hernandez et al., under review). Some studies focused on the impact of TM on this esterase activity in soil and showed that free cations such as Pb^{2+} or Cu^{2+} inhibited the enzyme activity (Satyanarayana and Getzin, 1973). Here, the general decrease of activity with the addition of Sb are consistent with the latter study, even if the response is not dose-dependent with the range of concentration we used. However, comparison should be performed with care as the authors used (1) a purified preparation of soil carboxylesterase and (2) a malathion as a substrate, while we used 4-nitrophenyl butyrate as substrate in soil:H₂O suspension analysis.

Catalase activity decreased in all Sb treatments in the presence of SCGc. With SCG, this oxidoreductase activity increased at low Sb (while it decreased in OM- treatments), but it was similar to control in the other Sb treatments. Catalase is an intracellular enzyme, so it reflects soil microbial activity (enzymatic activity from living organisms) through microbes' response to oxidative stress by lysing hydrogen peroxide into water (Pan et al., 2010). A loss of activity could be either due to a loss of microbial biomass or a reduction of oxidative stress. Furthermore, Dehydrogenase activity – another intracellular enzyme activity associated to microbial activity and biomass – was generally not affected by SCG, SCGc and Sb. Consequently, in our conditions, the treatments had no large effect on microbial activity and biomass compared with controls, and SCGc decreased Sb-induced oxidative stress while SCG increased it.

Protease activity was generally not affected by SCG, SCGc and Sb.

β -Glucosidase increased in Sb-free soil treated with SCGc and was one point under control in all Sb treatments. With SCG, no clear pattern was observed (under control with no Sb, above at low Sb, under at medium Sb and no effect at high Sb).

Acid phosphatase was one of the most impacted enzyme, with an important increase of activity at all Sb doses with both SCG and SCGc. Our results contrast with those by Jain et al. (2016), who reported an increase of acid phosphatase activity in biochar-treated soils under a maize cultivation. These contradictory results could be explained by several factors such as (i) soil physicochemical properties, (ii) biochar properties (source, production protocol...) and (iii) the interaction between them.



SCG SCGc OM-

Alkaline phosphatase activity increased with SCGc in Sb-free and low Sb treatments while it only increased at low Sb dose for SCG condition. Both SCG and SCGc had no effect at higher Sb doses. In our conditions, we found that SCGc was highly colonized by bacteria and fungal hyphae (possibly AMF). This microbial colonization could also explain the present increase of phosphatase activity as these organisms are known to be key actors of phosphorus solubilization (Smith and Smith, 2011).

Urease activity increased in all treatments but in a less extent with SCGc. This increase of activity was previously observed in different condition for biochar (Jain et al., 2016) and for SCG. Urease enzymes participate in the N cycle through the ureolysis reaction $(\text{NH}_2)_2\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{NH}_3$ and have been shown to increase because of the presence of *Rhizobium* sp. and other plant growth promoting rhizobacteria (Babu et al., 2015). The plant model we chose (*Pisum sativum* L.), a leguminous producing large amount of nodules, could be a suitable explanation of the enhanced urease activity.

As for SCG/SCGc-free soil, no clear dose-response curve was evidenced in our conditions, preventing the use of a specific enzyme as an indicator of Sb toxicity.

Finally, until now, no study investigated the relationship between SCG, SCGc and Sb and their combined effects on soil enzyme activities, making this field of research widely open for further experiments to strengthen and discuss the present results.

6.3.2. Biochar colonization with microorganisms

At the end on this experiment, we extracted the SCGc from the soil (Figure 6.7) and washed it 4 times with deionized water to study its composition and residual enzyme activity.

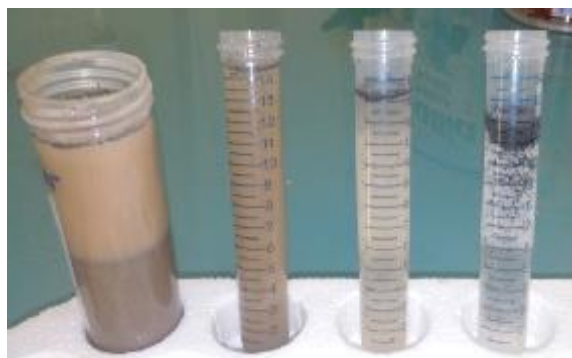
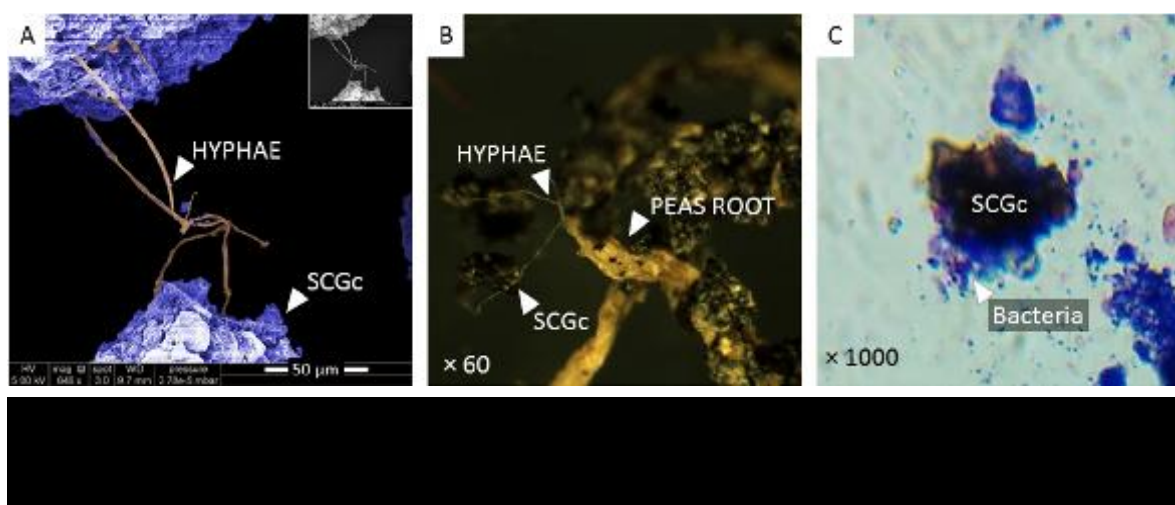


Figure 6.7: SCGc extraction from soil.

From left to right: soil:H₂O (1/1) mix; sampling supernatant; repeated washing. The floating biochar is extracted and stored for analysis.

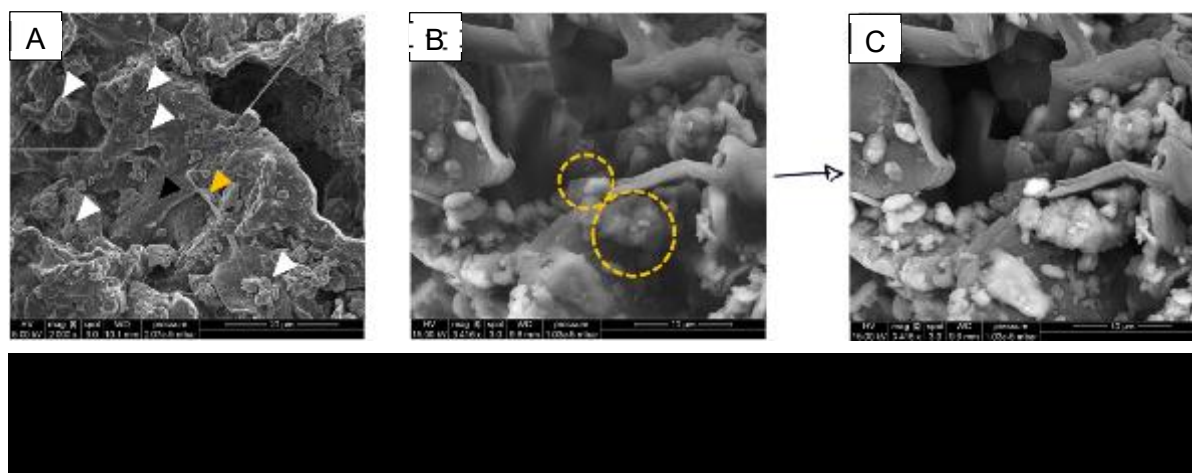
6.3.2.1. Presence on microorganism in SCGc (biochar)

Figure 6.8 illustrates scanning electron microscopy (A), optic microscopy (B) of fungal structures on washed biochar, and stained gram \pm bacteria (Gregersen, 1978) bound around H₂O-washed biochar (C). Spent coffee ground biochar (SCGc) was well colonized by AMF and soil bacteria, permitting to increase soil structure by contributing to aggregate formation, compared to the control. This colonization was also observed between *Rhizophagus irregularis* and different types of biochar (Hammer et al., 2014) where the authors concluded that AMF were able to uptake nutrient from biochar, increasing therefore plant biomass.



6.3.2.2. Possible presence of Sb in soil particles adsorbed in SCGc

Then, using Scanning electron microscopy coupled with X-ray analysis, we found that SCGc particles adsorbed small soil residues, even after the washing process and these residues contained heavy elements (Figure 6.9). These heavy atoms could be Sb, unfortunately the spectrum measurement did not permit to validate this hypothesis because the SCGc structure is extremely rich in calcium, which has almost the same energy as Sb, hiding its possible presence in small particles.



6.3.2.3. Residual activity in biochar

To study if SCGc could be a good amendment in agriculture, part of the samples was sterilized after the washing phase (sterilization with a Bench Top Steam sterilizer, Nüve®, program OT 32 VS - steam and vacuum sterilization during 30 min at 134°C). Then, dehydrogenase activity was measured as an indicator of living microorganisms (Figure 6.10).

We found that even after steam and vacuum sterilization, dehydrogenase activity was still present, and significantly higher than at T1. This suggests that SCGc tridimensional structure offered shelter to the soil microorganisms. These outcomes present a good potential for the development of commercial product, which could need numerous cleaning and drying steps in their preparation.

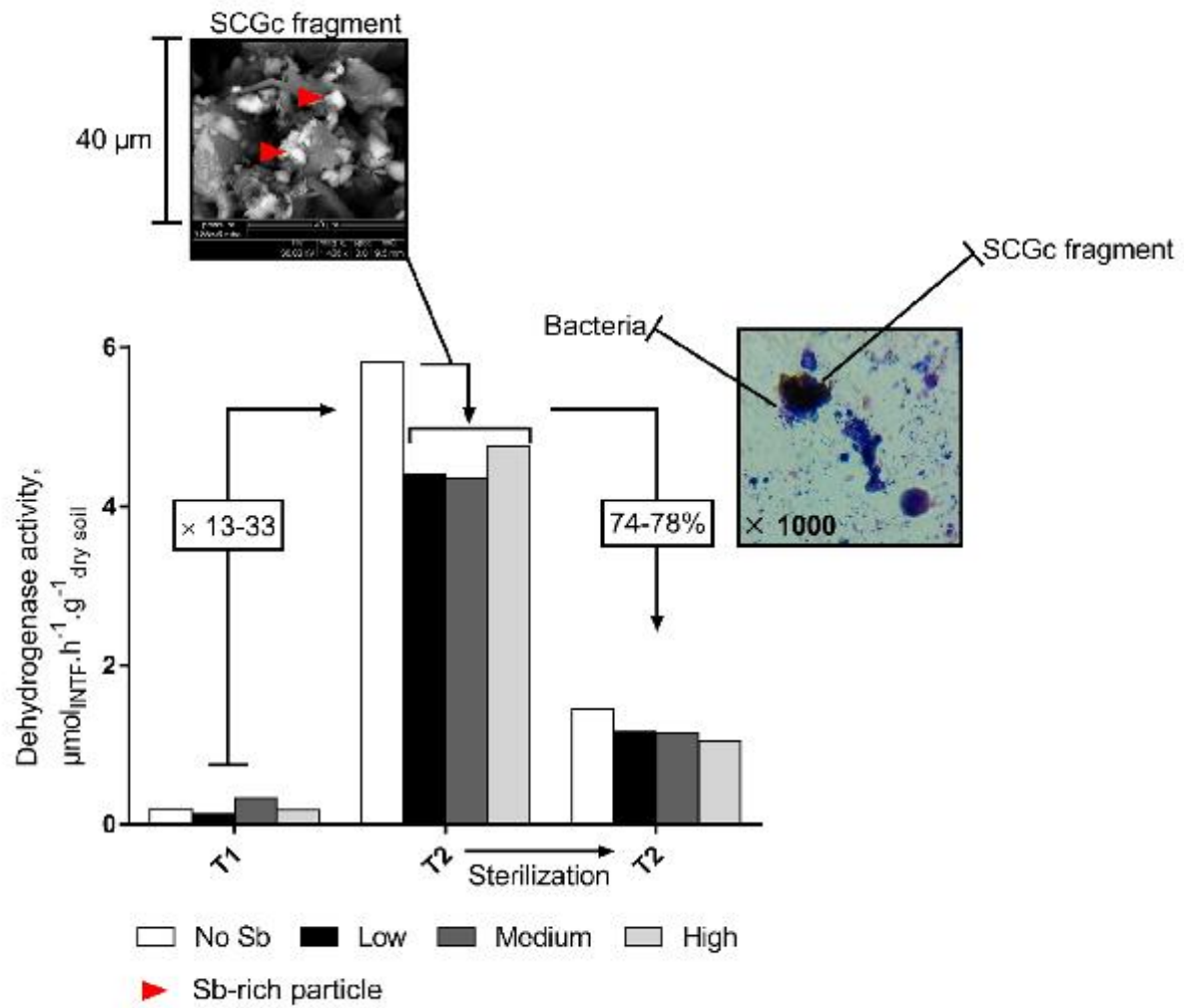


Figure 6.10: Dehydrogenase activity before and after sterilization

6.4. Conclusion

The objective of the present study was to compare the influence of Spent Coffee Ground (SCG) and Spent Coffee Ground biochar (SCGc) on soil enzyme activities and antimony accumulation in plants in the presence of a Sb gradient.

Our findings indicates that [SCGc was colonized by AMF and soil bacteria](#), suggesting its potential pool of nutrients. Such result could permit to develop interesting ‘activated’ amendment for degraded soils. Indeed, [SCGc conserved microbial enzymatic activity even after sterilization](#), meaning that its porosity might offer shelter to microorganisms.

Then, in the control soil, the strong increase of the IBRv2 index showed that [enzyme activities were strongly affected by Sb spiking](#). However, this effect seemed to be concentration dependent, with higher influence at low concentration. [The use of SCGc decreased the IBRv2 index in all Sb treatments](#), suggesting its capacity to alleviate Sb stress. However, the results for each enzyme suggested complex interactions between Sb, SCG/SCGc and microbial communities, making this approach difficult to generalize for Sb monitoring in environmental studies.

Moreover, Sb content in edible organs is still under process, meaning that for now we cannot conclude on the interest or sanitary risk of using SCG or SCGc on Sb contaminated soils.

6.5. Supplementary materials

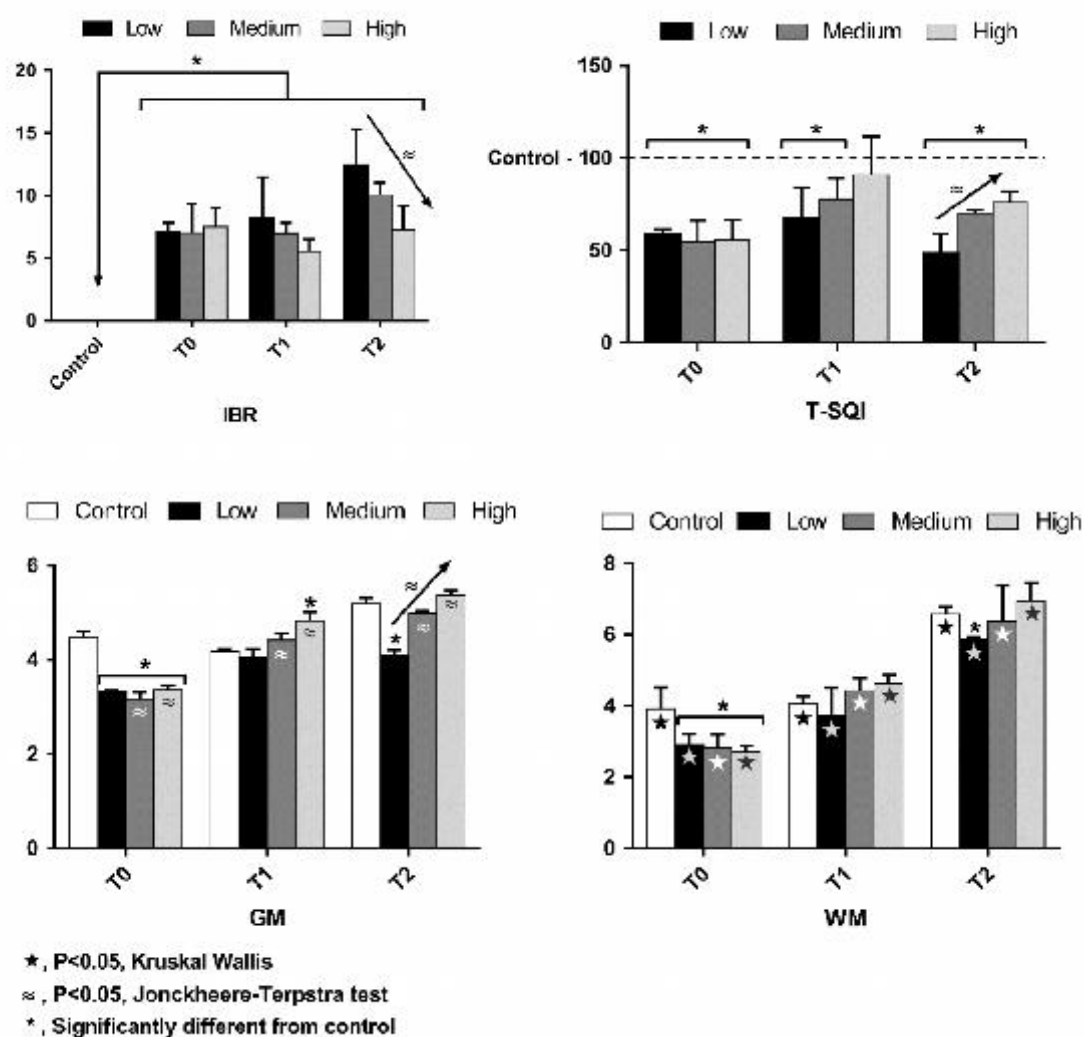


Figure 6.11: Indexes of control soils (SCG- and SCGc-free) depending on Sb concentrations and sampling period (T0, T1 & T2). The control soil (no bar or white bar depending on the index) represents the natural soil. It is set to zero in IBR and T-SQI calculation. Histograms represent mean \pm standard deviation (n=4) of each index.

Chapter 7. DISCUSSION

In this thesis, the role of various biofertilizers and bioamendments (lombricompost, arbuscular mycorrhizal fungi and biochar) was investigated in the transfer from soil to edible plants of two major urban inorganic contaminants (cadmium and lead) and a hazardous emerging one (antimony) (Li et al., 2013). Their influence on human bioaccessibility of these metal(loid)s (TM) has been studied because such biofertilizers and bioamendments are already commercialized even if their relationship with TM has not fully been studied. Most of the results have been discussed in each chapter, so the present one aims to discuss further some points, especially the interest and risks of these biofertilizers inputs in urban agriculture.

First, and before everything else, pot experiments as performed in this thesis are a usual and efficient way to simplify and study complex natural systems. However, some studies indicate that they probably generate measurement biases (Joner and Leyval, 2001; Sýkorová et al., 2007); therefore, our trials would need to be implemented in field to strengthen the results, discussions and recommendations developed herein.

7.1. Species and varieties have to be chosen wisely

Vegetables shown different pattern of TM accumulation, depending on the soil (as described earlier) and the plant species (Säumel et al., 2012; Akhter, 2012; Pierart et al., 2015) which is consistent with the differences we observed between lettuce and leek accumulation and between sandy soil and sandy-clay soil (Chapter 3: Pierart et al., 2016 under review). It is generally accepted that TM accumulation rate is as follow: legume/fruit/seed < root vegetable < leafy vegetable.

Indeed, fruits/seeds are the main reproductive organs, suggesting that a physiological barrier prevent TM to accumulate near the dissemination organs. Root vegetables are in contact with all the soil contaminants and the contamination might come not only from the accumulation of TM in plant organs, but also of their sorption in root surfaces, enriched with OH and COOH groups, able to complex TM, while the inner part of the root organs contain generally less TM (Queirolo et al., 2000). With transmission electronic microscopy coupled with X ray analysis (MET JEOL JEM 1400-EDS, centre de micro-caractérisation Raymond Castaing, UMS 3623, Toulouse, France) we confirmed that soil particles rich in heavy atoms were bound to leek root surface and extraradical fungal hyphae (Figure 7.1), even after washing. This suggests that the results obtained for TM content in roots might represent not only the TM absorbed but also the TM adsorbed externally.

To investigate more deeply the chemical speciation of the present elements, TOF-SIMS microscopy would be an interesting approach for intracellular analysis. However, this technic is only semi quantitative. For quantitative analysis, laser ablation mass spectrophotometry could be a suitable tool. In that case, it is possible to measure precisely TM contents in each nanometric layer of a sample, to distinguish TM adsorbed on root surface, TM stored in the epidermis (which both of them would be took out by peeling) and TM accumulated in inner tissues.

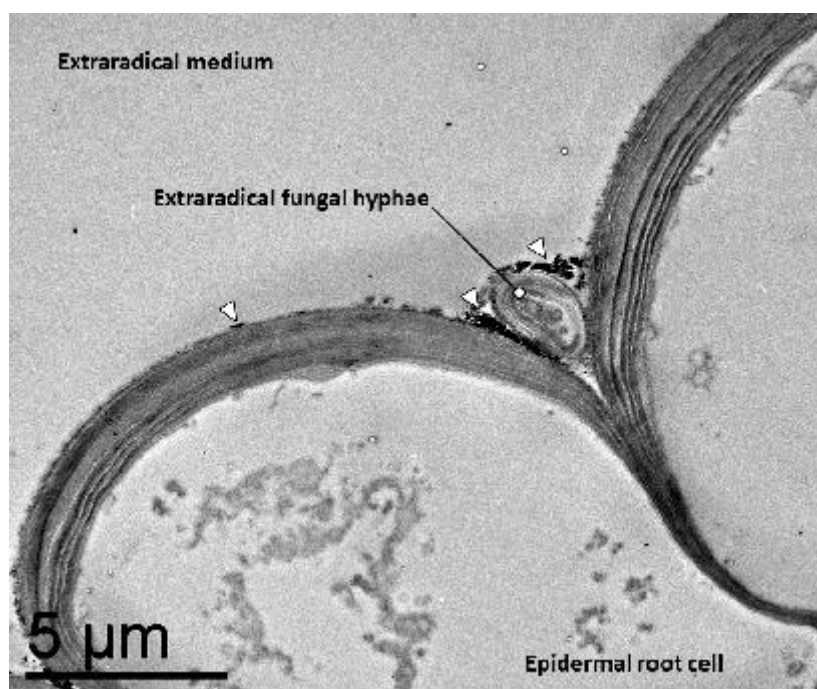


Figure 7.1: Picture of leek root cells in transmission electronic microscopy coupled with X ray analysis. White arrows (Δ) indicates accumulation of heavy elements bound to root and fungal surface.

Leafy vegetables present the highest potential risk of TM accumulation because they are facing both soil pollution (through translocation from roots to leaves as shown for Cd in [Chapter 3](#) for example) and aerial pollution through deposition of metal-rich particles (Uzu et al., 2010; Xiong et al., 2014, 2016a), they cannot be peeled and they are generally more difficult to wash properly.

However, some authors suggest that the difference in TM accumulation was more plant species dependent than plant type (leafy vegetable, root vegetable, fruits...) dependent (Säumel et al., 2012) and others even found phytoaccumulation variations of TM occur at the cultivar scale (Michalska and Asp, 2001). Consequently, the precaution principle suggests to grow preferentially fruit vegetables in urban area, and to wash the harvest carefully and peel vegetables when it is possible to limit the ingestion of thin particles of soil and decrease thereby the contaminant concentration up to 75-100% (Singh and Kumar, 2006) ([Figure 7.2](#)).

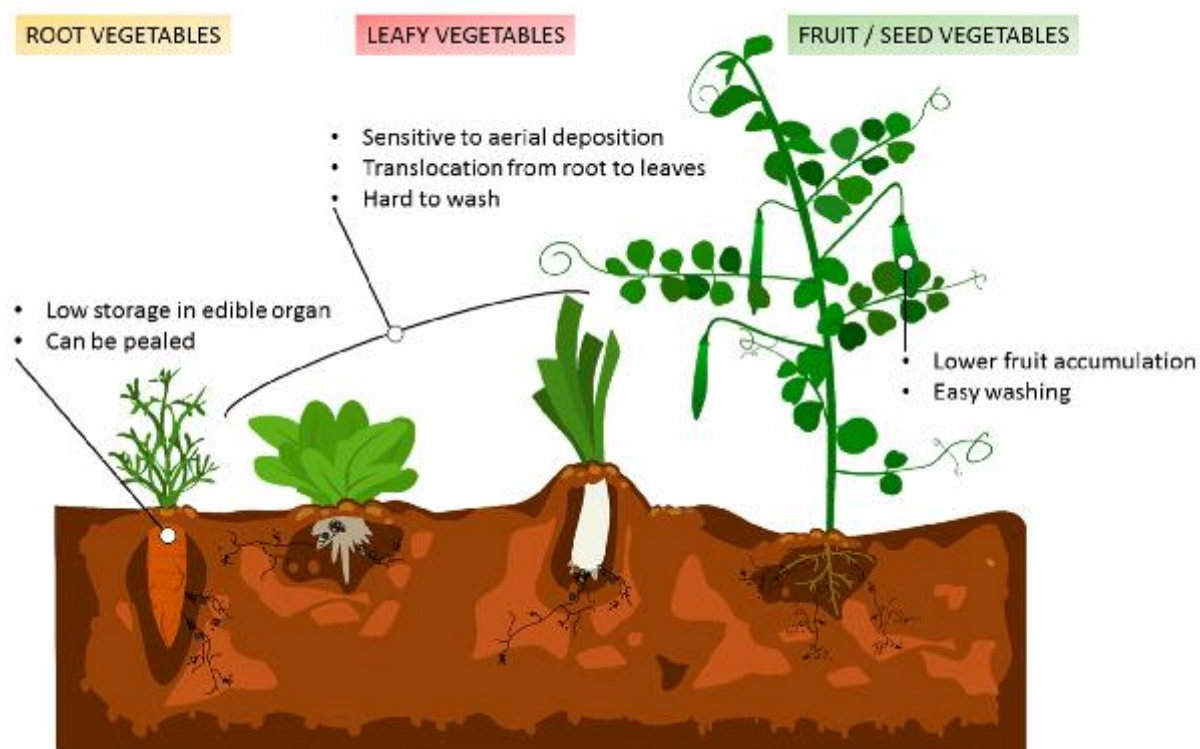


Figure 7.2: Choosing the best plant type facing a hazardous contamination

7.2. Is it risky or safe to use organic matter amendments in UPA?

In agriculture, and not only in urban and peri-urban agriculture (UPA), the use of compost is a widespread technic to increase total soil organic matter (SOM) with the final objective to increase yield. Actually, SOM increase soil fertility (inorganic nutrients are released by SOM mineralization and are available for microorganisms and plants). However, as described in [Chapter 1](#), addition of organic matter can influence TM mobility in soil (Ruttens et al., 2006; Karami et al., 2011; Murray et al., 2011).

7.2.1. OM addition and TM mobility

Indeed, in [Chapter 3](#) and [Chapter 4](#), we found that the addition of a commercial lombricompost (OM) influenced TM mobility in soil. Cd and Pb mobility decreased (EDTA extraction), which might be due to the increase of soil negative surfaces. Cadmium is known to be relatively mobile in soil, with a strong affinity to OM but a weak binding to clay minerals (Prokop et al., 2003). Hence, its phytoavailability is considered to be higher than Sb or Pb (Beesley et al., 2010). Sb has a great affinity to oxide, silicate and clay minerals in soil (Wilson et al., 2010) and is considered to have a low mobility (Murciego et al., 2007). Furthermore, Sb sorption has been estimated to be higher in mineral soils than in organic rich soils (Wilson et al., 2010),

which is consistent with our findings as the studied soil present a low organic matter content and a low Sb mobility.

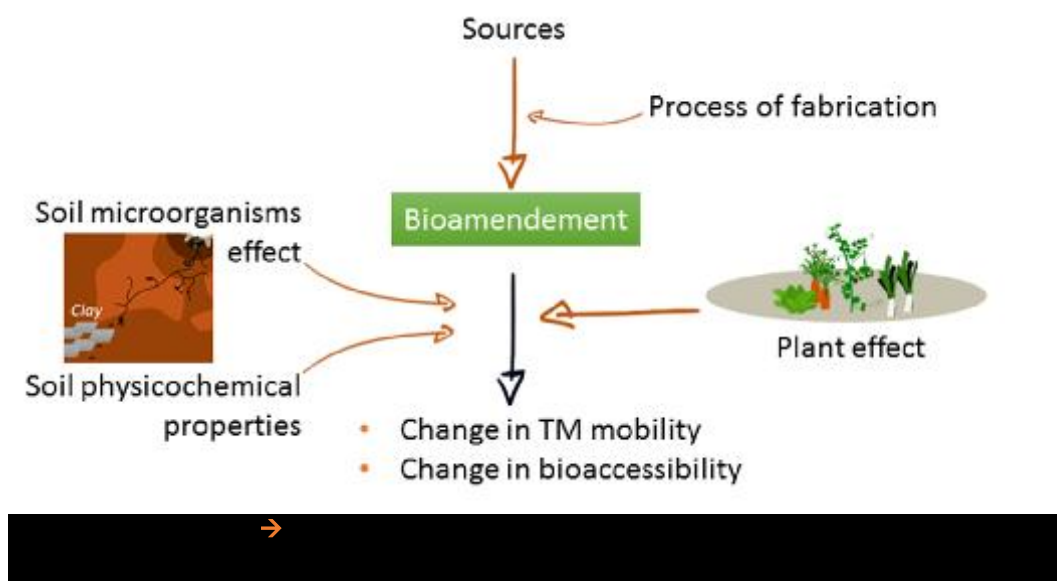
However, the results obtained on the exchangeable phase (estimated by the chemical CaCl_2 extraction) are more contrasted, with combined effects of plant, contaminant origin, and soil physicochemical properties.

These variations of TM mobility in soil because of OM addition were generally not correlated with TM accumulation in plant. TM mobility can change due to local pH modification and solubilization, or because of complexation with organic acids excreted by plants or microorganisms (Jones, 1998; Violante et al., 2010), but it doesn't mean that these complexes are phytoavailable. Indeed, it has been shown that TM availability to plants rely on the physicochemical properties of the complexing agent, which could explain why we found no correlation between Sb mobility in soil and Sb accumulation in plant, as also suggested by Hammel et al (Hammel et al., 2000).

7.2.2. OM addition and human bioaccessibility

Monitoring human bioaccessibility is an interesting method to ensure food quality (Pelfrene, 2016; Xiong et al., 2016b) as it makes a link between environment, health and society. Concerning TM, bioaccessibility in plants depends of the element type as follow: Cd (~90%) > Pb (~60%) > Sb (~20%), which is in the same range as previously reported (Leveque, 2014; Xiong, 2015). OM addition had no effect on these fractions. We found no literature about the effect of compost on the bioaccessibility of TM in plants. However, a study focused on the effect of biochar on the bioaccessibility of polycyclic aromatic hydrocarbons and heavy metal in turnip showed that such organic amendment can reduce human bioaccessibility (Khan et al., 2015). Consequently, once again these effect seem to be dependent on the composition of the amendment, giving simple recommendation difficult to make. Thus, their use should be studied with care when soils are heavily contaminated with highly bioaccessible elements such as cadmium.

Added to these interconnected effects, compost can be prepared from a large variety of sources, such as plant residues, food residues, animal manure, and sewage sludges. We only tested lombricompost herein while compost physicochemical properties vary as much as its sources (Doan et al., 2014) and so do its effects on TM phytoavailability (Ruttens et al., 2006; Madrid et al., 2007; Fang et al., 2016) (Figure 7.3). Consequently, the main difficulty remains that these mechanisms are soil and amendment dependent (Sterrett et al., 1996; Brazauskienė et al., 2008; Fang et al., 2016), making general recommendations tricky. Moreover, from a global point of view, increase of SOM in the field can also modify erosion and water retention, changing in consequence metal transfers. The use of modelling approach could be interesting to dig further these analysis and propose efficient recommendations.



7.3. What about biofertilizers like mycorrhizal fungi?

Arbuscular mycorrhizal fungi are naturally present in soil, and they are in symbiosis with more than 94% of the angiosperms (Brundrett, 2009). Their role in TM mobility (and particularly Pb and Cd) has been described since 1980 (Chapter 1 - Table 1.2), showing the complexity of response as no general pattern can be developed. Available literature on Sb fate related with AMF is scattered as reviewed in Chapter 1. Recently, a study focused on this relationship has been published (Wei et al., 2016) and revealed that *Funneliformis mosseae* increases Sb uptake by plant when added in a natural soil (*i.e.* already containing AMF). Here, we tested the bio-augmentation, which consists in the isolation, multiplication and application of the local AMF community (and not only one species). This technic can easily be developed in UPA projects as it only requires trap crops, having a good mycorrhization rate such as leek. It also has the economic interest to save the purchase of commercial fungal fertilizer. However, our results showed that bio-augmentation had generally no effect on Sb mobility in soil, but decreased Pb and Cd exchangeable fraction (depending on the soil considered). At the plant scale, bio-augmentation decreased total Pb, increased Cd, and had no significant effect on Sb accumulation. However plant and soil effect were observed. This is consistent with studies showing that the fungal mediated uptake appears to be depend on the plant considered (Aloui, 2009; Janoušková et al., 2005; Leyval et al., 2002) and partly on the fungal strain (Liu et al., 2014).

In addition, phytoaccumulation of TM has been shown to be site-specific (Hough et al., 2004), but also season and fertilizer dependent (Florijn et al., 1992; Tyksiński and Kurdubaska, 2005). Chen et al. (Chen et al., 2016) showed that fungal community can be impacted by NO_3^- content in soil, meaning that the composition of the fertilizer has to be considered. Indeed, their impact on fungal diversity could explain the variation of phytoaccumulation, as TM accumulation depends on the fungal community (Hassan et al., 2013). Hence, as shown in [Chapter 4](#), using local AMF community could be a suitable technic to propose to gardeners to limit TM accumulation in plants. But, its efficiency remains dependent on environmental conditions (soil, plant, fertilization...) and the fungal community associated with plants can evolve seasonally (Dumbrell et al., 2011). This highlights the need to study precisely the role of the main ubiquitous species in TM solubilization and plant uptake in an attempt to find efficient couple [specific AMF species-specific edible plant] to cultivate on contaminated soil. For example, similar study could be carried out with commercial biofertilizers instead of local bioaugmented inoculum to compare the results as it has already partly been done in different conditions (Cozzolino et al., 2010; Firmin et al., 2015; Maček et al., 2016; Trotta et al., 2006).

Another complex effect to consider is the interaction of AMF with the rest of the soil fauna. Indeed, the soil and especially the rhizosphere is composed of macro and micro communities, which interact and generate combined effects on TM mobility. For example, it has been shown that together earthworms and AMF changed Cd speciation in soil (Aghababaei et al., 2014). Consequently, using global monitoring instead of focusing on one or another compartment could permit to obtain a large view of the effects of TM in soil and their possible mobility. The approach used in [Chapter 6](#) with enzymatic activity monitoring is already used for the study of metal contamination (Lessard et al., 2013), but also pesticide effects on soil vitality (Bello et al., 2014; Sanchez-Hernandez et al., under review). This technic presents the great interest to study at the same time, with fast analysis, multiple organisms through their biochemical metabolism so the global soil life can be evaluated at once. However, in the case of antimony, various studies intended to find enzymes which could be relevant indicators of Sb toxicity in soil, however, as in our case, results are complex, making this tool difficult to use (An and Kim, 2009).

7.4. Could biochar application in urban gardens reduce TM impact on crops quality?

Coffee is the second most traded aliment in the world (Kim et al., 2014) and it generates large amount of wastes (spent coffee grounds, SCG) which are intensively studied to develop recycling and valorization pathways (Campos-Vega et al., 2015). They are organic materials composed of more than 50% fibers, complex lignin structures and embedded tannin material composed of polyhydroxy polyphenol functional groups currently observed in humic substances and able to adsorb heavy metals (Fiol et al., 2008). These wastes can be brought directly to soil, but some studies indicate a potential phytotoxicity due to volatile compounds and organic acids released in the soil (Limousy et al., 2013).

Nevertheless, it has been shown that SCG contains S=O ligands able to form stable complex with Pb and decrease its phytoavailability (Kim et al., 2014). As Sb chemistry indicates a high affinity to sulfides, we suggest that the use of SCG, in tolerable dose to avoid plant toxicity, could be an efficient way to decrease Sb mobility in soil. This might not be as efficient with charred spent coffee ground (SCGc) because sulfurs are burnt during the pyrolysis (Kim et al., 2012). However, the use of SCGc presents other agronomical interest such as water and nutrient retention, soil structuration, and as shown in [Chapter 6](#), it can give shelter to microbial and fungal community.

The main difficulty would be to develop low cost, efficient and stable biochar production methods at the scale of UPA projects because for now, biochar can be purchased, but its cost is high or may be home made, as traditional charcoal. In the last case, its heterogeneity (in terms of production process and wastes used for production) influences greatly its characteristics, efficiency and potential toxicity (Jha et al., 2010; Barrow, 2012; LeCroy et al., 2013). Biochar is currently a key topic and have good application potential for contaminated soils as shown by the numerous studies and reviews on the subject (Sohi et al., 2010; Barrow, 2012; Lehmann and Joseph, 2015; Yang et al., 2016). Consequently, we recommend to follow the advances in this field.

7.5. Some recommendations

7.5.1. *Could modelling approach be used as management tools?*

When a land is sought for UPA projects, a global survey of the area should be mandatory to ensure health security. Indeed, as shown by Dumat et al (Dumat et al., 2015), urban gardens are sometimes set up on non-contaminated areas, but with water sources contaminated by TM, like arsenic, for example. However, as discussed earlier, it is barely impossible to test each plant, OM amendment and biofertilizers before giving access to gardeners, who will grow food sometimes overpassing the risks, even when aware of them (Dumat et al., 2015). This is why the development of preventive measures before opening new gardens through modelling approach of TM at the soil-microorganism-plant interface is a major field of study.

In our condition (*i.e.* pot soil-AMF-plant system) we did not find any correlation between TM mobile fractions and TM accumulation in plants permitting to develop a simple linear model as found in other studies (Uzu et al., 2009). Lin et al. also showed that the initial Cd^{2+} concentration and diffusion processes were the dominant factors in modelling Cd phytoavailability (Lin et al., 2015).

Furthermore, in the case of Cd and Pb, a recent study showed that root uptake is better represented with an active pathway than a passive absorption (Lugli and Mahler, 2016).

In terms of bioaccessibility and human health risk, which are also time consuming and complicated methods to set up, we showed that the bioaccessible fraction varied with contaminant origin. Various studies intended to develop a model predicting TM bioaccessibility with soil characteristics. However, while Pelfrene (Pelfrene, 2016) succeeded in representing mathematically TM bioaccessible fractions using simple soils analyses (P_2O_5 , CaCO_3 , pH, Fe, Al), Zhu et al (Zhu et al., 2015) concluded that a model developed in one kind of soil cannot be extrapolated easily to another soil.

Consequently, field trials are still recommended to increase the dataset representing the diversity of soil, plant and contaminant interactions to strengthen these mathematical approaches and make them efficient decision tool¹.

¹ However, even the best model won't be able to predict punctual contamination, as it is the case with fireworks for example (Moreno et al., 2010) !

7.5.2. Choosing the right place to grow food

At the early stage of the creation of new UPA areas, it is crucial to evaluate the contamination sources surrounding the place. In France, the ICPE regulation (Installations Classées pour la Protection de l'Environnement – industries/facilities classified for environmental protection) permits to know risky areas allowing to decrease the risk of contaminant exposure.

Then, when no information is available, soil analysis can be performed as proposed earlier. If there is no geogenic or ancient anthropic contamination in soil and water, air quality need also to be checked in urban areas, with also the influence of wind transport, building and hedge interceptions.

Actually, since the industrial revolution, air quality in urban areas has been of major concern. Every year, small particles thresholds are overpassed, especially in big cities (such as Mexico, Paris, Ganhzou...) (Zhu et al., 2012). These particles are usually rich in metals and can accumulate in crops (Uzu et al., 2010, 2009; Xiong, 2015) (Figure 7.4).

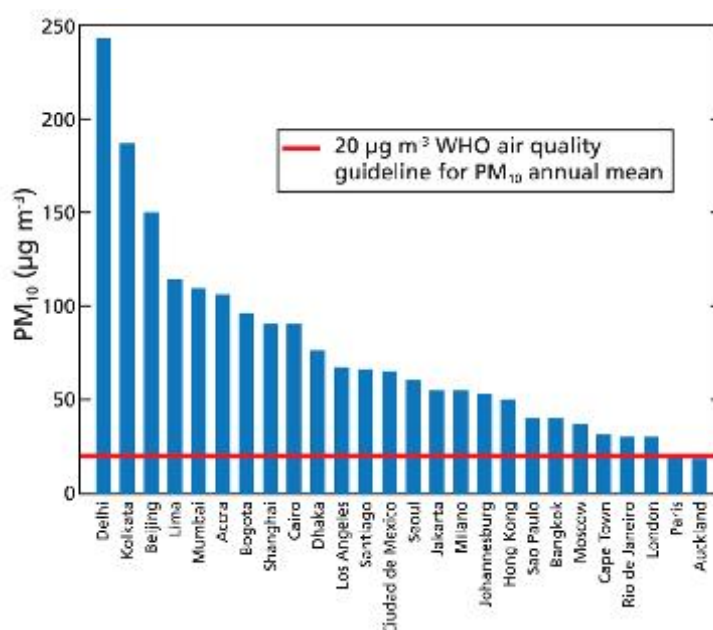
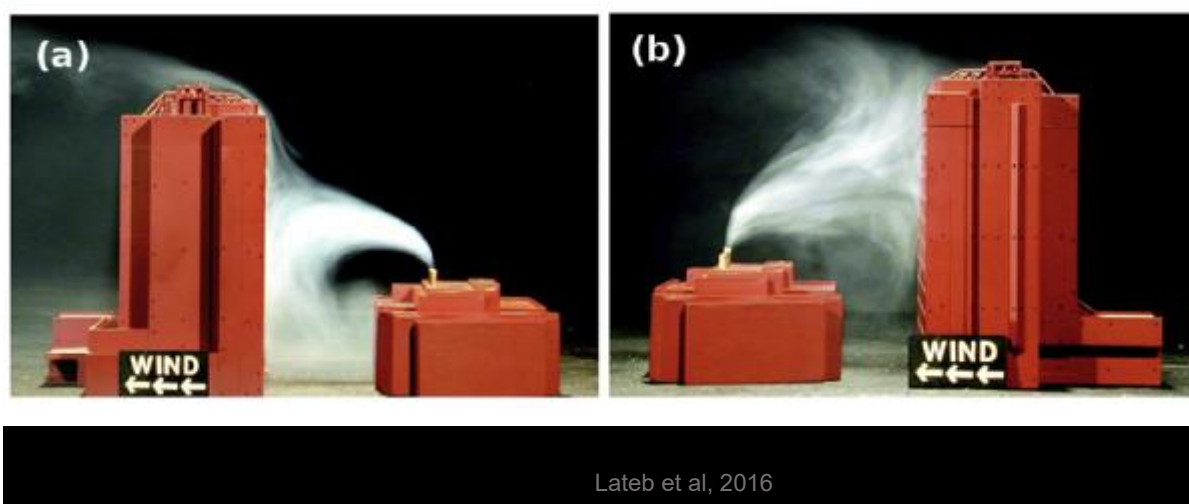


Figure 7.4: Annual (2012) mean of PM10
(Particles with diameters $\leq 10 \mu\text{m}$), Zhu et al, 2012.

In unsafe areas, considerations might be given to grow crops under shelter (to prevent from air contamination) and in pot/container filled with clean soil, compost or artificial substrate in the case of hydropony (to prevent from soil contamination).

However, one of the interests of urban agriculture is to use abandoned urban areas such as balcony, rooftops, roadsides, and wastelands among others. Accordingly, urban architecture could be used as an efficient tool to choose the lowest aerial deposition areas (Borrego et al., 2006). In their study of Cd phytoaccumulation, Säumel et al showed that the presence of plants constituting a barrier between roadside and crop can reduce TM in edibles (Säumel et al., 2012). Hence, modelling the dispersion of TM in urban areas could give clues to decide where UPA project can be safely set up (Lateb et al., 2016) (Figure 7.5).



7.5.3. Increase citizens' global knowledge on environment-health link

Crop quality in urban and peri-urban areas is the result of numerous factors. Soil ecosystems are complex, with number of biogeochemical interaction and human presence affects also these phenomena. In these areas, land use generally generates conflicts between urbanization and the will to conserve green spots in cities. Hence, the development of UPA projects faces multiple difficulties to succeed.

Facing this reality and the need to bring clear answers to citizens willing to make sustainable agriculture and consume local healthy vegetables, the concept of simplicity and collaborative multidisciplinary works are pertinent tools. Simplicity is a theory developed by Jeffrey Kluger² with the objective to describe and analyze the complexity of a problem with simple analytic tools, to make it intelligible.

For example, the fate of TM in soil is highly complex, because of the amount of interactions occurring therein. Facing this problem, we could have used sequential extraction (Kumar, 2016) or even combine this technic with stochastic modelling (Hu and Cheng, 2016). These approaches could have result in a more precise description of soil geochemistry but they also

² *Simplicity: Why Simple Things Become Complex (and How Complex Things Can Be Made Simple)*, 2008, J. Kluger

generate measurement artefacts (Calmano et al., 2001; Rapin et al., 1986; Wallmann et al., 1993) and they are costly, time consuming and complex to set up. Indeed, the objective was to use efficient tools to give simplex answers that gardeners could appropriate.

Accordingly, we chose to use only two chemicals (CaCl_2 and EDTA) as they are often efficiently used to describe mobile and phytoavailable TM in soil (Meers et al., 2007). However, as the results obtained present many tendencies but only few significant data, it could be interesting to test other chemicals extracting the same TM fractions such as DTPA or NH_4NO_3 for example (Omaka et al., 2015; Sakan et al., 2016).

Then, Informing gardeners and working in collaboration with them and politic institutions (Dumat et al., 2015) generates collective intelligence propitious to develop successful and safe urban agriculture projects. Such projects generally improve not only citizens' quality of life (Gonzalez et al., 2016; Martin et al., 2016) but also cities' environment (Aerts et al., 2016). Even if the discovery of a contamination generates legitimate fears and worries among the population, their will to conserve arable lands can still drive to the development of sustainable projects (Dumat et al., 2015) as summarized in Figure 7.6.

Consequently, informing gardeners about soil functioning, soil-plant-atmosphere transfer of nutrients and contaminants is crucial to rationalize and manage the risks. This is one of the objectives of the *Réseau Agriville* (see Chapter 1, section 1.2.4), a platform offering information and pedagogic/scientific resources to various UPA actors.

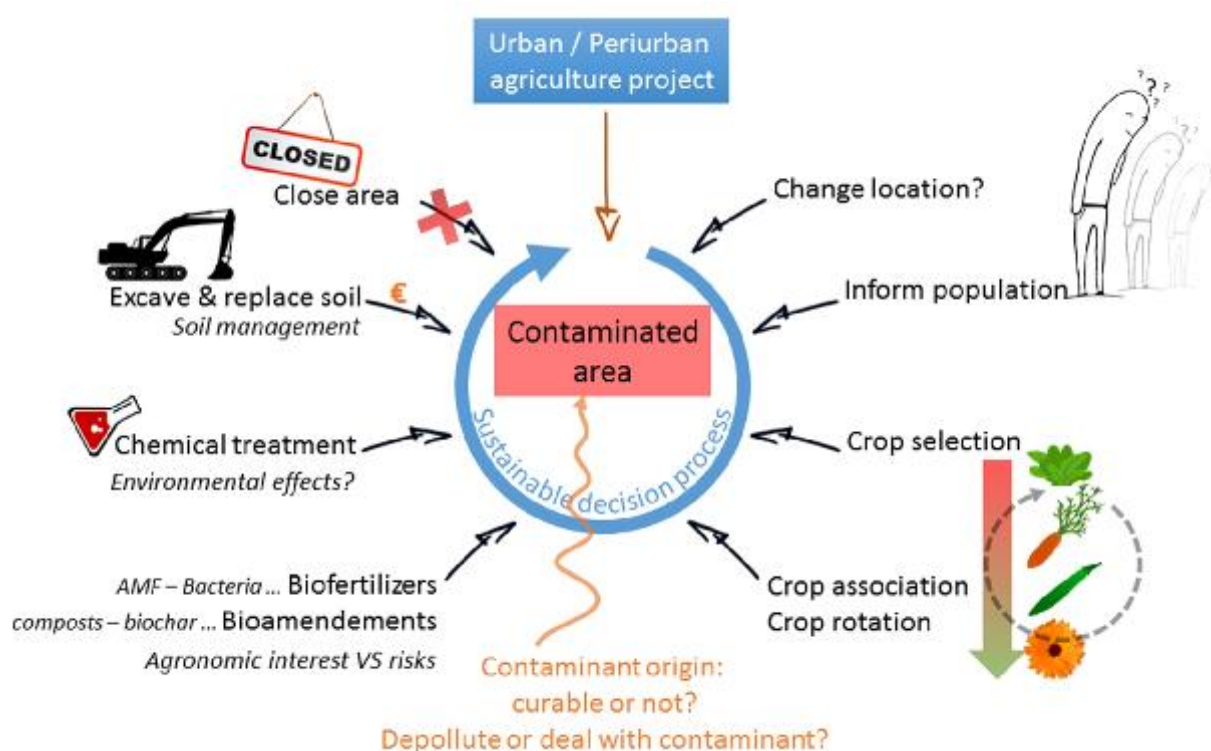


Figure 7.6: Management of a contamination in urban agriculture project

Chapter 8. GENERAL CONCLUSION & PERSPECTIVES

This conclusion is followed by its French version, on page 178.



7.4 billion People;



54% of them (of us?) are living in urban and peri-urban areas;

> 66% in 2050 according to the United Nations;

“The management of urban areas has become one of the most important challenge of the development of the 21th century (...) The success or failure of building sustainable cities will be an important factor permitting the development program to succeed for the post-2015”

John Wilmoth, Head of the population division, United Nations.

Urban areas concentrate such a proportion of the population that it is astonishing to consider that they can also be so hostile (noise, higher temperature than in the field, social inequalities, air, soil and water contamination...). **This is why cities of tomorrow¹ need to be though both sustainable and resilient to welcome the two-thirds of the 9.7 billion People estimated to swarm around the earth in 2050.**

Urban and peri-urban agriculture (UPA) projects have been gaining great interest since the last decades, with multiple will such as social binding, biological food production, ecological conservation, economical resilience... But this infatuation is actually facing risks of contamination, weakly known coming from industrial development, traffic, wastes, and all the historic pollutions that are being discovered progressively. Thus UPA could be an opportunity to develop sustainable cities if contaminations are managed carefully.

Accordingly, the objective of this thesis was to assess the role and the interactions of biofertilizers (Arbuscular Mycorrhizal Fungi, AMF) and bioamendments (lombricompost, biochar) used and/or usable in UPA in the transfer of anthropic or geogenic, major (Cd, Pb) and emerging (Sb), inorganic contaminants from soil to edible plants currently grown in UPA (lettuce, leek and peas), with a sanitary focus on their human bioaccessibility, especially for Sb, for which no legal threshold exists in vegetables.

¹ If you have not seen it already, you should go watch the movie [Demain](#), of Cyril Dion and Mélanie Laurent

Therefore, the study of metal(loid) (TM) transfer from soil to edible plants is crucial in UPA to ensure that dedicated areas and gardener's practices are safe enough to grow food and consume it.

Here, the main objectives were to:

- Assess the phytoavailability and bioaccessibility of TM from different contamination origin (geogenic vs anthropic) on different edible plants,
- Study the impact of biofertilizer (AMF) and organic matter amendments (lombricompost, biochar) on TM transfers from soil to plant and within plant (translocation and compartmentation),
- Study the impact of these amendments/fertilizers and Sb contaminant on soil life through enzymatic survey,
- Evaluate the influence of microbial community on TM fate at the soil-microorganism-plant interface,
- Study the influence of these physicochemical and biological parameters on human bioaccessibility of TM in soil and edible organs for health risk evaluation.

A multi-disciplinary approach with a set of experiments presenting a complexity gradient to represent the soil-microorganism-plant interface was used to answer the former scientific questions. The major results were:

- ▶ TM mobility and phytoavailability in soil is complex because many parameters interact: soil physicochemical properties, TM type and source, plant type (species, cultivar), fungal community and organic matter composition. For example: AMF and OM addition decreased Cd and Pb mobility, but had no clear effect on Sb mobility. Furthermore, TM mobility was soil/contaminant origin (anthropic > geogenic) and plant dependent (leek > lettuce) with interactions between these parameters.
- ▶ It is noteworthy that TM phytoaccumulation wasn't directly linked to their mobility and phytoavailability in soil, suggesting an active uptake more than a passive one. The accumulation was plant specific (lettuce > leek), and organ specific (root > leaf) for all studied TM. Furthermore, it was both TM type and origin specific (anthropic >> geogenic), except for Pb for which accumulation was similar in the two studied soils.

- ▶ AMF and OM effects on TM phytoaccumulation were complex, with no evident pattern because of all the interactions presented above. Most of the results evidenced only trends, highlighting the need to strengthen this study with either more replicates or field work. The most relevant results were that AMF increased Cd in lettuce on BZC soil but had no effect on NTE soil, which was the opposite for Pb. Moreover, AMF had no effect on Sb phytoaccumulation. OM addition tended to decrease TM in plants, but most of the results were only trends, except for Cd phytoaccumulation in leeks cultivated in BZC soil which significantly decreased with OM. For the first time, we highlighted that a coupled effect of AMF and OM seemed to decrease TM in edible parts (*i.e.* by decreasing the translocation factor). Unfortunately, our experimental set up did not give statistical significance, except for Cd in NTE soil. Additionally, these variations of accumulation were generally observed in roots, suggesting that TM were mainly stored in roots or bound to roots and fungal walls. This latter hypothesis was strengthened by microscopic observations of soil residues bounded to these structures even after washing. Consequently, peeling root vegetables could be a suitable recommendation in the case of TM contamination instead of only washing them.
- ▶ We showed that TM bioaccessibility in soil was strongly influenced by their origin (anthropic > geogenic) and for the first time that AMF had no effect on these fractions. Lombricompost increased Cd bioaccessibility in soil (but had no clear effect on Pb and Sb). This suggests that TM released recently are more bioaccessible during digestion than old contaminants. These differences in bioaccessibility were correlated to TM mobility in soil and their solubility (availability in soil solution), which could indicate that their chemical speciation influenced their bioaccessibility.
- ▶ TM bioaccessibility in plants depended on the element type as follow: Cd (~90%) > Pb (~60%) >> Sb (~20%). OM addition had no effect on these fractions in plants. AMF increased Sb bioaccessibility under both anthropic and geogenic contamination, which has not been observed before. A plant effect was also evidenced, with higher bioaccessibility in lettuce than in leek. Interestingly, contaminant origin influenced their bioaccessibility in plant (higher in anthropic contaminated soil) but a plant effect also interacted as these variations were only measured in lettuce and not in leek.
- ▶ With hydroponic condition, we simplified the system by taking out eventual soil interactions. This approach permitted to found that Sb speciation influenced how AMF (mix of fungal species isolated from the former Pb-Sb contaminated soils) could participate in Sb phytoaccumulation. However the response was plant specific as we observed an increase

of TM accumulation in carrot regardless of the chemical species, but a decrease in lettuce with an influence of the Sb chemical form at the organ scale (AMF had no effect on KSb accumulation in leaves while it decreased it with Sb_2O_3). Furthermore, we evidenced here for the first time that AMF enhanced both the translocation factor and the bioaccumulation factor of Sb, except in lettuces spiked with Sb_2O_3 in which we observed a significant decrease of TF. This suggests a selective absorption and storage depending on the chemical species rather than only an absorption depending on the oxidative state.

- ▶ Fungal diversity played a key role in TM mobility in soil and phytoaccumulation as described previously, with a composition dependent on soil and plant (this composition might also be seasonal according to the literature, which complicates even more the whole system). Some concerns were also evidenced, as OM addition and bioaugmentation with local AMF decreased fungal diversity associated with plants, so these amendments have to be used with care in the case of TM contamination in cultivated soils.
- ▶ Sb influence on soil microbial activity through enzymatic assays was strong, as Sb spiking increased significantly the IBRv2 index revealing important variations of enzyme activities. This effect seemed to be concentration dependent, with higher influence at low Sb contents. These original results highlight the potential use of the IBRv2 index to develop soil standard quality values.
- ▶ For the first time, the present study highlighted how the use of charred spent coffee ground (SCGc, *i.e.* biochar) buffered efficiently Sb toxicity compared to SCG or control treatments. However, some enzymes were still affected by Sb contamination (carboxylesterase, urease, and acid phosphatase). Interestingly, this bioamendment was well colonized by AMF and soil bacteria, and it conserved living microbial enzymatic activity after sterilization, meaning that biochar offered shelter to soil microorganisms. These findings support its great potential as a microflora-stimulating material and a source of nutrients for recovering degraded soils.

PERSPECTIVES

The present work, through its findings, limitations and biases, generated many fields of prospects:

The experimental set up

First of all, as stated in [Chapter 7](#), we focused here on two simplified models (hydropony and real soil) in pot experiments. It would be highly recommended, if not mandatory to set up similar protocol in the field to avoid pot biases affecting plant growth, fungal and microbial community, and eventually plant metabolism (such as TM uptake for example). However, to evaluate individual role of each actor, simpler models are also interesting tools, such as hairy root systems presented in [Chapter 5](#).

The plant used

Then, we showed a plant effect on TM mobility & accumulation. This could be due to the plant species used, but also to the cultivars and/or the crop sequence itself (lettuce → leek). To determine the predominant effect (if there is one), it could be interesting to compare the opposite sequence with the same plants but also with other species and cultivar. This would generate interesting data to add to existing databases such as BAPPET².

The biofertilizers & bioamendments applied

The observed effect of TM mobility and phytoaccumulation has been shown to be both fungal community and OM-origin dependent according to former studies (developed in [Chapter 4](#) and [7](#)). Consequently, further research is needed to characterize the effects of the most commonly used OM amendments and AMF commercial fertilizers in urban gardens to develop management strategies in contaminated urban areas and beyond. Moreover, we focused on AMF as main actors of soil biogeochemical processes to simplify the system and evaluate their own role, but numerous studies indicate that rhizospheric bacterial communities are known to influence TM and nutrient mobility at the soil-plant interface. The new generation sequencing technics such as Illumina MiSeq present a great potential to study all microorganism communities in soil, with the downside that taxonomic databases are still incomplete and the method efficiency itself seems to be species dependent.

² BAPPET - BAse de données sur les teneurs en Eléments Traces métalliques de Plantes Potagères

Using math as a decision tool

Even if there is still a long way before being able to represent mathematically the complexity of each scenario and interaction without field investigation and data collection, modelling these processes is of tremendous interest to limit expensive field study and to reduce the use of chemicals and plastic in laboratory analysis. When efficient, they could be used during decision process when UPA projects are developed for example.

Biochar

The results of [Chapter 6](#) about the use of spent coffee ground based biochar (SCGc) as a shelter for microorganism stimulate future research to activate SCGc with selected microorganisms able to solubilize or stabilize TM, pesticide or other contaminants to create a recycled usable tool in the phytoremediation or phytostabilization processes.

--- Take home message ---

Finally, the best recommendation might be to complete the classical sentence *eat at least five fruits and vegetables per day* ... with ... *from different origins*, to limit repeated ingestion of contaminant

This is the end, beautiful friend...

The Doors, The End, 1967.

French version of the conclusion & perspectives



Conclusion générale, version Française



7.4 Milliards d'habitants;



54% d'entre eux (d'entre nous?) vivent en zone urbaine ou péri-urbaine;

> 66% en 2050 selon les Nations Unies;

“La Gestion des zones urbaines est devenue l'un des défis de développement les plus importants du 21^e siècle (...) Le succès ou l'échec de la construction de villes durables sera un facteur important pour la réussite du programme de développement pour l'après-2015”

John Wilmoth, Directeur de la division de la population aux Nations Unies.

Les zones urbaines concentrent une telle proportion de la population qu'il est étonnant de remarquer qu'elles peuvent être si hostiles (bruit, température plus élevée qu'à l'extérieur, inégalités sociales, contamination de l'air, de l'eau, du sol...). Voilà pourquoi les villes de demain³ ont besoin d'être d'avantage durables et résilientes pour accueillir les deux tiers des 9.7 milliards de personnes estimées à fourmiller autour du globe en 2050.

Les projets d'agriculture urbaine et péri-urbaine (AUP) ont nettement gagné en intérêt ces dernières décennies, au travers d'objectifs multiples tels que le lien social, la production d'aliments biologiques, la protection de l'environnement ou encore la résilience économique. Mais cet engouement fait face à des risques encore faiblement connus de contaminations provenant du perpétuel développement industriel, du trafic, et des déchets générés par notre mode de vie. De fait, les projets d'AUP pourraient être l'occasion de développer des villes durables, à condition que les contaminations soient gérées avec précaution.

L'objectif de cette thèse était d'évaluer le rôle et l'interaction des biofertilisants (champignons mycorhiziens arbusculaires, AMF) et bioamendements (lombricompost, biochar) utilisés et/ou utilisables en AUP dans le transfert de contaminants inorganiques majeurs (Cd, Pb) et émergents (Sb), d'origine anthropiques ou géogéniques, du sol vers les plantes comestibles actuellement cultivées en AUP comme la laitue, le poireau et le pois ; ceci avec une attention

³ Si vous ne l'avez pas vu, Allez voir le film [Demain](#), de Cyril Dion et Mélanie Laurent

particulière sur l'aspect sanitaire via l'étude de la bioaccessibilité humaine de ces ETM, en particulier pour Sb, pour lesquels aucun seuil légal n'existe actuellement pour les végétaux cultivés.

Par conséquent, les transferts des ETM du sol aux plantes comestibles doivent être surveillés en AUP afin d'assurer que les espaces dédiés soient suffisamment sains et les pratiques agricoles des jardiniers raisonnées pour cultiver de la nourriture (sans risque pour l'environnement) et la consommer sans risque sanitaire.

Ici, les principaux objectifs étaient les suivants:

- Évaluer la phytodisponibilité et la bioaccessibilité des ETM d'origine différente (géogénique vs anthropique) dans des plantes comestibles,
- Étudier l'impact des biofertilisants (AMF) et de bioamendements (lombricompost, biochar) sur les transferts des ETM à partir du sol vers les plantes (translocation et compartimentation),
- Étudier l'impact de Sb et de ces amendements / fertilisants sur la vie du sol par l'étude de l'activité enzymatique,
- Évaluer l'influence des communautés fongiques sur le devenir des ETM à l'interface sol-microorganisme-plante,
- Étudier l'influence de ces paramètres physico-chimiques et biologiques sur la bioaccessibilité humaine des ETM dans le sol et les organes comestibles des plantes, pour l'évaluation des risques sanitaires.

Une approche multidisciplinaire composée d'un ensemble d'expériences organisées autour d'un gradient de complexité a été utilisée pour représenter l'interface sol-microorganisme-plante et répondre à ces questionnements.

Les principaux résultats sont les suivants :

- La mobilité des ETM et leur phytodisponibilité dans le sol est complexe car de nombreux paramètres interagissent: les propriétés physico-chimiques du sol, le type d'ETM mais aussi leur source, le type de plante (espèce et probablement cultivar), la composition de la communauté fongique et de la matière organique. Par exemple: l'ajout d'AMF et de lombricompost a diminué la mobilité de Cd et Pb, mais n'a eu aucun effet évident sur la mobilité de Sb. En outre, la mobilité des ETM était dépendante (i) du sol / de l'origine des contaminants (anthropique > géogénique) et (ii) de la plante (poireau > laitue) ; avec des interactions entre ces paramètres.

- ▶ Il est à noter que la phytoaccumulation des ETM n'est pas directement lié à leur mobilité et phytodisponibilité dans le sol dans nos conditions, ce qui suggère une absorption active plutôt que passive. L'accumulation des ETM est spécifique de la plante (laitue > poireau), et de l'organe (racine > feuille) pour l'ensemble des ETM étudiés. En outre, la phytoaccumulation des ETM est à la fois spécifique au type d'élément mais aussi à leur origine (anthropique >> géogénique), sauf pour le plomb dont l'accumulation était similaire dans les deux sols étudiés.

- ▶ Les effets observés de l'ajout d'AMF et OM sur la phytoaccumulation des ETM sont complexes, du fait des interactions présentées ci-dessus. La plupart des résultats ont seulement mis en évidence des tendances ; soulignant la nécessité de renforcer cette étude par un travail de terrain, ou éventuellement avec d'avantage de réplicas afin de renforcer les analyses statistiques. Les résultats les plus pertinents ont montré que l'ajout d'AMF augmente le Cd dans la laitue sur sol sablo-limoneux, mais n'a aucun effet sur sol sableux, ce qui était à l'opposé des résultats obtenus pour le Pb. Par ailleurs, l'ajout d'AMF n'a eu aucun effet sur la phytoaccumulation de Sb. Par contre, l'ajout de lombricompost a eu tendance à diminuer les ETM dans les plantes, mais la plupart des résultats étaient seulement des tendances, à l'exception de la phytoaccumulation du Cd dans le poireau cultivé sur sol sablo-limoneux, qui a diminué de manière significative avec l'ajout de lombricompost.

- ▶ Pour la première fois, nos résultats montrent que l'apport couplé d'AMF et de lombricompost semble diminuer les ETM dans les parties comestibles (en diminuant le facteur de translocation). Malheureusement, les designs expérimentaux mis en place ne l'ont pas mis en évidence de façon significative, excepté pour le cadmium dans les poireaux cultivés sur sol sableux. En outre, ces variations d'accumulation ont généralement été observées majoritairement dans les racines, ce qui suggère que les ETM étaient soit stockés dans ces organes, soit liés aux parois racinaires et fongiques. Cette hypothèse a été renforcée par des observations en microscopie électronique à transmission de résidus de sol liés à ces structures, même après lavage des racines. Par conséquent, pour réduire l'exposition humaine, peler les légumes racines pourrait être une recommandation appropriée dans le cas de contamination aux ETM plutôt que de simplement les laver.

- ▶ Nous avons montré que la bioaccessibilité des ETM dans le sol a été fortement influencée par l'origine des contaminants (anthropique > géogénique), et pour la première fois que que l'ajout d'AMF n'a aucun effet sur ces fractions. A l'opposé, le lombricompost a augmenté la bioaccessibilité du Cd dans le sol (mais n'a eu aucun effet évident sur Pb et Sb). Ceci suggère que les ETM récemment libérés dans l'environnement sont d'avantage bioaccessibles lors de la digestion que les contaminants métalliques anciens. Ces différences de bioaccessibilité étaient corrélées à la mobilité des ETM dans le sol et leur solubilité (disponibilité dans la solution du sol), ce qui pourrait indiquer que leur spéciation chimique influence directement leur bioaccessibilité.

- ▶ Dans nos conditions, nous avons confirmé que la bioaccessibilité des ETM dans les plantes dépend du type d'élément comme suit: Cd (~ 90%) > Pb (~ 60%) >> Sb (~ 20%). L'ajout de lombricompost n'a eu aucun effet sur ces fractions dans les plantes. Pour la première fois, nous rapportons que l'apport d'AMF augmente la bioaccessibilité de Sb. Un effet plante a également été mis en évidence, avec une bioaccessibilité plus élevée dans la laitue que dans le poireau. De plus, l'origine de la contamination a influencé sur sa bioaccessibilité dans les plantes (plus élevée dans les sols présentant une contamination anthropique). Cependant, un effet plante semble aussi intervenir car ces variations ont été mesurées seulement dans la laitue.

- ▶ En hydroponie, le système a été simplifié afin de limiter les éventuelles interactions avec le sol. Nous avons observé comment la spéciation chimique de Sb a influencé la façon dont le mélange d'espèces fongiques (isolées à partir des sols contaminés précédemment étudiés) pourrait participer à sa phytoaccumulation. Toutefois, la réponse était spécifique de la plante : augmentation de l'accumulation en ETM chez la carotte indépendamment de l'espèce chimique ; diminution chez la laitue avec une influence de la forme chimique de Sb à l'échelle de l'organe (la mycorhization n'a eu aucun effet sur KSb dans les feuilles alors qu'elle a diminué l'accumulation de Sb₂O₃). Par ailleurs, nous montrons ici pour la première fois que l'ajout d'AMF a augmenté à la fois le facteur de translocation et le facteur de bioaccumulation, excepté dans les laitues traitées avec Sb₂O₃ dans lesquelles nous avons observé une diminution significative du facteur de translocation. Ces résultats suggèrent une absorption et un stockage sélectifs dépendant de la spéciation chimique des ETM plutôt que seulement leur degré d'oxydation. Des biotransformations dans la plante pourraient également modifier la spéciation des ETM et leur bioaccessibilité.

- ▶ La diversité des communautés fongiques du sol a joué un rôle clé dans la mobilité des ETM et leur phytoaccumulation décrite précédemment. Cette composition semble dépendre du sol et des plantes (elle peut également varier de façon saisonnière d'après la littérature, ce qui complique encore d'avantage l'ensemble du système). Par ailleurs, des risques ont été mis en évidence quant à l'ajout de lombricompost et la bioaugmentation avec des propagules d'AMF locales. En effet, ces deux pratiques ont diminué la diversité fongique associée aux plantes. Par conséquent, ils doivent être utilisés avec précaution dans le cas de contamination en ETM dans les sols cultivés.

- ▶ L'influence de Sb sur l'activité microbienne du sol (étudiée grâce à des dosages d'activité enzymatiques) a été forte. En effet, le dopage du sol en Sb a augmenté de façon significative l'indice IBRv2 révélant d'importantes variations d'activités enzymatiques. Cet effet semble dépendre de la concentration en Sb, avec une influence plus élevée à des teneurs faibles en Sb. Ces résultats originaux mettent en évidence l'utilisation potentielle de l'indice IBRv2 dans le développement des valeurs standards de qualité des sols.

- ▶ Pour la première fois, cette étude montre comment l'utilisation de biochar de marc de café a efficacement atténué la toxicité de Sb par rapport aux traitements SCG (marc de café cru) et sans matière organique. Cependant, certaines enzymes étaient encore affectées par la contamination en Sb (carboxylestérase, uréase et phosphatase acide). Par ailleurs, ce bioamendement a été bien colonisé par les champignons et bactéries du sol, et il a conservé une activité enzymatique microbienne (vivante) après stérilisation. Ces résultats illustrent son potentiel pour la remédiation des sols dégradés, en tant que matériau offrant un abri efficace aux microorganismes du sol tout en étant une source de nutriments favorables à leur développement.

PERSPECTIVES

Le présent travail, au travers de ses conclusions, limites et biais, a généré de nombreuses perspectives:

Le dispositif expérimental mis en place

Tout d'abord, comme il a été discuté au [chapitre 7](#), nous nous sommes concentrés ici sur deux modèles simplifiés (hydroponie et sol réel) dans des expériences en pots. Il serait hautement souhaitable, sinon obligatoire de mettre en place des protocoles similaires en plein champ pour éviter les biais des expérimentations en pot affectant de nombreux paramètres physiologiques tels que la croissance des plantes, des champignons et de la communauté microbienne, et éventuellement le métabolisme des plantes comme l'absorption des ETM par exemple. Cependant, pour évaluer le rôle individuel de chaque acteur, des modèles encore plus simples, tels que les systèmes hairy-root présentés au [chapitre 5](#), sont aussi des outils intéressants.

La plante utilisée

A plusieurs reprises, nous avons montré un effet plante sur la mobilité et l'accumulation des ETM. Cela pourrait être dû aux espèces utilisées, mais aussi aux cultivars et à la séquence de culture elle-même (laitue → poireau). Pour déterminer l'effet prédominant (s'il y en a un), il pourrait être intéressant de comparer l'ordre inverse avec les mêmes plantes, mais aussi d'autres espèces et cultivars. Cela générerait des données intéressantes à ajouter aux bases de données existantes telles que BAPPET⁴.

Les biofertilisants et bioamendements appliqués

Comme cela a été développé dans les [chapitres 4 et 7](#), nous avons montré que la mobilité et la phytoaccumulation observées des ETM sont dépendantes à la fois de la communauté fongique et de l'origine de la matière organique (selon des études antérieures). Par conséquent, des recherches supplémentaires seraient nécessaires pour caractériser les effets des biofertilisants et bioamendements commerciaux les plus couramment utilisés dans les jardins urbains. Cela permettrait d'élaborer des stratégies de gestion raisonnée dans les zones urbaines contaminées et au-delà. De plus, nous nous sommes concentrés ici sur les AMF en tant que principaux acteurs du sol dans les processus biogéochimiques afin de simplifier le système et évalué leur propre rôle. Cependant de nombreuses études montrent que les communautés bactériennes rhizosphériques peuvent influencer la mobilité des ETM et des

⁴ BAPPET - BAs de données sur les teneurs en Eléments Traces métalliques de Plantes Potagères

éléments nutritifs à l'interface sol-plante. Les nouvelles techniques de séquençage haut débit tels que l'Illumina MiSeq présentent un grand potentiel pour étudier l'ensemble des communautés de micro-organismes dans le sol, avec l'inconvénient toutefois que les bases de données taxonomiques sont encore incomplètes et que l'efficacité de la méthode elle-même semble dépendre des espèces microbiennes étudiées.

Utiliser les mathématiques comme un outil d'aide à la décision

Même s'il y a encore un long chemin avant d'être en mesure de représenter mathématiquement la complexité de chaque scénario sans travail de terrain et collecte de données préalables, la modélisation de ces processus biologiques présente un intérêt considérable pour limiter les études de terrain coûteuses... Lorsqu'ils seront assez robustes, ces outils pourront être utilisés dans les processus de décision lors du développement de projets d'AUP par exemple.

Le biochar, un vaste champ de recherche

Les résultats du [chapitre 6](#) ont montré le potentiel du biochar de marc de café comme stimulateur et abri propice aux microorganismes. Ceci ouvre la voie à la recherche de méthodes pour activer ce biochar avec des micro-organismes sélectionnés, capables de solubiliser ou stabiliser les ETM, les pesticides ou d'autres contaminants afin de créer un outil recyclé utilisable dans les processus de phytoremédiation ou phytostabilisation.

--- Le mot de la fin ---

Finalement, la meilleure des recommandations pourrait être de compléter le conseil santé *mangez au moins cinq fruits et légumes par jours* ... par ... *d'origines différentes*, pour limiter l'ingestion répétée de contaminants.

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TITLE:

Role of arbuscular mycorrhizal fungi and bioamendments in the transfer and human bioaccessibility of Cd, Pb, and Sb contaminant in vegetables cultivated in urban areas

ABSTRACT:

Urban agriculture (UA) and pollution are two worlds more inter-connected every day, creating one of the main challenges of sustainable cities as persistent metal(loid) contamination increases as much as the interest for urban agriculture.

Biofertilizers and bioamendments used in UA (arbuscular mycorrhizal fungi, compost, and biochar) can influence the mobility of contaminants in soil. This study aims to better understand the fate of anthropic or geogenic, major (Cd, Pb) and emerging (Sb), inorganic contaminants in soil-plant-biofertilizer systems and their human bioaccessibility.

While contaminant mobility in soil is affected by biofertilizers, their origin influences also their bioaccessibility. The fungal community seems crucial in this phenomenon but is impacted by compost addition. Hence, using these biofertilizers in contaminated soils has to be thought wisely because of the multiple interactions affecting contaminant's phytoavailability.

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KEYWORDS:

Mycorrhization, Trace Elements, Transfer, Bioaccessibility, Enzymatic Activity, Biochar

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TITRE :

Rôle des champignons mycorhiziens à arbuscules et des bioamendments dans le transfert et la bioaccessibilité humaine de Cd, Pb et Sb vers les végétaux cultivés en milieu urbain.

RESUME :

Pollution et agriculture urbaine (AU) sont deux mondes interconnectés soumettant les villes au défi de la durabilité, dans un contexte où la pollution aux metalloïdes augmente au moins autant que l'intérêt pour l'agriculture urbaine.

Les biofertilisants / bioamendements utilisés en AU (champignons mycorhiziens à arbuscules, compost, biochar) peuvent influencer la mobilité des polluants du sol. Cette étude vise à mieux comprendre le devenir de contaminants inorganiques géogéniques et anthropiques, majeurs (Cd, Pb) ou émergents (Sb), dans des systèmes sol-plante-biofertilisant et leur bioaccessibilité pour l'homme.

Si la mobilité des polluants est modifiée par les biofertilisants, le type de source influence aussi leur bioaccessibilité. La communauté fongique semble cruciale dans ces phénomènes mais est impactée par l'ajout de compost. Ainsi, l'utilisation de ces biofertilisants sur sol pollué est à raisonner du fait des interactions multiples affectant la phytodisponibilité des polluants.

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MOTS-CLES :

Mycorhization, Eléments traces métalliques, Transfert, Bioaccessibilité, Activité enzymatique, Biochar

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